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DSCR1-mediated TET1 splicing regulates miR-124 expression to control adult hippocampal neurogenesis

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Abstract

Whether epigenetic factors such as DNA methylation and microRNAs interact to control adult hippocampal neurogenesis is not fully understood. Here, we show that Down syndrome critical region 1 (DSCR1) protein plays a key role in adult hippocampal neurogenesis by modulating two epigenetic factors: TET1 and *miR-124*. We find that *DSCR1* mutant mice have impaired adult hippocampal neurogenesis. DSCR1 binds to *TET1* introns to regulate splicing of *TET1*, thereby modulating TET1 level. Furthermore, TET1 controls the demethylation of the *miRNA-124* promoter to modulate *miR-124* expression. Correcting the level of TET1 in *DSCR1* knockout mice is sufficient to prevent defective adult neurogenesis. Importantly, restoring *DSCR1* level in a Down syndrome mouse model effectively rescued adult neurogenesis and learning and memory deficits. Our study reveals that DSCR1 plays a critical upstream role in epigenetic regulation of adult neurogenesis and provides insights into potential therapeutic strategy for treating cognitive defects in Down syndrome.

Keywords adult hippocampal neurogenesis; Down syndrome; DSCR1; TET1; miR-124

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Neuroscience

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Introduction

Adult neurogenesis is the process of generating new neurons in the adult brain. Newly born neurons arise in the two specific regions of the brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Gage, 2000; Zhao *et al*, 2008; Kriegstein & Alvarez-Buylla, 2009). The process of adult neurogenesis is dynamic and is similar to that of embryonic neurogenesis, in which both extrinsic and intrinsic factors, including epigenetic regulators, maintain the neural precursor cells and regulate their differentiation (Ming & Song, 2011; Goncalves *et al*, 2016). Following proliferation and differentiation of neural stem cells in the SGZ of the dentate gyrus, these newborn neurons have been shown to integrate into the existing neural circuits, which might contribute to both learning and memory (Deng *et al*, 2010). Importantly, defects in adult hippocampal neurogenesis have been observed in various animal models of neuropsychiatric diseases, including schizophrenia, depression, Parkinson's disease, Alzheimer's disease, and neurodevelopmental disorders such as Down syndrome (Contestabile *et al*, 2007; Guidi *et al*, 2008; Apple *et al*, 2017). However, the precise cellular and molecular mechanisms underlying adult hippocampal neurogenesis and their links to neurological disorders are not well understood.

Epigenetic factors, including DNA methylation and small non-coding RNAs (microRNAs), are crucial for adult neurogenesis in the brain (Kosik & Krichevsky, 2005; Ma *et al*, 2009). The methylcytosine dioxygenase enzyme, ten-eleven translocation 1 (TET1), is a demethylase reported to regulate the expression of genes involved in both embryonic and adult neurogenesis (Ito *et al*, 2010; Dawlaty *et al*, 2011). *TET1* knockout mice have reduced number and impaired capacity for neural progenitor cell proliferation in the adult SGZ, as well as impaired spatial learning and memory (Zhang *et al*, 2013). These data suggest that TET1 likely plays a critical role in controlling gene expression involved in adult neurogenesis associated learning and memory.

In addition to DNA methylation, microRNAs can regulate the expression of genes involved in various biological processes, including adult neurogenesis (Schratt *et al*, 2006; Han *et al*, 2016). *miR-124* is abundantly expressed in neurons and is involved in neural differentiation. In the SVZ region, *miR-124* expression is initiated in the proliferating neural progenitor cells and is maintained throughout adult neurogenesis (Lagos-Quintana *et al*, 2002; Cao *et al*, 2007). Thus, inhibition of *miR-124* activity impairs adult neurogenesis and increases ectopic astrocytes (Cheng *et al*, 2009). Moreover, overexpression of *miR-124* results in loss of neural stem cells and blocks neurogenesis in the SVZ region (Akerblom *et al*, 2012). Despite the importance of *TET1* and *miR-124* pathways in the

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process of adult neurogenesis, it remains unclear how the expression of *TET1* and *miR-124* is regulated. Moreover, the possibility that interplay between these two epigenetic pathways can modulate adult neurogenesis has not been investigated.

DSCR1 (Down syndrome critical region 1, also called *RCAN1*), located on human chromosome 21, is highly expressed in the brain and is especially enriched in hippocampal neurons (Fuentes *et al*, 1995). DSCR1 is conserved among animals and is upregulated in patients with Down syndrome (Fuentes *et al*, 2000; Arron *et al*, 2006). Animal models have shown that DSCR1 is required for learning and memory, suggesting that altered DSCR1 expression might contribute to the intellectual disability in DS (Chang *et al*, 2003; Dierssen *et al*, 2011; Shaw *et al*, 2015). Importantly, while DSCR1 is abundantly expressed in the hippocampus, whether DSCR1 plays a role in adult hippocampal neurogenesis is not known.

In this study, we demonstrate that DSCR1 is required for adult hippocampal neurogenesis, and further identify the mechanisms underlying how DSCR1 regulates this process. We show that DSCR1 binds to and modulates *TET1* splicing, which subsequently controls *miR-124* expression by regulating the methylation status of the *miR-124* promoter. Loss of *DSCR1* leads to increased TET1 levels, resulting in *miR-124* promoter hypomethylation and increased *miR-124* expression. *DSCR1* transgenic mice display opposite changes, albeit they also have defects in adult hippocampal neurogenesis. Strikingly, correcting the *DSCR1* dosage alleviates both the impaired adult hippocampal neurogenesis and the defective learning and memory seen in a Down syndrome mouse model (Ts65Dn). Together, our results reveal that precise regulation of DSCR1 and that interplay between TET1 and *miRNA-124* are crucial for normal adult hippocampal neurogenesis. These findings further highlight potential therapeutic targets for the treatment of patients with Down syndrome and other neurological disorders associated with adult neurogenesis.

Results

Adult hippocampal neurogenesis is impaired in *DSCR1* knockout and transgenic mice

Since DSCR1 expression is enriched in the hippocampus and *DSCR1* mutant animals exhibit deficits in learning and memory (Fig EV1; Hoeffer *et al*, 2007; Martin *et al*, 2012), we hypothesized that DSCR1 might modulate adult hippocampal neurogenesis. To this end, we investigated neuronal proliferation, differentiation, and maturation during the process of adult hippocampal neurogenesis in *DSCR1* mutants (Fig 1). We first examined the proliferation of neural progenitor cells in the SGZ of 4-month-old *DSCR1* mutant mice by administrating bromodeoxyuridine (BrdU). BrdU was injected intraperitoneally twice a day for 5 days, and brain sections were collected after 1, 10, and 21 days, which were then used for double labeling experiments. Actively proliferating progenitors identified by BrdU and Ki67, makers of dividing cells, were significantly reduced in the SGZ of both *DSCR1* knockout and transgenic mice (Fig 1A and B). We next performed double labeling with BrdU and DCX (a marker of cell differentiation), or BrdU and NeuN (a marker of maturation), in order to assess whether differentiation or maturation of progenitor neurons was altered in *DSCR1* mutants. The SGZ of *DSCR1* mutant mice showed a significantly reduced number of $BrdU^+/DCX^+$ and $BrdU^+/NeuN^+$ neurons (Fig 1C–F), implying that DSCR1 likely influenced multiple stages of adult hippocampal neurogenesis. Interestingly, both *DSCR1* knockout and transgenic mice showed similar defects, suggesting that precise regulation of DSCR1 levels is required for normal adult hippocampal neurogenesis.

As the observed decrease in neural differentiation could have originated from a decrease in the number of proliferating cells, we further assessed the effects of DSCR1 on adult neurogenesis in the hippocampus by isolating neural stem cells from adult dentate gyrus and preparing neurospheres *in vitro* (Fig EV2A–E). Consistent with the results presented in Fig 1, the number and size of the neurospheres acquired from the hippocampi of *DSCR1* mutants were significantly reduced compared with the controls. Secondary neurospheres originating from the individual primary neurons also indicated that the neurons from the *DSCR1* mutants have significantly diminished the self-renewal capability. However, the mutant neurospheres were still able to differentiate into neurons, astrocytes, and oligodendrocytes, which were confirmed by immunostaining using cell markers for each type of cells: Tuj1 for neurons; GFAP for astrocytes; and Olig2 for oligodendrocytes (Fig EV2F). These results suggest that DSCR1 affects the proliferation and self-renewal of the neural stem cells, but not the differentiation potential of the stem cells.

DSCR1 expression during adult hippocampal neurogenesis

Next, we examined *DSCR1* expression during adult hippocampal neurogenesis. To this end, fluorescence-activated cell sorting (FACS) together with quantitative RT–PCR was performed (Fig EV3). First, to label the proliferating neurons in the hippocampus, we injected mice with 5-ethynyl-2′-deoxyuridine (EdU). We then isolated and sorted the cells based on a combination of EdU and other known markers for neural progenitor cells ($EdU^+/SOX2^+$), neuroblasts (EdU^+/DCX^+), and mature neurons ($EdU^+/NeuN^+$; Fig EV3A–F). Next, *DSCR1* levels in these sorted cells were assessed via qRT–PCR (Fig EV3G). Higher level of *DSCR1* was detected in mature neurons, while lower level of DSCR1 was detected in neuroblast cells relative to those observed in neural progenitor cells.

DSCR1 controls expression of miR-124

miR-124 is enriched in the brain and upregulated during adult neurogenesis (Papagiannakopoulos & Kosik, 2009). Furthermore, altering the expression of *miR-124* resulted in impaired adult neurogenesis in the SVZ of the lateral ventricles (Cheng *et al*, 2009; Akerblom *et al*, 2012). However, the underlying mechanism regarding how *miR-124* expression is controlled during adult neurogenesis is not known. Since DSCR1 is also highly expressed in the brain and required for normal adult neurogenesis (Fig 1), we hypothesized that DSCR1 and *miR-124* might act in the same biological pathway in the hippocampus during adult neurogenesis. To test this hypothesis, we first monitored different forms of *miR-124* present in the dentate gyrus of *DSCR1* mutants via qRT–PCR analysis of pri-*miR-124*, pre-*miR-124*, and mature *miR-124* (Fig 2A). *DSCR1* deletion increased the levels of all three forms of *miR-124*, while upregulation of *DSCR1* decreased the levels of all three forms of *miR-124*. These results suggest that DSCR1 can control the transcription of *miRNA-124*, but it is unlikely to be involved in the processing of *miR-124*. This prompted us to further examine whether DSCR1

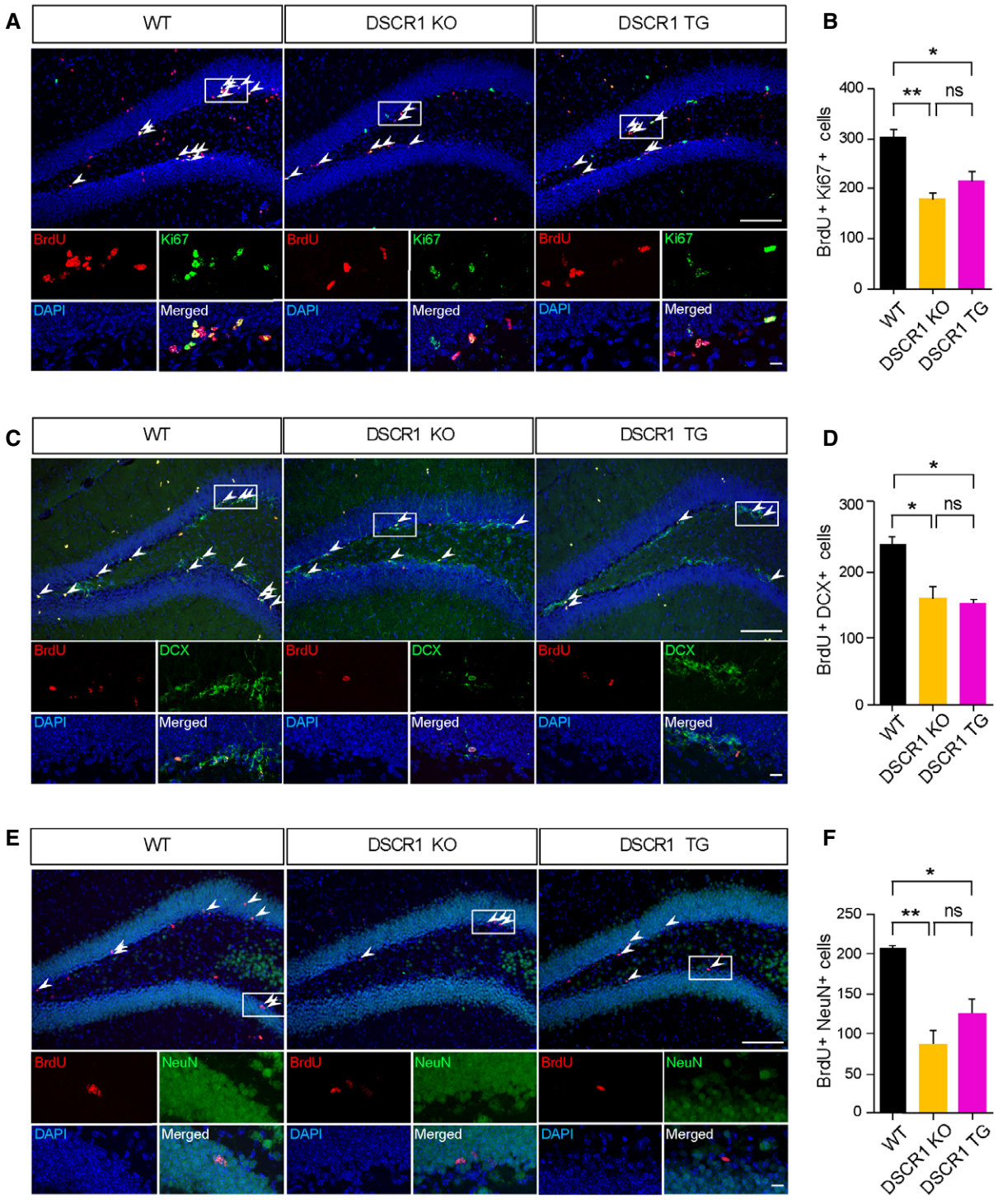

Figure 1. *DSCR1* mutants show impaired adult neurogenesis.

A, B Actively dividing cells labeled by BrdU and Ki67 are reduced in the SGZ of *DSCR1* mutants compared to that of wild-type mice. Brain sections were prepared 1 day after BrdU injection. The white box area is magnified in the lower panels: DAPI (blue), Ki67 (green), and BrdU (red). Arrow heads indicate BrdU and Ki-67 double-positive cells in the SGZ.

C, D Differentiating cells are identified by staining with BrdU and DCX. Brain sections were prepared 10 days after BrdU injection.

E, F Maturation of progenitor cells is assessed by staining with BrdU and NeuN. Brain sections were prepared 21 days after BrdU injection. Each hippocampal section was 40 μm in thickness, and a total of 24 sections were obtained from one hippocampus.

Data information: Scale bars: 100 μm in the large panel and 10 μm in the magnified images. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ animals for each condition, $^{*}P < 0.05$, $^{**}P < 0.01$.

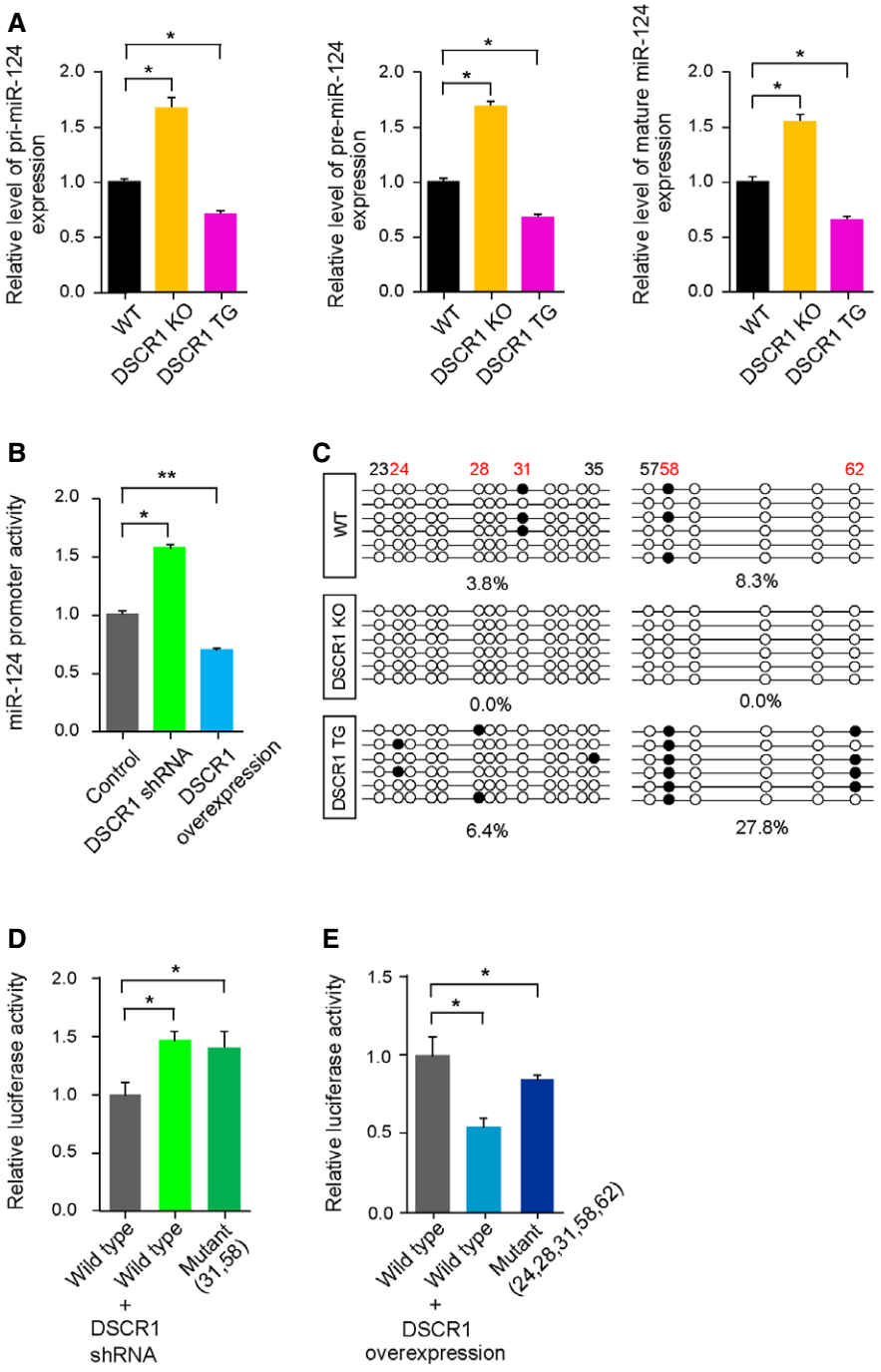

Figure 2. DSCR1 regulates *miR-124* by mediating methylation in the promoter of *miR-124*.

A *miR-124* expression is altered in *DSCR1* mutants. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ animals for each condition, $^{*}P < 0.01$.

B Promoter activity of *miR-124* in Neuro2 A cells with DSCR1 reduction or overexpression. Firefly luciferase reporter under the control of *miR-124* promoter is measured. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ for each condition, $^{*}P < 0.05$, $^{**}P < 0.01$.

C Bisulfite sequencing analyses of the *miR-124* promoter. Each CpG site is indicated, and the methylation status of two different regions is shown. Open and filled circles represent unmethylated and methylated CpG sites, respectively. The percentage of methylated CpGs among total number of CpGs is also shown.

D Neuro2A cells containing *DSCR1* shRNA show increased activity of *miR-124* promoter measured. The *miR-124* promoter was inserted in front of the *luciferase* reporter. Site-directed mutation of two methylation sites (31 and 58) in the promoter shows the luciferase activity similar to that of DSCR1 reduction. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ for each condition, $^{*}P < 0.05$.

E Overexpression of DSCR1 reduced the luciferase activity, while removing all methylation sites in the *miR-124* promoter increases it. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ for each condition, $^{*}P < 0.05$.

could regulate the promoter activity of *miR-124*. We generated a luciferase reporter under the control of *miR-124* promoter, which was then transfected to Neuro2A cells together with either *DSCR1* shRNA or *DSCR1* transgene. Luciferase assays indicated that cells with DSCR1 reduction displayed an increase in luciferase activity, while the cells overexpressing DSCR1 demonstrated a decrease in luciferase activity (Fig 2B). These results indicate that DSCR1 regulates the activity of the *miR-124* promoter.

Next, we performed bisulfite sequencing to test the possibility that DSCR1 could regulate *miR-124* promoter activity by altering the DNA methylation status of the promoter. Interestingly, a complete loss of CpGs methylation in the *miR-124* promoter was observed in the hippocampi of *DSCR1* knockout, while *DSCR1* upregulation showed an increase in the number of CpG methylation sites compared with the control (Fig 2C). We then tested whether changing the methylation status of the *miR-124* promoter was sufficient to alter its promoter activity. First, we blocked methylation at the CpG site by mutating two methylation sites detected in the normal *miR-124* protomer. Results of the luciferase assays revealed that this construct displayed higher luciferase activity when expressed in Neuro2A cells, similar to when *DSCR1* levels were reduced (Fig 2D). Next, we blocked all of the identified CpG methylation sites found in *DSCR1* transgenic hippocampus by site-directed mutagenesis of Cs to Ts. This results in significantly enhanced *miR-124* promoter activity compared with upregulation of *DSCR1* expression alone (Fig 2E), albeit the luciferase activity was still slightly lower than in cells without any methylation of the promoter. As such, it is possible that DNA methylation still occurred at different CpG sites within the promoter. Together, these results demonstrate that DSCR1 controls *miR-124* expression by modulating CpG methylation levels in the miR-124 promoter. It is also plausible that binding of transcription factors might be affected by changes in DNA methylation.

DSCR1 regulates *TET1* mRNA transcription

How does DSCR1 affect the DNA methylation level in *miR-124* promoter? DSCR1 does not contain the conserved domains present in demethylases; thus, we reasoned that DSCR1 might not directly regulate DNA methylation, but rather it might act indirectly by modulating the activity of another enzyme. Given that TET1 is a demethylase shown to regulate adult hippocampal neurogenesis, we tested whether DSCR1 could modulate the expression of TET1. We first examined whether DSCR1 controlled *TET1* expression in the hippocampus using qPCR. The results of our qRT-PCR analysis, as presented in Fig EV4, indicated that *TET1* mRNA transcript was increased in the hippocampus of *DSCR1* knockout mice, but it was significantly reduced in *DSCR1* transgenic mice. However, the level of the pre-*TET1* mRNA transcript was not altered in *DSCR1* mutants (Fig EV4B). We then assessed the strength of the *TET1* promoter by using luciferase reporter assays. The results indicated that different levels of *DSCR1* did not alter the *TET1* promoter strength (Fig EV4C). Lastly, we examined whether DSCR1 could alter the stability of the *TET1* mRNA transcript. We treated Neuro2A cells with reduced or overexpressed *DSCR1* with actinomycin D, a transcription inhibitor, and traced the decay of the *TET1* mRNA transcripts over the next 15 h using qRT–PCR. The *TET1* mRNA transcript levels gradually declined over time regardless of the *DSCR1* level, indicating that DSCR1 had no apparent effect on the stability of the *TET1* mRNA (Fig EV4D). Together, these data suggested that DSCR1 controlled the transcription of the *TET1* mRNA but had no effect on the processing or the stability of the *TET1* mRNA.

DSCR1 regulates splicing of the *TET1* mRNA

We then hypothesized that DSCR1 might regulate the splicing of the *TET1* pre-mRNAs. It is important to note that DSCR1 contains an RNA recognition motif (RRM) domain; however, the role of this domain has not been previously investigated (Strippoli *et al*, 2000). To test our hypothesis, we first assessed whether DSCR1 could directly bind to the *TET1* introns. *TET1* consists of 13 exons and 12 introns. We randomly selected the 8th and 9th introns of *TET1* and the 8th exon of *TET1* for this analysis by generating three different biotin-labeled RNAs via *in vitro* transcription. These biotin-labeled RNAs were incubated with Neuro2A lysates, and the RNAs were then precipitated using streptavidin-conjugated beads (Fig 3A). Western blot analysis showed that DSCR1 bound to the intron 8 and 9 sequences of *TET1*; however, DSCR1 did not interact with the biotinylated exon 9 of *TET1*. *TET1* introns 11, 12 and *GAPDH* introns 3 and 4 were also used for the binding assay. These *TET1* introns interacted with DSCR1, confirming that DSCR1 binds to *TET1* introns. However, introns 3 and 4 of *GAPDH* did not bind to DSCR1 (Fig EV4E–G), indicating that DSCR1 specifically binds to *TET1* introns. Splicing of pre-mRNAs begins with spliceosome assembly, which requires the U1 snRNP to recognize an intron, followed by U2 snRNP binding to strengthen the formation of the pre-spliceosome. To determine whether DSCR1 could affect the assembly of the spliceosome on the *TET1* pre-mRNA, we monitored the interaction between DSCR1 and the *TET1* intron 8 in the presence of U1 snRNA and U2 snRNA (Fig 3A). The binding affinity of DSCR1 to the intron 8 of *TET1* decreased with increasing dosage of the U1 snRNA and U2 snRNAs, suggesting that DSCR1 could modulate splicing of the pre-*TET1* mRNA by interfering with the binding of the spliceosome machinery at *TET1* introns.

Next, we examined whether the RRM domain of DSCR1 mediates its interaction with *TET1* introns. We expressed a Flag-tagged DSCR1 with or without the RRM domain and assessed its binding to the *TET1* intron. Figure 3B shows that DSCR1 without the RRM domain was not able to interact with the *TET1* intron. To further verify our results, we prepared a fragmented luciferase reporter that was separated by an insertion of *TET1* intron 8 and intron 9. We reasoned that if DSCR1 expression does indeed modulate splicing by binding to *TET1* introns, reducing the amount of DSCR1 should enhance luciferase activity by allowing increased splicing, whereas overexpression of DSCR1 should suppress splicing (Fig 3C). Indeed, Neuro2A cells transfected with the fragmented luciferase reporter together with the *DSCR1* shRNA showed significantly higher luciferase activity, whereas *DSCR1* overexpression resulted in reduced luciferase activity (Fig 3D). We also generated a fragmented luciferase expression vector separated by the introns 3 and 4 of the *GAPDH* gene as a control (Fig 3D). Figure 3D shows that luciferase activity of this reporter was not altered in cells containing different levels of DSCR1, suggesting that DSCR1 specifically interferes with the splicing of *TET1* mRNA. Furthermore, consistent with the observed changes in *TET1* mRNA levels (Fig EV4A), we found that the level of the TET1 protein was higher in *DSCR1* knockout mice but lower in *DSCR1* overexpressing transgenic mice (Fig 3E).

Next, we tested whether altering TET1 levels could affect *miR-124* expression. Neuro2A cells transfected with *TET1* shRNA showed significantly reduced levels of miR-124 (Fig EV5A–C). To further confirm that

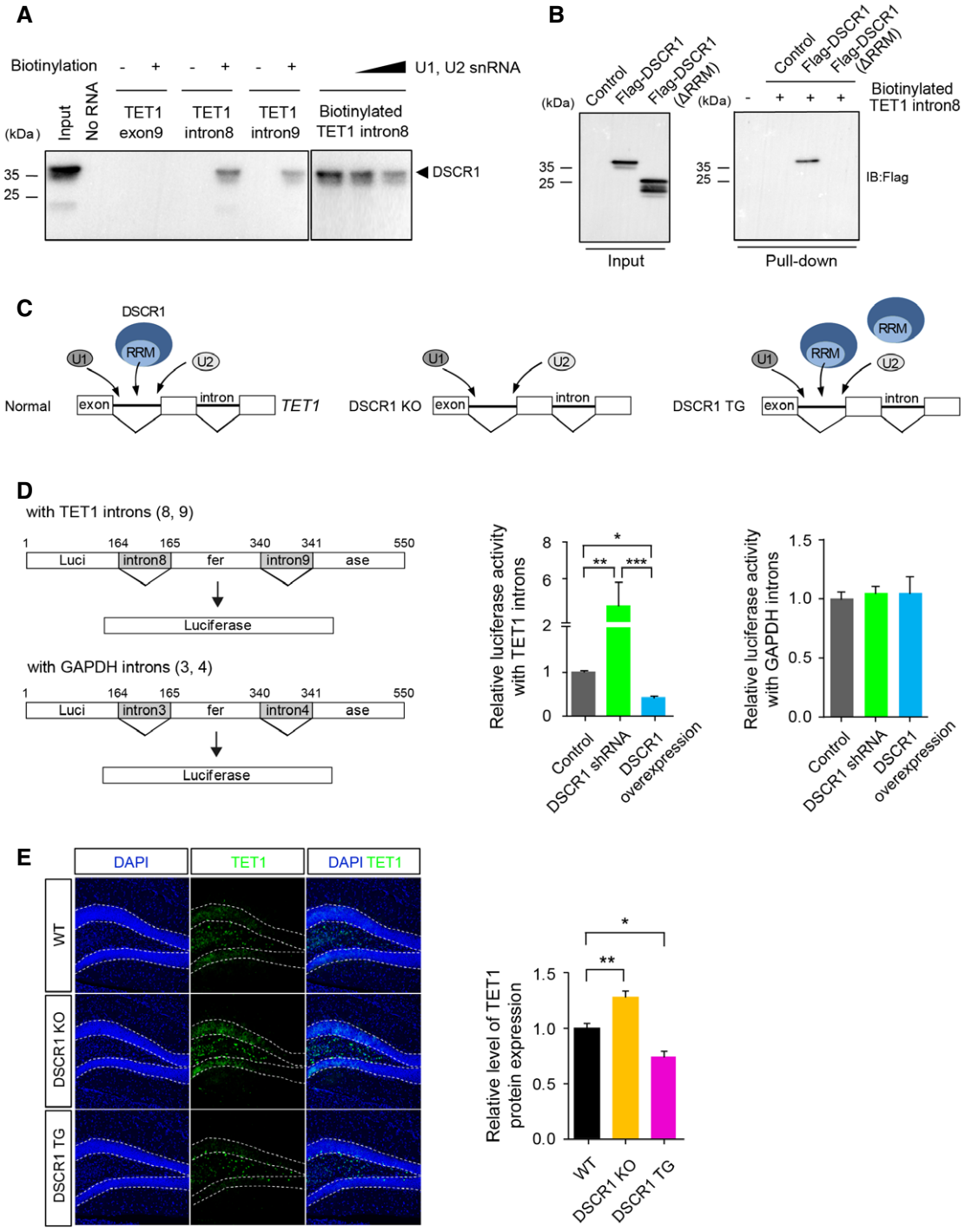

Figure 3. DSCR1 regulates *TET1* splicing.

A DSCR1 binds to *TET1* introns. The binding affinity of DSCR1 to the intron 8 of *TET1* decreased with increased dosage of U1 snRNA and U2 snRNA.

B DSCR1 missing RNA recognition motif (ΔRRM) does not bind to the *TET1* intron 8.

C, D Schematic diagram of the luciferase reporter separated by *TET1* introns 8 and 9 or *GAPDH* introns 3 and 4. DSCR1 reduction increases the activity of this luciferase construct, while DSCR1 overexpression decreases its activity. In contrast, altering DSCR1 levels did not affect luciferase activity of the construct containing *GAPDH* introns. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ for each condition, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

E TET1 protein expression in the dentate gyrus region of hippocampus of *DSCR1* mutants. TET1 is increased in *DSCR1* knockout, while it is decreased in *DSCR1* transgenic mice. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ animal for each condition, $^{*}P < 0.05$, $^{**}P < 0.01$.

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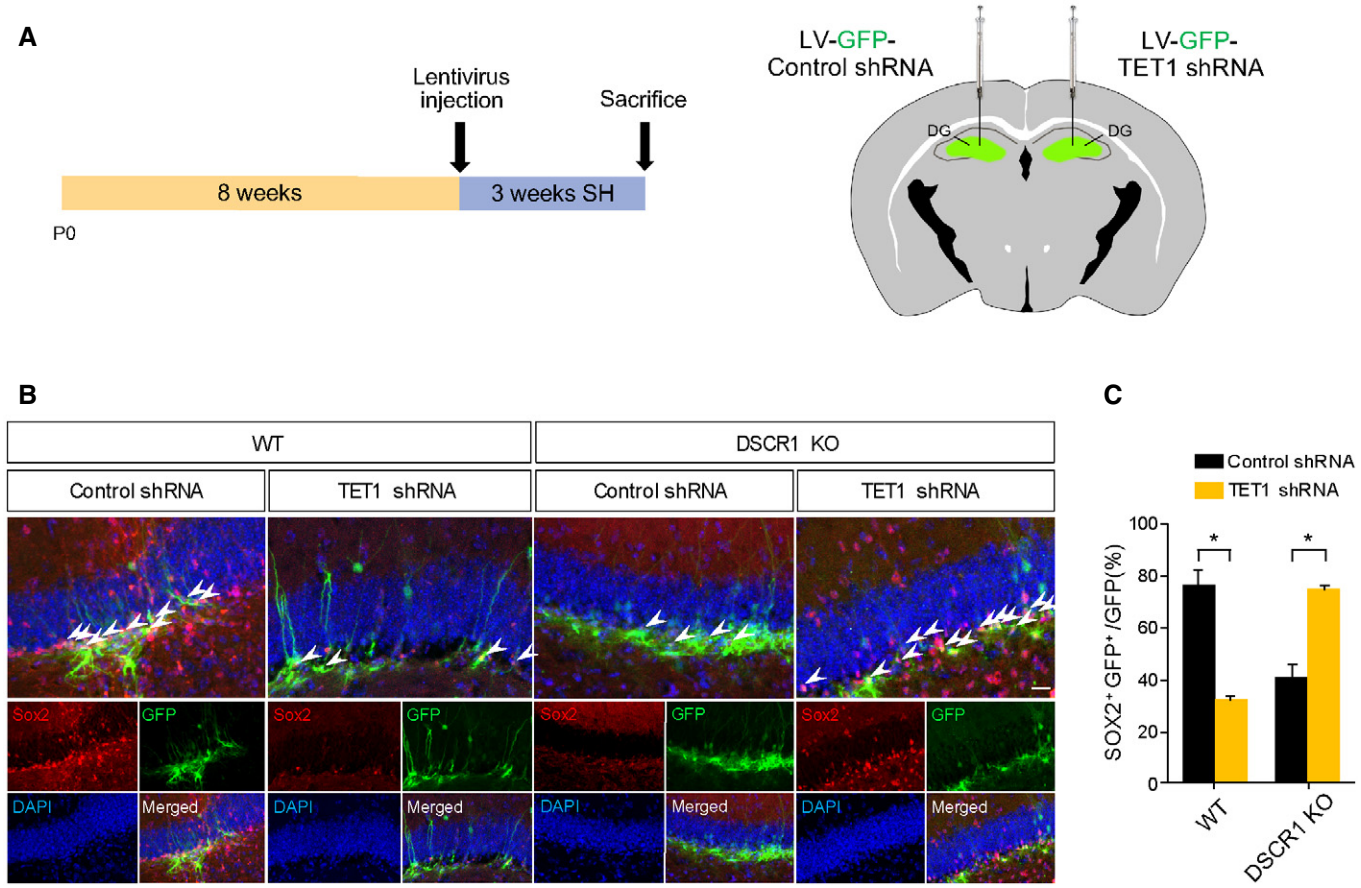

Figure 4. TET1 reduction restores abnormal adult neurogenesis in *DSCR1* knockout mouse.

A Schematic of the experimental timeline (left). Lentivirus containing *TET1* shRNA and *GFP* or random shRNA and *GFP* is injected into the contralateral dentate gyrus of hippocampus. Two-month-old wild-type or *DSCR1* knockout mice were used for analyses.

B Three weeks after lentivirus injection, neural progenitor cells are identified by double staining of GFP and Sox2 in dentate gyrus region. The white arrow heads indicates Sox2 and GFP double-positive cells. Scale bar, 10 μm.

C The number of GFP and Sox2 double-positive cells is restored to that of wild type. Scale bar: 10 μm. Values are shown as mean ± SEM and tested for statistical significance by paired *t*-test. $N = 3$ animal for each condition, $^*P < 0.05$.

TET1 could indeed regulate *miR-124* expression by altering the methylation of *miR-124* promoter, we monitored the methylation status of *miR-124* promoter using bisulfite sequencing (Fig EV5D and E). As anticipated, the methylation levels of *miR-124* were increased in cells containing *TET1* shRNA, confirming that TET1 could down-regulate the transcription of *miR-124*. To verify whether TET1 binds to the promoter region of *miR-124*, we performed ChiP experiment using N2A cells over-expressing *TET1* Flag. The result clearly showed that TET1-Flag bound to the promoter region of *miR-124* (Fig EV5F).

Reduction of TET1 prevents impaired adult neurogenesis in the hippocampus of *DSCR1* knockout mice

Having demonstrated that *DSCR1* knockout mice have impaired adult neurogenesis and elevated *TET1* expression in the hippocampus, we next tested whether restoring *TET1* expression in the hippocampus of these mice would rescue the defect in adult neurogenesis. To this end, we stereotaxically injected a lentivirus encoding *GFP* and the *TET1* shRNA into the dentate gyrus in one hemisphere of *DSCR1* knockout mice, and a lentivirus encoding *GFP* and a random shRNA were injected into the contralateral dentate gyrus (Fig 4A). At 3 weeks post-injection, the dentate gyri were assessed for adult neurogenesis (Fig 4B). The percentage of cells expressing both GFP and Sox2, a marker for neural progenitor cells, among all of the GFP positive cells was determined. The percentage of double-positive GFP and Sox2 cells was significantly higher in the dentate gyrus infected with the lentivirus encoding *GFP* and the *TET1* shRNA compared with the cells on the contralateral side (Fig 4C), suggesting that reducing TET1 levels in *DSCR1* knockout cells could alleviate the impaired adult neurogenesis. Together, these findings further confirmed that DSCR1 could control TET1 expression levels, which is required for adult hippocampal neurogenesis.

Defects in adult neurogenesis, as well as learning and memory present in Ts65Dn mice, are prevented by reducing the *DSCR1* dosage

Prior reports have shown that the Down syndrome mouse model (Ts65Dn) displays defective adult hippocampal neurogenesis (Reeves *et al*, 1995; Holtzman *et al*, 1996). Interestingly, both global

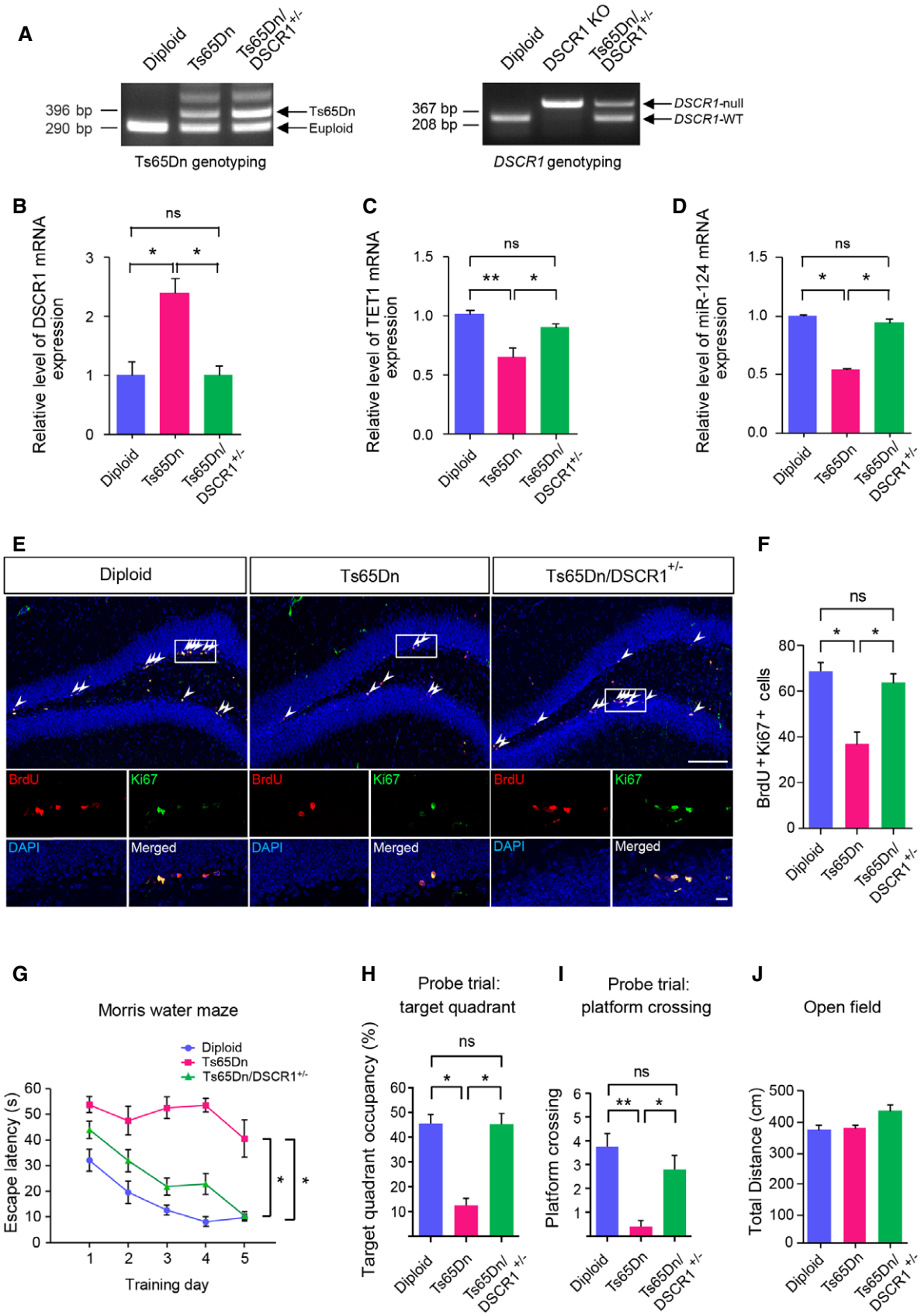

Figure 5.

Figure 5. Correcting *DSCR1* dosage prevents impaired adult neurogenesis and learning and memory found in Ts65Dn mouse.

A Genotype of Ts65Dn/*DSCR1*$^{+/-}$ mouse was confirmed by detecting markers for Ts65Dn and *DSCR1* KO as shown previously (Ryeom *et al*, 2003; Baek *et al*, 2009; Duchon *et al*, 2011).

B Expression of the *DSCR1* mRNA in Ts65Dn mouse was increased about 2.5-fold compared to that of wild type. However, Ts65Dn/*DSCR1*$^{+/-}$ mouse showed normal level of *DSCR1* mRNA transcript. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 3$ for each condition, $^{*}P < 0.05$.

C, D As expected, the levels of *TET1* and *miR-124* are decreased in Ts65Dn mouse; however, Ts65Dn/*DSCR1*$^{+/-}$ mouse restores the *TET1* mRNA and *miR-124* expression. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. *ß-actin* was used for normalization. $N = 3$ (diploid), 3 (Ts65Dn), and 4 (Ts65Dn/DSCR1$^{+/-}$) mice, $^{*}P < 0.05$, $^{**}P < 0.01$.

E, F The number proliferating progenitor neurons identified by BrdU and Ki-67 double staining is restored in the SGZ of Ts65Dn/*DSCR1*$^{+/-}$ mouse compared to that of Ts65Dn mouse. The white box area is magnified in the lower panels: DAPI (blue), Ki67 (green), and BrdU (red). Arrow heads indicate BrdU and Ki-67 double-positive cells in the SGZ. Scale bars: 100 μm in the large image and 10 μm in magnified image. Each hippocampal section was 40 μm in thickness, and total 6 hippocampi were used for analysis. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 6$ (control), 5 (Ts65Dn), and 3 (Ts65Dn/*DSCR1*$^{+/-}$) animals, $^{*}P < 0.05$.

G–J Ts65Dn has learning and memory defects, but Ts65Dn/*DSCR1*$^{+/-}$ mouse clearly rescues learning and memory defects to control levels. Values are shown as mean ± SEM and tested for statistical significance by one-way ANOVA followed by Bonferroni *post hoc* test. $N = 8$ (control), 5 (Ts65Dn), and 10 (Ts65Dn/*DSCR1*$^{+/-}$) animals, $^{*}P < 0.001$ (G, H). $^{*}P < 0.05$ (I), $^{**}P < 0.01$ (I). (J) Open-field analysis shows no defects in movement of tested animals. Values are shown as mean ± SEM, $N = 6$ (control), 5 (Ts65Dn), and 3 (Ts65Dn/*DSCR1*$^{+/-}$) animals.

Source data are available online for this figure.

CpG methylation and TET1 levels are also reduced in patients with DS (Jin *et al*, 2013; Sailani *et al*, 2015). These results, along with our findings, led us to hypothesize that altered DSCR1 levels might be the cause of defective adult neurogenesis and impaired learning and memory manifested in these model mice. To test this hypothesis, we normalized *DSCR1* dosage in Ts65Dn mice by crossing Ts65Dn mice to *DSCR1* knockout mice (Fig 5A). Since we know that DSCR1 acts upstream of the *TET1* and *miRNA-124* pathways, which regulate adult hippocampal neurogenesis, we next investigated whether Ts65Dn/*DSCR1*$^{+/-}$ mice have restored *TET1* and *miRNA-124* levels. As expected, Ts65Dn mice showed low levels of *TET1* and *miRNA-124*; however, Ts65Dn/*DSCR1*$^{+/-}$ mice have normal *TET1* and *miRNA-124* levels (Fig 5B–D). The resulting Ts65Dn/*DSCR1*$^{+/-}$ mice and littermates were examined for adult neurogenesis as well as their learning and memory capacity (Fig 5E–J). As shown in Fig 1, at 1-day post-injection of BrdU, cryo-sectioned hippocampi of these mice were double stained using BrdU and Ki67 to determine the number of neural progenitor cells (Fig 5E and F). The number of progenitor cells was clearly lower in the Ts65Dn mice, while Ts65Dn/*DSCR1*$^{+/-}$ mice demonstrated a restored number of progenitor cells, similar to that of diploid mice (Fig 5E and F). These results reveal that an increased dosage of *DSCR1* can contribute to the impaired adult hippocampal neurogenesis observed in Ts65Dn mice.

We next determined whether this genetic rescue of the *DSCR1* copy number was sufficient to alleviate the defective learning and memory phenotypes observed in Ts65Dn mice. To this end, we performed the Morris water maze (MWM) assay for spatial learning and memory, as shown in Fig EV1. Strikingly, Ts65Dn/*DSCR1*$^{+/-}$ mice showed normal learning and memory, indicating that DSCR1 indeed played a critical role in spatial learning and memory (Fig 5G–J). Together, these results demonstrated that normalizing *DSCR1* dosage in Ts65Dn mice could alleviate impaired adult hippocampal neurogenesis via *TET1* and *miRNA-124* pathways, and this also improved the learning and memory defects present in Ts65Dn mice.

Discussion

DSCR1 is located on chromosome 21 and is upregulated in patients with DS (Fuentes *et al*, 1995). It is reported that DSCR1 causes a delay in neural differentiation in the neocortex (Kurabayashi & Sanada, 2013); however, the role of DSCR1 during adult hippocampal neurogenesis has not been fully explored. In this study, we reported a novel role for DSCR1 in controlling adult hippocampal neurogenesis in mice. We showed that DSCR1 regulated *TET1* expression and adult neurogenesis by modulating *TET1* splicing. Additionally, correcting *TET1* expression in *DSCR1* knockout mice was able to rescue their defects in adult neurogenesis. This study further revealed that TET1 could regulate *miR-124* expression by altering the methylation status of the *miR-124* promoter, providing mechanistic insights into how the interplay between DNA methylation and miRNA pathways regulates adult hippocampal neurogenesis (Fig 6).

We provided several lines of evidence to support the finding that DSCR1 and TET1 act in the same pathway to modulate adult neurogenesis. First, we showed that DSCR1 could directly bind to introns within *TET1* via the RRM domain of DSCR1 (Fig 3A and B). Second, reducing DSCR1 expression increased TET1 expression by promoting splicing of *TET1* (Fig 3C and D). The presence of the U1 and U2 snRNPs impeded DSCR1 binding to the *TET1* introns, revealing that DSCR1 could compete with the factors required for pre-spliceosome formation, thus affecting TET1 expression. Third, we found that TET1 expression was altered in the hippocampus of *DSCR1* mutant mice (Fig 3E). The absence of *DSCR1* increased both *TET1* mRNA transcript and protein, while *DSCR1* overexpression decreased TET1 expression. Lastly, correcting *TET1* levels in *DSCR1* knockout mice was sufficient to rescue the defects in adult hippocampal neurogenesis (Fig 4). Consistent with this interpretation and similar to *DSCR1* mutants, *TET1* knockout mice demonstrated impaired adult neurogenesis, and reduced numbers of neural progenitor cells and their proliferation in the dentate gyrus (Zhang *et al*, 2013).

TET1 catalyzes the oxidation of 5mC to 5-hmC, and this serves as the initial step of active DNA demethylation in mammals (Tahiliani *et al*, 2009). We demonstrated that altering TET1 expression affected the CpG methylation status of the *miR-124* promoter and *miR-124* expression (Figs 2C–E, and EV5D and E). It is well established that miRNAs are involved in the epigenetic regulation of neurogenesis. One of the most abundant miRNAs in the brain is *miR-124*, and the involvement of *miR-124* in adult neurogenesis in the SVZ has been previously reported (Lagos-Quintana *et al*, 2002;

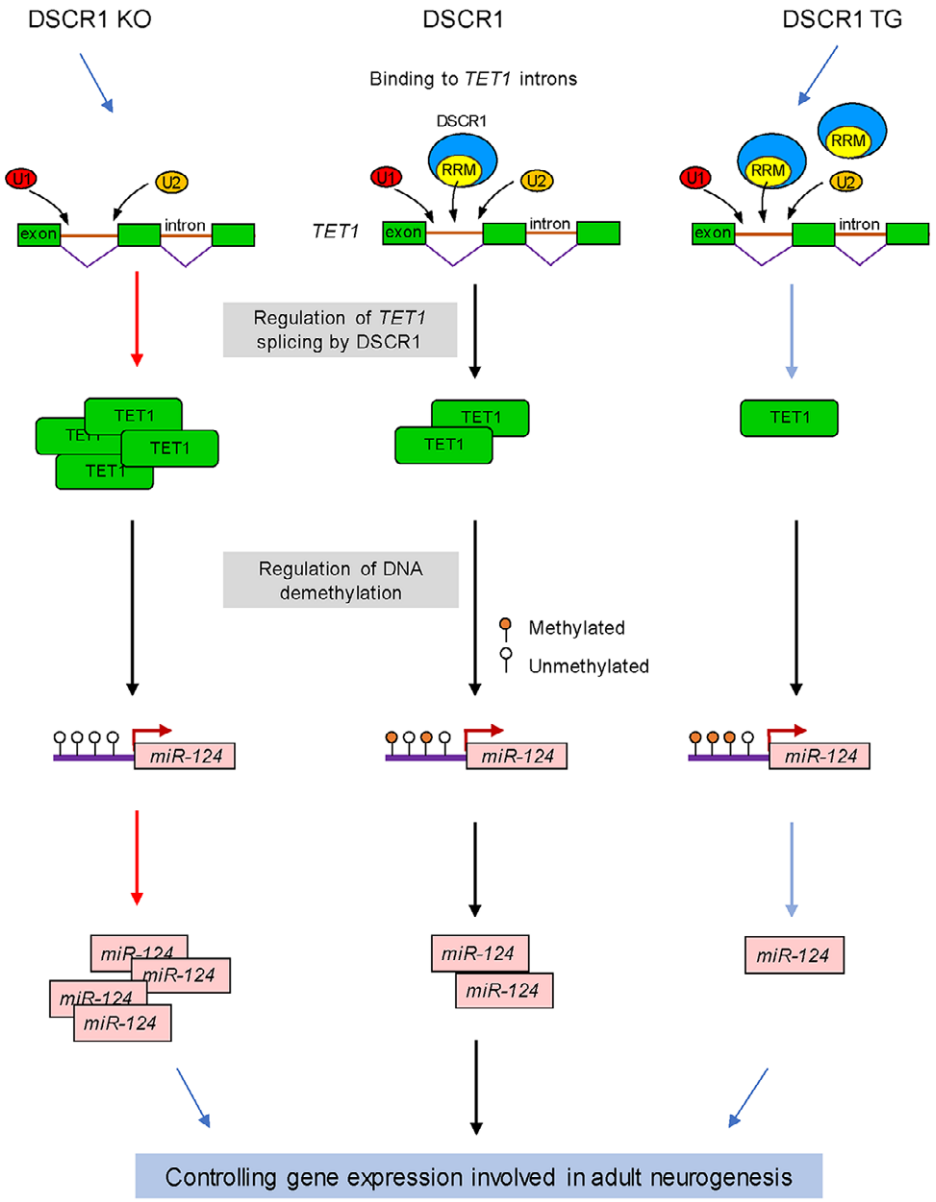

Figure 6. Model for regulation of adult hippocampal neurogenesis by DSCR1.

DSCR1 interferes with binding of U1 and U2 snRNPs to regulate splicing of *TET1* and the level of TET1. TET1 then affects demethylation of *miR-124* promoter, thereby modulating *miR-124* expression. Optimum level of *miR-124* controls expression of genes necessary for adult neurogenesis. In the absence of *DSCR1*, there is increased TET1 expression due to reduced interference in splicing by DSCR1, leading to decreased methylation of the *miR-124* promoter, and altered gene expression affecting adult neurogenesis. Too much DSCR1, on the other hand, competes with the U1 and U2 snRNPs to inhibit normal splicing of *TET1*, leading to reduced TET1 level. This then leads to increased methylation of *miR-124* promoter, reduced *miR-124* expression, and altered gene expression affecting adult neurogenesis.

Cao *et al*, 2007), although its precise role is somewhat unclear. One study showed that reducing or overexpressing *miRNA-124* generated impaired neurogenesis in the SVZ of mice (Cheng *et al*, 2009). Conversely, another study reported that blocking *miR-124* expression maintained neural stem cells in the SVZ and ectopic expression of *miR-124* produced an increased number of neurons (Akerblom *et al*, 2012). Reasons for the discrepancies between these studies are unclear, although it might be due to the different delivery strategies used (e.g., lentiviral or antisense RNA). Our work supports the conclusion that a tightly regulated level of *miR-124* in the hippocampus is essential for normal adult hippocampal neurogenesis, since both knockout and overexpression of *DSCR1* exhibited defective adult hippocampal neurogenesis while displaying increased and decreased *miR-124* expression, respectively. Taken together, our findings suggest that DSCR1 levels and the subsequent regulation of TET1 regulate *miR-124* expression and adult neurogenesis. In the future, it will be important to test whether restoring *miR-124* expression rescues the impaired learning and memory observed in *DSCR1* mutant mice.

DS, which is due to an extra copy of chromosome 21, can present with various clinical features. The most common features include intellectual disability, motor deficits, congenital heart disease,

craniofacial dysmorphology, accelerated aging, and early occurrence of Alzheimer's disease and neuropathologies (Wells *et al*, 1994; Head *et al*, 2012). At the molecular level, global DNA hypermethylation and down-regulation of *TET1* have been reported in DS (Jin *et al*, 2013; Sailani *et al*, 2015), although the underlying cause of these changes is poorly understood. There is also a reduced number of proliferating cells in the dentate gyrus of DS animal models (Contestabile *et al*, 2007) as well as defective learning and memory in Ts65Dn mice. In this study, we demonstrated that DSCR1 could control TET1 expression and adult hippocampal neurogenesis, and we discovered that reducing of the dosage of *DSCR1* in Ts65Dn mice prevented defects in both adult hippocampal neurogenesis and learning and memory. Collectively, these results suggest that perturbation in DSCR1 levels alone is sufficient to impair adult hippocampal neurogenesis and learning and memory in Ts65Dn mice. However, although DSCR1 is known as a regulator of calcineurin, it can also directly regulate multiple pathways in the nervous system (Fuentes *et al*, 2000). For example, the fly homolog of *DSCR1*, *nebula*, can directly interact with the adenine nucleotide translocator (ANT) to modulate mitochondrial function (Chang & Min, 2005). Another report has also shown that DSCR1 can directly interact with the Fragile X mental retardation protein (FMRP) (Wang *et al*, 2012). Although we cannot exclude the possibility that the function of DSCR1 in calcineurin regulation might also play a role in adult neurogenesis, our data suggest that DSCR1 likely regulates adult neurogenesis by direct binding to the introns in *TET1*. This is supported by data indicating that deletion of the RRM domain of DSCR1 abolished its ability to bind to *TET1* mRNA transcript to modulate splicing, and that restoring the level of *TET1* in *DSCR1* knockout mice was also sufficient to rescue defective adult hippocampal neurogenesis. It is however important to note that DSCR1 can regulate NFAT via calcineurin (Arron *et al*, 2006; Lee *et al*, 2010), and *miR-124* inhibits the NFAT pathways (Kang *et al*, 2013). Furthermore, BDNF, another extrinsic factor involved in adult neurogenesis, can activate NFAT pathways (Groth & Mermelstein, 2003). Future work will therefore be necessary to clarify whether DSCR1/TET1 and DSCR1/calcineurin pathways act together or in parallel to regulate adult neurogenesis. On the other hand, the rescue in learning and memory phenotype likely resulted from dual functions of DSCR1 in regulating adult hippocampal neurogenesis and calcineurin signaling, both of which are important for learning and memory (Casas *et al*, 2001).

In summary, we demonstrate that altered levels of DSCR1 impair adult hippocampal neurogenesis. DSCR1 mediates splicing of *TET1* mRNAs by binding to its introns, thereby regulating TET1 expression. We show that TET1 controls DNA methylation levels in the *miR-124* promoter, which subsequently determines *miR-124* expression. Lastly, we find that correcting *DSCR1* copy number in Ts65Dn mice rescues both impaired adult neurogenesis and defective learning and memory. Together, this study highlights the biological pathways that are responsible for establishing adult hippocampal neurogenesis, which is associated with learning and memory. It is also important to note that a large number of studies have attempted to understand the genotype to phenotype correlations in DS; however, it still remains unclear which genes in trisomy 21 are responsible for some of the phenotypes. Excitingly, our results show that correcting the dosage of a single gene, *DSCR1*, is sufficient to prevent both the impaired adult hippocampal neurogenesis and defective learning and memory observed in Ts65Dn mice. Hence, our findings not only provide new insights into mechanisms regulating adult hippocampal neurogenesis, but also have important therapeutic potentials for treating cognitive deficits in DS.

Materials and Methods

Animals

Animals were used in accordance with protocols approved by the Animal Care and Use Committees of the Ulsan National Institute of Science and Technology. *DSCR1*$^{-/-}$ and *DSCR1* transgenic mice were obtained from K. Baek at Sungkyunkwan University. All mice used in this paper are 4-month-old males and have the C57BL/6 strain background. Ts65Dn was obtained from the Jackson Laboratory.

BrdU administration

5-bromo-2-deoxyuridine (BrdU, 150 mg/kg body weight, Sigma-Aldrich) was intraperitoneally injected to a mouse twice a day at intervals of 12 h for 5 days. Mice were sacrificed at 1, 10, and 21 days after the last injection for experiments, and perfusion was performed with phosphate-buffered saline (PBS 0.1 M, pH 7.4) and 4% paraformaldehyde (PFA). Isolated brains were then fixed with 4% PFA at 4°C overnight and post-fixed 2 days with 30% sucrose for cryoprotection. Brain was rapidly embedded and sectioned at 40 μm in thickness using a cryostat (Leica).

Immunohistochemistry

Brain sections were washed three times with PBS for 5 min each and incubated with sodium citrate buffer (10 mM, pH 6) for 30 min at 80°C. The sections were cooled to room temperature (RT) and incubated with blocking buffer (0.1 M PBS, 0.3% Triton X-100, 5% normal horse serum) for 1 h. The sections were incubated in 1 M HCl for 30 min at RT and rinsed three times with PBS for 5 min each. The sections were then incubated with primary antibodies overnight at 4°C. After several washes, Alexa Fluor-conjugated secondary antibodies were applied for 2 h at RT. Images were taken using a Zeiss LSM 780 confocal microscope. We used several antibodies to detect BrdU and specific proteins in the brain sections: BrdU (1:300, Abcam), NeuN (1:300, Millipore), Ki67 (1:500, Abcam), DCX (1:300, Santacruz), TET1 (1:100, Abcam), SOX2 (1:100, Thermo Fisher Scientific), and GFP (1:300, Abcam).

Neurosphere assay

Neurosphere culture was performed as previously described (Guo *et al*, 2012; Walker & Kempermann, 2014). Briefly, adult hippocampi were dissected out, and cells were dissociated with the enzyme mix (Papain 2.5 U/ml, Sigma-Aldrich; Dispase 1 U/ml, Sigma-Aldrich). Isolated cells were cultured in Neurobasal Medium with 2% B27, 1× GlutaMAX, 50 units/ml Penicillin/Streptomycin, 20 ng/ml EGF, and 20 ng/ml FGF-2. After 10–12 days, spheres were dissociated into single cells with treatment of accutase and plated into a 24-well plate to analyze the primary neurosphere formation. Neurospheres were then collected and dissociated into single cells, and plated until the formation of secondary neurospheres. Zen

image analysis program (Zeiss) was used to count the number of neurospheres and measure the diameter of neurospheres. To analyze the differentiation capability of neurospheres, the dissociated neurospheres were cultured in the differentiation medium for 7 days and then stained using cell markers for each type of cells: Tuj1 (1:500, Abcam) for neuron; GFAP (1:300, Sigma-Aldrich) for astrocyte; and Olig2 (1:500, Santacruz) for oligodendrocytes.

EdU labeling and fluorescence-activated cell sorting

5-ethynyl-2′-deoxyuridine (EdU, 50 mg/kg body weight, Sigma-Aldrich) was intraperitoneally injected to a mouse (6–8 weeks old) twice a day at intervals of 12 h for 3 days. Mice were sacrificed at 1, 10, and 21 days after the last injection for experiments. Brains were isolated, and 5 hippocampi were dissected out. Dentate gyrus from the hippocampus was then isolated and minced using a scalpel blade for approximately 1 min until no large pieces remained. Minced tissues were transferred to a pre-warmed enzyme mix (Papain 2.5 U/ml and Dispase 1 U/ml) and incubated for 15 min at 37°C. Tissues were then dissociated mechanically using pipette and further incubated for 10 min at 37°C. Next, 8 ml of neurosphere culture medium (2% B27, 1× GlutaMAX, 50 units/ml Penicillin/Streptomycin, 20 ng/ml EGF, and 20 ng/ml FGF-2) was added to dilute the enzyme mix, and the prep was centrifuged at 130 × *g* for 5 min. Supernatant was removed, and cells were then washed twice in 10 ml of neurosphere culture medium. The tissue suspension was then filtered through a cell strainer (Corning) to acquire single-cell suspensions to perform fluorescence-activated cell sorting (FACS). Isolated cells were fixed and permeabilized with 4% PFA, 0.1% saponin in DPBS together with RNasin plus RNAse inhibitor (1:100, Promega) on the ice for 30 min. For Edu staining, Click-iT Edu Flow Cytometry Assay Kits with Alexa Fluor 488 picolyl azide (Thermo Fisher Scientific) was used according to the manufacturer's protocol and followed by primary antibody staining: SOX2 (1:100, Thermo Fisher Scientific), DCX (1:100, Santacruz), NeuN (1:100, Millipore) with staining buffer (0.5% Tween-20, 1% BSA in DPBS) at RT for 30 min. For secondary antibody staining, Alexa Fluor 647-conjugated Goat anti-Mouse IgG (H+L; 1:300, Thermo Fisher Scientific) was used. Cells were sorted with FACSAria Fusion (BD Biosciences). We put an effort to stabilize the RNA by adding the RNasin Plus RNase inhibitor into all buffer (fixation, washing, staining, and sorting) used in FACs. The composition of the buffer is as follows: fixation buffer: 4% PFA, 0.1% saponin, 1:100 RNasin Plus RNase inhibitor in PBS; washing buffer: 0.2% BSA, 0.1% saponin, 1:100 RNasin Plus RNase inhibitor in PBS; staining buffer: 1% BSA, 0.1% saponin, 1:25 RNasin Plus RNase inhibitor in PBS; and sorting buffer: 0.5% BSA, 1:25 RNasin Plus RNase inhibitor in PBS.

Immunoblotting

Tissues and cells were lysed in RIPA buffer with protease inhibitors (Roche). Lysates were denatured with the SDS sample buffer and separated in the SDS polyacrylamide gels, which were then transferred to the PVDF membrane (GE Healthcare). To detect proteins, several primary antibodies were used: TET1 (1:1,000, Genetex), GAPDH (1:1,000, Santa Cruz), DSCR1 (1:1,000, Santa Cruz), and FLAG (1:1,000, Santa Cruz). HRP-conjugated goat against mouse or rabbit secondary antibodies (1:5,000, Promega) was used.

RNA isolation and qPCR

Total RNAs were extracted from hippocampus or FACS-sorted neurons using Trizol, and cDNAs were prepared by High-Capacity RNA-to-cDNA Kit (Life Technologies), which was used for real-time quantitative PCR. PowerUp SYBR Green Master Mix (Life Technologies) was used according to the manufacturer's instructions for LightCycler 480 II (Roche). Data analysis was performed by the comparative Ct method (Rao *et al*, 2013). The relative expression of genes was normalized using GapdH by $2^{-\Delta C_t}$.

Gene-specific primers used for qPCR are as follows:

DSCR1 forward	TCATCGACTGCGAGATGGAG
DSCR1 reverse	TGGTGTCCTTGTCATATGTTCTG
TET1 pre-mRNA forward	CGCCATCACACCATGCAAA
TET1 pre-mRNA reverse	GCCATCTGCTGCCCTCTTCT
TET1 mature mRNA forward	GAAGGAAGGGAAGAGCTCTCAGG
TET1 mature mRNA reverse	AGCCGTCGAACAGTGATGGT
pri-miR-124 forward	CATCCTCCCTCTCTTCCATC
pri-miR-124 reverse	TTAAATAAGGTCCGCTGTG
pre-miR-124 forward	AGGCCTCTCTCTCCGTGTTCA
pre-miR-124 reverse	CAGCCCCATTCTTGGCATTC
mature miR-124 forward	GCGAATGCATTAAGGCACGCGG
mature miR-124 reverse	GATAAGCTCGCGAGGGTCCGAGGTATTC
GapdH forward	GCCATCAACGACCCTTCATT
GapdH reverse	GCTCCTGGAAGATGGTGATGG
U6RNA forward	CGCTTCGGCAGCACATATAC
U6RNA reverse	AAAATATGGAACGCTTCACGA

Morris water maze test

Hidden platform and probe trial were performed as previously described (Nunez, 2008; Ma *et al*, 2013). Briefly, for hidden platform test, four trials with different starting directions were measured. Each trial lasted for 1-min and had 2-min intervals between the trials. Mouse was tested every day for 5 days. For probe trial, mice that completed training for 5 days were tested for 30 s. Observation of animals was recorded and analyzed by SMART system (HARVARD apparatus).

Open-field test

Each mouse was located in the center of an acryl box (40 × 40 × 40 cm), and its behavior was recorded for 15 min using a video tracking system. The total distance travelled was analyzed by SMART system (HARVARD apparatus).

Virus preparation and stereotaxic injection

TET1 shRNA or control shRNA was cloned into PLL3.7 lentiviral vector containing CMV-EGFP. The lentiviral vector and a packaging

vector were co-transfected into HEK293T cells. After 48 h, supernatants containing the virus were filtered through 0.22 μm filter and then concentrated by ultracentrifuge at 80,000 *g* for 1.5 h at 4°C. Eight-week-old mice were anesthetized by isoflurane, and the virus was stereotaxically injected into the hippocampus dentate gyrus region using stereotaxic injection apparatus. Mice were then sacrificed at 21 days after the virus injection.

Luciferase assay

The promoter of *miR-124* or *TET1* was cloned in front of *firefly luciferase* to measure its promoter strength. This construct and pRL-TK plasmid (*renilla luciferase* control reporter vector, Promega) were co-transfected into N2A cells. Luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega). Activity of firefly luciferase was normalized to that of renilla luciferase.

Biotinylation and biotin–streptavidin pull down assay

In vitro transcription with T7 polymerase (Roche) in the presence of biotinylated UTP was used to synthesize biotinylated *TET1* intron 8, intron 9, exon 9, U1 snRNA, or U2 snRNA. To analyze whether DSCR1 binds to *TET1* intron or exon, these RNAs with or without biotinylation were incubated with Neuro2A lysates in protein lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, and 0.5% sodium deoxycholate). After 30 min incubation at RT, pre-cleared streptavidin agarose beads (Thermo Fisher Scientific) were added and further incubated at 4°C overnight on a rotary shaker. After washing with lysis buffer three times, resin bound proteins were eluted and then analyzed for DSCR1 binding using immunoblotting.

Chip assay

Chip assay was performed as previously described (Nelson *et al*, 2006). Briefly, N2A cells overexpressing *TET1*-Flag were crosslinked by formaldehyde and collected. Cells were sheared by sonication, and then, shared chromatin was immunoprecipitated with 5 μg of control IgG (Santa Cruz) or anti-Flag (Santa Cruz) antibodies. 10% Chelex 100 resin (Sigma) was used to extract DNA; then, qPCR was performed using the primers to detect the promoter region of miR-124 (forward primer: 5F-ACC CAC TTC TCC CAG GAT CT and reverse primer: 3R-GAG GGT TGT GCC AAG AAA AA).

RNA stability measurement

To analyze the stability of *TET1* mRNA transcripts, Neuro2A cells containing *DSCR1* reduction or overexpression were treated with actinomycin D (Sigma, 5 μg/ml), a transcription inhibitor, and decay of the *TET1* mRNA transcripts was traced for the next 15 h using qRT–PCR.

Bisulfite sequencing

Hippocampi of wild-type and *DSCR1* mutants were used for genomic DNA extraction and bisulfite modification, which were performed according to instructions in the EZ DNA Methylation-Direct Kit (Zymo Research). The bisulfite PCR primers used for *miR-124* promoter region are listed below. Bisulfite-modified DNA was then amplified by PCR using the Epitaq HS DNA polymerase (TaKaRa), and the purified PCR product was cloned into pMD20-T for sequencing. Six clones from each condition were randomly chosen and amplified by colony PCR using EmeraldAmp GT PCR Master Mix (TaKaRa) for further analysis. Finally, amplified PCR products were sequenced using primers: M13-forward (GTTTTCCCAGTCACGAC) and M13-reverse (CAGGAAACAGCTATGAC). Bisulfite sequencing data were quantified by the percentage of methylated CpGs to the total number of CpGs. The promoter region of *miR-124* was divided into 4 for sequencing.

bisulfite miR-124 forward (1)	GCAAGCTTTTTAAGTTATTAAAGAAAAGTAGG
bisulfite miR-124 reverse (1)	TAGGATCCAATCAAATAAAATAAAAAAAA
bisulfite miR-124 forward (2)	GCAAGCTTTATGGTTTTTATTTTTTATTTTT
bisulfite miR-124 reverse (2)	TAGGATCCTACCAAAATCCTCTAAATAAACTC
bisulfite miR-124 forward (3)	GCAAGCTTATTGAGAAAAGAGGATTGGAGTTA
bisulfite miR-124 reverse (3)	TAGGATCCAAAAACCACATCTACTAACAATTCC
bisulfite miR-124 forward (4)	GCAAGCTTGGATTTATTTTTAATTTTTTGTTTT
bisulfite miR-124 reverse (4)	TAGGATCCATACAACTTAAAAATCCAACCCTAC

Statistical analysis

Statistical significance was measured by Student's *t*-test, one-way ANOVA followed by Bonferroni *post hoc* test using GraphPad Prism 6.0 software (GraphPad Software).

Expanded View for this article is available online.

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Author contributions
CC designed and performed most experiments; TK conducted initial splicing experiments; K-TM and KTC conceived this project; and K-TM supervised this study and wrote the manuscript with contribution from CC and KTC.

Conflict of interest
The authors declare that they have no conflict of interest.

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