SIRT1 suppresses self-renewal of adult hippocampal neural stem cells
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ABSTRACT
The balance between self-renewal and differentiation of adult neural stem cells (aNSCs) is essential for the maintenance of the aNSC reservoir and the continuous supply of new neurons, but how this balance is fine-tuned in the adult brain is not fully understood. Here, we investigate the role of SIRT1, an important metabolic sensor and epigenetic repressor, in regulating adult hippocampal neurogenesis in mice. We found that there was an increase in SIRT1 expression during aNSC differentiation. In Sirt1 knockout (KO) mice, as well as in brain-specific and inducible stem cell-specific conditional KO mice, the proliferation and self-renewal rates of aNSCs in vivo were elevated. Proliferation and self-renewal rates of aNSCs and adult neural progenitor cells (aNPCs) were also elevated in neurospheres derived from Sirt1 KO mice and were suppressed by the SIRT1 agonist resveratrol in neurospheres from wild-type mice. In cultured neurospheres, 2-deoxy-D-glucose-induced metabolic stress suppressed aNSC/aNPC proliferation, and this effect was mediated in part by elevating SIRT1 activity. Microarray and biochemical analysis of neurospheres suggested an inhibitory effect of SIRT1 on Notch signaling in aNSCs/aNPCs. Inhibition of Notch signaling by a γ-secretase inhibitor also largely abolished the increased aNSC/aNPC proliferation caused by Sirt1 deletion. Together, these findings indicate that SIRT1 is an important regulator of aNSC/aNPC self-renewal and a potential mediator of the effect of metabolic changes.

KEY WORDS: Adult neurogenesis, SIRT1, Self-renewal

INTRODUCTION
In the dentate gyrus (DG) of the hippocampus, adult neural stem cells (aNSCs) can undergo either proliferative self-renewal to maintain the aNSC reservoir or differentiate after several rounds of proliferation to supply new neurons (Bonaguidi et al., 2011). A proper balance between self-renewal and differentiation is essential for maintaining stable neurogenesis in the adult DG. The self-renewal of NSCs is maintained intrinsically by stem cell-specific ‘stemness’ genes (Ahmed et al., 2009), including those controlled by Notch signaling (Ables et al., 2011, 2010; Louvi and Artavanis-Tsakonas, 2006). The differentiation of NSCs results from the gradual inactivation of ‘stemness’ genes and the activation of pro-neuronal genes, a process under epigenetic regulation (Ma et al., 2010). However, the epigenetic factors involved in balancing aNSC self-renewal and differentiation remain to be fully clarified.

Adult hippocampal neurogenesis is tightly regulated by extrinsic physiological and pathological stimuli, such as exercise, dietary restriction and hypoxia (Ming and Song, 2005; Zhao et al., 2008). These stimuli cause metabolic stress that might influence aNSC self-renewal or differentiation, or both (Rafalski and Brunet, 2011), although the molecular mechanism linking metabolic changes and aNSC regulation remains unclear. The well-known metabolic sensor SIRT1 has recently been shown to affect aNSC differentiation in vitro and to reduce the number of newly differentiated neurons in vivo (Saharan et al., 2013). However, whether SIRT1 also regulates aNSC self-renewal in vivo and whether the reduced number of newborn neurons is due to reduced self-renewal or reduced neuronal differentiation remains to be clarified. In this study, we found both in vitro and in vivo evidence that SIRT1 directly influences aNSC self-renewal, in addition to its role in regulating aNSC differentiation (Hisahara et al., 2008; Prozorovski et al., 2008; Saharan et al., 2013).

To elucidate the role of metabolism-sensitive molecules in adult neurogenesis, we focused on SIRT1, which is a nicotinamide adenine dinucleotide (NAD)+-dependent histone deacetylase (Finkel et al., 2009; Haigis and Guarente, 2006; Haigis and Sinclair, 2010). The requirement of NAD+ for deacetylation makes SIRT1 an ideal mediator of the cross-talk between metabolism and epigenetic regulation. Deacetylation of histones and non-histone substrates by SIRT1 regulates metabolism, genome stability, cell survival and inflammation (Feige and Auwerx, 2008; Gan and Mucke, 2008; Oberdoerffer et al., 2008; Vaziri et al., 2001). Previous studies have implicated SIRT1 in the neuron/astrocyte fate choice during differentiation of embryonic and adult NSCs (Hisahara et al., 2008; Prozorovski et al., 2008; Saharan et al., 2013). However, before aNSC fate choice, it is unclear whether SIRT1 also regulates aNSC self-renewal as a sensor of metabolic stress. Using genetic ablation and pharmacological manipulation of SIRT1 both in vivo and in neurosphere cultures, we discovered a cell-autonomous action of SIRT1 in suppressing aNSC/aNPC self-renewal, in part through the inhibition of Notch signaling. We also provide in vitro evidence that SIRT1 can serve as a metabolic sensor for regulating aNSC/aNPC self-renewal.

RESULTS
SIRT1 expression increases during aNSC differentiation
To determine whether SIRT1 is expressed in the adult DG, we used SIRT1-specific antibodies to stain brain sections of adult wild-type (WT) and SIRT1 germine KO mice. We found that Sirt1 was highly expressed in the nuclei of hippocampal granule cells in WT mice, whereas no SIRT1 signal was detected in SIRT1 KO mice (Fig. 1A), confirming the specificity of anti-SIRT1 antibodies. In cultured
neurospheres derived from adult hippocampi, co-immunostaining for the SIRT1 and NSC/NPC markers nestin and Sox2 showed that SIRT1 was expressed in nestin+/Sox2+ cells (Fig. 1B). By identifying the developmental stages of aNSC differentiation (modified from Ming and Song, 2011), we found that SIRT1 was expressed in the nuclei of Sox2+ aNSCs/aNPCs in the subgranular zone (SGZ), but at a much lower level than that found in neighboring (Rbfox3– Mouse Genome Informatics) neurons in the granule cell layer (Fig. 1D,E). This low SIRT1 level in aNSCs was further confirmed by the low SIRT1 signal in the nuclei of glial fibrillary acidic protein (Gfap)+/Sox2+ radial glia-like (RGL) cells in WT mice and in GFP-labeled aNSCs in Gfap-Gfp transgenic mice (supplementary material Fig. S1B,C). A relatively low level of SIRT1 was also observed in GFAP+ astrocytes in the hilus (supplementary material Fig. S1D). Thus, SIRT1 expression is low in aNSCs and high in mature neurons.

To further examine SIRT1 expression pattern in the heterogeneous population of aNSCs/aNPCs and immature neurons in DG, we used the established markers mini-chromosome maintenance protein (Mcm2) and doublecortin (Dcx) to identify amplifying aNPCs and immature neurons (Fig. 1F,H,I). This low SIRT1 level in aNSCs was further confirmed by the low SIRT1 signal in the nuclei of glial fibrillary acidic protein (Gfap)+/Sox2+ radial glia-like (RGL) cells in WT mice and in GFP-labeled aNSCs in Gfap-Gfp transgenic mice (supplementary material Fig. S1B,C). A relatively low level of SIRT1 was also observed in GFAP+ astrocytes in the hilus (supplementary material Fig. S1D). Thus, SIRT1 expression is low in aNSCs and high in mature neurons.

Fig. 1. SIRT1 expression was low in aNSCs but high in aNPCs and immature neurons. (A) Immunostaining of SIRT1 in brain sections from adult WT and KO mice. (B) Immunostaining of neurospheres derived from WT and KO mice for SIRT1, nestin and Sox2. (C) Schematic diagram of markers for cells at different developmental stages of aNSC differentiation (modified from Ming and Song, 2011). (D) Immunostaining of SIRT1, NeuN and Sox2 in the DG of adult WT and Sirt1 KO brain sections. Arrows, Sox2+ cells with low SIRT1 level; arrowheads, Sox2+ cells with relatively high SIRT1 level. (F) Immunostaining of SIRT1, Mcm2 and Sox2. Bold arrows, Sox2+/Mcm2+ aNSCs; arrowheads, Sox2+/Mcm2+-amplifying aNPC; thin arrows, more committed Sox2−/Mcm2− amplifying aNPCs. (H) Immunostaining of SIRT1, Mcm2 and Dcx. Bold arrows, Mcm2+/Dcx− aNPCs; arrowheads, Mcm2+/Dcx− committed aNPC; thin arrows, Mcm2+/Dcx− immature neurons. (E,G,I) Quantitation of SIRT1 fluorescent intensity in different cell types (E, n=64-112, ***P<0.001; G, n=25-74, ***P<0.001, **P=0.002; I, n=66-86, ***P<0.001, *P=0.039; all Student’s t-test). Dotted circles outline the nuclei. Scale bars: 50 µm (A), 25 µm (B,D,F,H).
immature neurons, respectively (Ming and Song, 2011) (Fig. 1C). Immunostaining intensity measurements of SIRT1 showed that SIRT1 was higher in MCM2+ amplifying aNPCs in the SGZ than in Sox2+/MCM2− aNSCs (Fig. 1F,G). Moreover, a much stronger SIRT1 signal was observed in MCM2+/DCX+ immature neurons than in MCM2+ amplifying aNPCs (Fig. 1H,I) (see more examples in supplementary material Fig. S1E-G). Thus, SIRT1 was expressed at a low level in aNSCs, but at a higher level in amplifying aNPCs and in immature neurons.

The expression pattern of SIRT1 during aNSC differentiation suggests that SIRT1 is involved in either promoting differentiation or suppressing self-renewal of aNSCs/aNPCs. As shown below, our evidence indicates that the increase of SIRT1 level suppresses aNSC self-renewal.

**Sirt1 deletion increases adult hippocampal neurogenesis in vivo**

Because SIRT1 was endogenously expressed in aNSCs/aNPCs, we further explored whether SIRT1 plays a role in adult hippocampal neurogenesis in vivo. Daily 5-Bromo-2′-deoxyuridine (BrdU) injection was given for 7 days to adult (7-9-week old) WT and KO mice, and the mice were sacrificed 1 day after the last injection. We found that the number of BrdU+ cells in the DG of KO mice was significantly higher than in WT mice, suggesting increased neurogenesis in KO mice (Fig. 2A,B). Next, we crossed Sirt1floxflox mice with the nestin-Cre transgenic line to specifically delete Sirt1 in the nervous system. These conditional knockout (CKO) mice expressed a non-functional mutant SIRT1 protein owing to the deletion of exon 4 of the Sirt1 coding region (supplementary material Fig. S2A) (Li et al., 2007). We found that these CKO mice had apparently normal brain anatomy, although the brain size was smaller than that of control (CTR) mice (supplementary material Fig. S2B). The same BrdU labeling method applied to these CKO mice revealed a markedly higher number of BrdU+ cells in the CKO mice than in control mice (Fig. 2C,D). Moreover, the number of cells stained positive for Ki67 (Mki67 – Mouse Genome Informatics), a marker for proliferating cells, was also significantly higher in KO mice (Fig. 2E,F) and CKO mice (Fig. 2G,H), indicating increased proliferation of aNSCs/aNPCs. These results support the notion that SIRT1-deletion elevates hippocampal neurogenesis via increasing the proliferation of aNSCs/aNPCs.

**Sirt1 deletion elevates self-renewal of aNSCs/aNPCs in vivo**

We further examined whether SIRT1 suppresses aNSC proliferation by determining the identity of BrdU-labeled cells. Cells labeled with BrdU after 7 days of BrdU administration (Fig. 3A, upper panel) should contain both proliferating aNSCs/aNPCs and differentiated cells that had undergone cell cycle exit. The increased number of BrdU+ cells could result from the increase of either proliferating or differentiated cells. As T-box brain gene 2 (Tbr2; Eomes – Mouse Genome Informatics) is expressed in aNPCs (Hodge et al., 2008), we used triple-immunostaining with Sox2, Tbr2 and BrdU to distinguish aNSCs (BrdU+/Sox2+/Tbr2−), aNPCs (BrdU+/Tbr2+ and differentiated cells (BrdU+/Sox2−/Tbr2+) (Fig. 3B, see also the diagram in Fig. 1C). We found that the increased number of BrdU+ cells in Sirt1 KO mice was due to an increased aNSC/aNPC population, whereas the numbers of differentiated cells were comparable between WT and KO mice (Fig. 3C). Correspondingly, among all BrdU+ cells the average percentage of aNSCs/aNPCs was higher and that of differentiated cells was lower in KO mice compared with WT mice (Fig. 3D). In order to further examine the effect of SIRT1 on the transition from aNSCs to aNPCs, we injected WT and KO mice with four pulses of BrdU at 2-h intervals and perfused the animals 2 h after the last injection (Fig. 3A, lower panel). We found that among all BrdU+ cells the average percentage of aNSCs was higher and that of aNPCs was lower in KO mice compared with WT mice (Fig. 3E), again indicating an elevated aNSC self-renewal rate in Sirt1 KO mice.

To further confirm these results, we chose two other markers, GFAP and DCX, to distinguish aNSCs (BrdU+/GFAP−/DCX− cells with radial processes), aNPCs (BrdU+/GFAP+/DCX−), differentiated...
neurons (BrdU+/GFAP−/DCX+) and astrocytes (BrdU+/GFAP+/DCX− cells with astrocyte morphology) among cells labeled after 7 days of BrdU administration (Fig. 3F). We found an increase in both the absolute number and the percentage of aNSCs/aNPCs among BrdU+ cells in KO mice compared with WT mice (Fig. 3G,H).

Moreover, we found very few BrdU+ astrocytes, and no significant differences in the absolute number and percentage of these cells between WT and KO mice (Fig. 3G,H), indicating that the increase of BrdU+ cells was not due to BrdU labeling of proliferating astrocytes. Taken together, these results show that SIRT1 deletion causes elevated self-renewal of aNSCs/aNPCs in vivo, implicating that endogenous SIRT1 suppresses aNSC/aNPC self-renewal in WT mice.

Most newborn neurons in the DG die within several days after birth (Sierra et al., 2010). To exclude the possibility that the increased BrdU-labeled cells were due to increased cell survival, we examined the number of labeled cells that survived for one month after the last BrdU injection (Fig. 4A), and found, instead of increased survival, a lower number of BrdU+ neurons in Sirt1 KO mice compared with WT mice (Fig. 4B,C). This is consistent with the reported role of SIRT1 in cell survival (Vaziri et al., 2001). Meanwhile, we also found a reduced number of aNSCs/aNPCs (BrdU+/Sox2+) one month after BrdU labeling in KO mice compared with WT mice (Fig. 4D), suggesting increased cell death or increased dilution of BrdU in the absence of SIRT1. Thus, the increased number of BrdU− cells in Sirt1 KO mice was not due to increased cell survival.

To determine the effect of an enhanced aNSC self-renewal on neuronal production, we further examined the absolute number of aNPCs and immature neurons by quantifying Tbr2+ and DCX+ cells. We found a higher number of Type 2a (Sox2+/Tbr2+), Type 2b
Loss of SIRT1 increases self-renewal of cultured aNSCs/aNPCs

We further tested SIRT1 function in aNSCs/aNPCs, using an independent approach of in vitro neurosphere assay (Reynolds and Weiss, 1992). Adult hippocampi were dissociated and aNSCs/aNPCs were isolated by gradient centrifugation and seeded at a low density (Fig. 6A). This allows for a more accurate assay for examining the self-renewal capacity of stem cells (Pastrana et al., 2011). We found that the number of primary neurospheres derived from hippocampi of adult Sirt1 KO mice was much higher than that of WT mice (Fig. 6B,C), indicating the existence of more aNSCs/aNPCs in the SGZ of KO mice. The diameters of secondary neurospheres were also significantly increased in WT mice in a dosage-dependent manner (Fig. 6D,E), although a marginal effect of resveratrol on other signaling pathways (Poulsen et al., 2013). Together, these in vitro results support the notion that endogenous SIRT1 suppresses aNSC/aNPC self-renewal in a cell-autonomous manner.

SIRT1 mediates the effect of metabolic stress caused by 2-DG

Because of the important role of SIRT1 in sensing metabolic stress in various tissues, we next investigated the involvement of SIRT1 in mediating the effect of metabolic stress induced by glucose restriction in cultured aNSCs/aNPCs. Substitution of glucose by 2-deoxy-D-glucose (2-DG), a non-metabolizable glucose analog, is widely used for inducing metabolic stress (Duan and Mattson, 1999; Stafstrom et al., 2008). We found that the expression of SIRT1 protein was only slightly increased when we substituted 2.5 mM glucose with 2.5 mM of 2-DG in the medium for 24 h (Fig. 7A,B). To evaluate the long-term effect of elevated self-renewal after Sirt1 deletion, the animals were sacrificed at 1 month after a 3-day tamoxifen injection (Fig. 5A, lower panel). Immunostaining with GFAP and DCX identifies RGL aNSCs (GFAP+/DCX+/YFP+ cells with radial processes), IPCs (GFAP+/DCX+/YFP+ cells with no process), newborn neurons (GFAP+/DCX+/YFP+ cells) and astrocytes (GFAP+/DCX+/YFP+ cells with astrocyte morphology) (Fig. 5E). Among YFP+ cells, we found a higher percentage of RGL aNSCs and a lower percentage of newborn neurons in the ER-CKO mice compared with control mice (Fig. 5F), consistent with an expansion of aNSCs due to increased self-renewal after Sirt1 deletion. Furthermore, no difference in the percentage of aNSC-derived astrocytes was observed (Fig. 5F), indicating that loss of SIRT1 did not affect lineage specification of aNSCs in vivo. This result differs from the reported role of SIRT1 in lineage specification of NSCs in vitro (Hisahara et al., 2008; Prozorovski et al., 2008; Saharan et al., 2013). We attribute this discrepancy to the fact that conditions for in vivo aNSC differentiation, in which the majority of aNPCs appears to differentiate into granule neurons (Bonaguidi et al., 2011; Ming and Song, 2005), are different from those used for in vitro studies. Taken together, these results support the notion that endogenous SIRT1 suppresses aNSC/aNPC self-renewal in a cell-autonomous manner.
Sinclair, 2010; Lin et al., 2004). We found that the NAD+/NADH ratio was indeed significantly increased under different dosages of 2-DG, which was largely due to the reduced NADH level (Fig. 7C-E). Thus, a mild metabolic stress induced by 2-DG could activate SIRT1 activity in aNSCs/aNPCs by the increased NAD+/NADH ratio.

Next, we evaluated whether SIRT1 mediates the effect of 2-DG on aNSC/aNPC proliferation. At lower concentrations (0.5 and 1 mM), we found that 2-DG significantly decreased the diameters of WT neurospheres but not those of Sirt1 KO neurospheres (Fig. 7F,G), consistent with a mediator role of SIRT1 in the action of metabolic stress on aNSC/aNPC proliferation. Severe metabolic stress induced by high concentration of 2-DG (2.5 mM for 7 days) also decreased the average diameter of neurospheres from WT mice. However, this was probably caused by SIRT1-independent effects due to severe energy deprivation resulting from high-level 2-DG accumulation in the cell, as a similar reduction of diameters was also observed for neurospheres from Sirt1 KO mice, although the reduction was smaller than that found in WT mice (Fig. 7F,G). Thus, activation of SIRT1 indeed could mediate the effect of mild metabolic stress induced by 2-DG on aNSC/aNPC proliferation in vitro.

**Involvement of Notch signaling in SIRT1 regulation of aNSC/aNPC self-renewal**

We next investigated the molecular mechanism underlying the phenotypes expressed by SIRT1 null aNSCs/aNPCs, as described above. We performed a genome-wide microarray analysis and compared the mRNAs in aNSCs/aNPCs of KO and WT secondary...
neurospheres, as well as mRNAs of WT neurospheres with and without the treatment with the SIRT1 activator resveratrol. We first determined genes the expression levels of which were altered as a result of Sirt1 deletion or resveratrol treatment (>1.5-fold). Genes that showed opposite changes resulting from Sirt1 deletion and resveratrol treatment were then identified as potential genes regulated by SIRT1 (Fig. 8A). A gene ontology (GO) analysis showed that many of these putative SIRT1-regulated genes, such as hexokinase 3 (HK3), malate dehydrogenase 1 (Mdh1) and nicotinamide N-methyltransferase (Nnmt), are involved in cell metabolic pathways (Fig. 8B; supplementary material Tables S3 and S4), thus supporting an important role of SIRT1 in the regulation of cell metabolism in aNSCs/aNPCs. In addition, GO analysis showed enrichment of many genes that are involved in the regulation of neurogenesis and cell proliferation, including EphA4, Sox7, Sox10, Fgfr3 and Erbb4 (Fig. 8C;D; supplementary material Table S3), further supporting the idea that SIRT1 regulates aNSC/aNPC proliferation and self-renewal. Interestingly, we found increased expression of many Notch signaling-related genes, including Notch ligand delta-like 4 (Dlk4), the Notch downstream target genes Hes5, Heyl and Hey2, as well as the Notch signaling regulatory genes mindbomb homolog 1 (Mib1) and manic Fringe (Mfng), in aNSCs/aNPCs from Sirt1 KO mice, and decreased expression of these genes in resveratrol-treated aNSCs/aNPCs from WT mice. This suggests that SIRT1 inhibits Notch signaling in aNSCs/aNPCs (Fig. 8E; supplementary material Table S3). Changes of transcripts for Notch signaling genes were further verified by real-time PCR (Fig. 8F).

Next, we inquired how SIRT1 regulates Notch signaling in aNSCs/aNPCs and whether Notch signaling is involved in the SIRT1 regulation of aNSC/aNPC self-renewal. In our microarray study, we did not identify Notch receptor genes by our screening criteria (>1.5-fold change and opposite changes caused by SIRT1 deletion and activation) (Fig. 9A), suggesting that the expression of Notch receptors was not affected by SIRT1 manipulation. However, we found that the levels of Notch intracellular domain (NICD), the proteolytic cleavage product of Notch1 receptor, both in proliferating and differentiating aNSCs/aNPCs were higher in cells derived from KO mice than those from WT mice (Fig. 9B). These results suggest that in aNSCs, SIRT1 could suppress Notch signaling by reducing the NICD level, similar to the effect of SIRT1 on Notch signaling in endothelial cells (Guarani et al., 2011). Nevertheless, we have not excluded the possibility of direct transcriptional regulation of Notch target genes by SIRT1.
although our analysis using chromatin immunoprecipitation-sequencing (ChIP-Seq) technique failed to demonstrate direct binding of SIRT1 to the promoter regions of Notch target genes (data not shown). Treatment with the specific γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), which inhibits the NICD generation and downstream activation of Notch effectors, significantly reduced the diameter of both WT and KO neurospheres, notably to a similar level (Fig. 9C,D). The finding that the effect of SIRT1 deletion was largely (although not completely) occluded by inhibiting Notch signaling suggests that SIRT1 suppression of aNSC/aNPC proliferation is in part accounted for by repressing Notch signaling.

SIRT1 regulation of aNSC lineage specification

Besides demonstrating the role of SIRT1 in aNSC self-renewal, we also investigated the reported effect of SIRT1 on lineage specification of NSCs in vitro (Hisahara et al., 2008; Prozorovski et al., 2008; Saharan et al., 2013) and potential involvement of Notch signaling. Cultured aNSCs/aNPCs from adult hippocampi were stained for the neuronal marker β-3-Tubulin (Tuj1; Tubb3 – Mouse Genome Informatics) after 7 days of in vitro differentiation. We found an increased percentage of Tuj1+ neurons in Sirt1 KO group (supplementary material Fig. S4), consistent with the role of SIRT1 in cell fate determination (Prozorovski et al., 2008; Saharan et al., 2013). Notch signaling inhibition by DAPT treatment in aNSCs/aNPCs derived from WT mice also increased the percentage of Tuj1+ neurons, but the effect was smaller than that of SIRT1 deletion, and DAPT treatment of aNSCs/aNPCs from SIRT1 KO mice did not further elevate the effect caused by SIRT1 deletion alone (supplementary material Fig. S4). Thus, during lineage specification in vitro, inhibition of Notch signaling or loss of SIRT1 could push differentiating aNSCs/aNPCs towards the neuronal fate, and the SIRT1 effect depends at least in part on Notch signaling.

Based on the results described above, we conclude that SIRT1 suppresses aNSC self-renewal before neuronal fate commitment and promotes non-neuronal (presumably astrocytic) fate during aNSC differentiation. SIRT1 appears to serve as an endogenous ‘stemness’ brake on ‘stemness’ genes such as Notch signaling genes, and a reduced SIRT1 level (or activity) leads to enhanced expression of ‘stemness’ genes and the elevation of self-renewal. Mild metabolic stress could thus fine-tune neurogenesis through SIRT1 signaling (Fig. 9E).

DISCUSSION

Based on in vivo analysis using multiple methods and several types of gene-deletion mice (germline KO, brain-specific CKO and inducible CKO), we have demonstrated a suppressive role of SIRT1 on aNSC self-renewal, a function that might involve SIRT1...
suppression of Notch signaling. Based on the known role of SIRT1 as a sensor for metabolic states, we further provided in vitro evidence that SIRT1 mediates the effect of metabolic stress on adult neurogenesis.

SIRT1 regulation of aNSC self-renewal versus differentiation
Saharan et al. (2013) recently reported an inhibitory function of SIRT1 on aNSC differentiation into neurons in vitro. Here, we report instead that SIRT1 suppresses aNSC self-renewal. These two findings are not contradictory, but reveal potential dual roles of SIRT1 during neurogenesis. It should be noted that, however, the conclusions of the two findings were based on different experimental conditions mainly used (in vitro versus in vivo). Saharan et al. (2013) showed that knockdown/knockout of SIRT1 increased the production of TuJ1+ neurons in vitro, a finding consistent with our in vitro observation (supplementary material Fig. S4). This finding of the suppressive role of SIRT1 on neuronal fate specification in vitro was used by Saharan et al. (2013) to explain their in vivo observation of increased neuronal production in Sirt1 CKO mice. However, we found that neuronal fate specification was not affected after Sirt1 deletion in vivo (Fig. 5E,F). Instead, the self-renewal/proliferation was elevated. The difference of SIRT1 effects on neuronal differentiation in vitro versus in vivo could be attributed to the fact that most progenitor cells in the adult hippocampus in vivo are committed to granule neurons and only a small population of astrocytes is generated (Bonaguidi et al., 2011; Ming and Song, 2005), a situation opposite to that found in vitro.

There are also differences in the findings of SIRT1 expression pattern and SIRT1 effects on aNSC proliferation between the two studies, and these could be accounted for by differences in the experimental approaches and analysis procedures. First, on the expression pattern of SIRT1 in aNSCs, Saharan et al. (2013) reported that SIRT1 is expressed in Ki67+ proliferating cells in the subventricular zone (SVZ) and SGZ, and is also highly expressed in mature granule neurons of DG in vivo. This expression pattern is consistent with our results (Fig. 1). However, they found a relatively
low level of SIRT1 expression in differentiated neurospheres in vitro, a finding seemingly inconsistent with SIRT1 expression in vivo observed in both studies. Again, this could be attributed to the in vitro conditions, in which large amount of astrocytes (with low levels of SIRT1 expression compared with neurons, see supplementary material Fig. S1D) were generated in differentiated neurospheres. Thus, the finding of reduced SIRT1 expression during aNSC differentiation in cultured neurospheres represents an in vitro phenomenon that is not found in vivo. Second, the absence of an in vivo effect of Sirt1 KO on aNSC proliferation reported by Saharan et al. (2013) was based on BrdU+ cell counts one week after a 2-day BrdU injection, when BrdU dilution and cell death may have occurred. By contrast, we have performed a 7-day continuous BrdU injection that was followed by immediate BrdU+ cell counting (Renault et al., 2009; van Praag et al., 1999) and found increased aNSC proliferation, a finding further supported by using the endogenous proliferation marker Ki67 (Fig. 2).

**Metabolic regulation of adult neurogenesis**

Dynamic regulation of adult hippocampal neurogenesis by environmental factors provides the life-long plasticity of hippocampal circuits that enables the animal’s adaptation to the changing environment. It has been suggested that aNSCs are able to sense and respond to changes in local and systemic energy states (Rafalski and Brunet, 2011; Folmes et al., 2012). Although SIRT1 is a well-known metabolic sensor and regulator in many tissues, it...
was unknown whether it serves a similar function in regulating aNSCs. In the present study, we found that mild metabolic stress induced by 2-DG treatment of aNSCs/aNPCs could increase the activity of SIRT1 in vitro, as suggested by the increased NAD+/NADH ratio (Fig. 7E). We also found an enrichment of SIRT1-responsive genes that are involved in the metabolic pathways of cultured aNSCs/aNPCs (Fig. 8B). Thus, SIRT1 may indeed serve as a metabolic sensor and a regulator of aNSCs/aNPCs. Conditions that change the metabolic state, such as hypoxia, exercise and caloric restriction, could influence hippocampal neurogenesis (Park and Lee, 2011; van Praag et al., 1999; Zhu et al., 2010), and SIRT1 expression level and SIRT1 activity are influenced by many physiological stimuli that may alter the metabolic state of the tissue (Boily et al., 2008; Chen et al., 2005; Cohen et al., 2004; Kotlai et al., 2010; Radak et al., 2013). Thus, the SIRT1 action shown here provides a potential explanation for the effect of environmental stimuli on adult hippocampal neurogenesis. Moreover, aNSCs/aNPCs are distributed near blood vessels in the adult SVZ and SGZ (Goldberg and Hirschi, 2009), and could thus quickly respond to energy changes by sensing the oxygen and nutrient signals from the blood vessels. We showed that SIRT1 could partially mediate the effect of 2-DG-induced metabolic stress on aNSC proliferation in vitro, but the in vivo evidence of SIRT1 involvement in metabolic control of adult neurogenesis remains to be further studied.

**SIRT1 regulation of Notch signaling**

As a master factor regulating NSC self-renewal, Notch signaling represses the expression of pro-neuronal genes through Notch targets, such as Hes1 and Hes5 (Louvi and Artavanis-Tsakonas, 2006). Our microarray analysis showed an enrichment of Notch signaling pathway genes among SIRT1-responsive genes (Fig. 8E). Moreover, we observed higher SIRT1 levels in aNPCs and immature neurons than in aNSCs (Fig. 1D-I). The increase of SIRT1 expression negatively correlated with the reduction of Notch signaling during aNSC differentiation (Ehm et al., 2010; Lugert et al., 2010), providing further evidence that SIRT1-dependent inhibition of Notch signaling occurs during adult hippocampal neurogenesis. Recently, SIRT1 was found to repress Notch signaling through its deacetylation and destabilization of NICD in endothelial cells (Guarani et al., 2011). We found a higher level of NICD in cultured aNSCs/aNPCs from KO mice compared with cells from WT mice, without changes in the expression of Notch receptors (Fig. 9A,B), suggesting a conserved mechanism of SIRT1 regulation of Notch signaling between endothelial cells and aNSCs. Furthermore, SIRT1 could also serve as a component of the histone-modifying co-repressor complex, which represses Notch target gene expression in mouse embryonic fibroblasts or mouse embryonic progenitor cells (Mulligan et al., 2011; Tiberi et al., 2012). Although we failed to detect direct epigenetic repression by ChIP-Seq, transcriptional regulation of Notch target genes by SIRT1 in aNSCs remains a possibility to be further examined. Furthermore, SIRT1 has many non-histone substrates, such as p53 and Foxo (Brunet et al., 2004; Vaziri et al., 2001), which are known to regulate aNSC self-renewal (Meletis et al., 2006; Renault et al., 2009). Thus, SIRT1-regulation of aNSC self-renewal might also involve transcription factors unrelated to the Notch signaling pathway.

Prozorovski et al. (2008) have shown that SIRT1 is involved in the fate specification of embryonic NSCs by promoting an astroglial lineage. This occurs at the expense of a neuronal lineage by interacting with Hes1 to repress the expression of the pro-neuronal gene Mash1 (Ascl1 – Mouse Genome Informatics). Interestingly, this facilitating effect of SIRT1-dependent Notch signaling during fate specification of NPCs is in contrast to our finding of SIRT1-dependent suppressive action of Notch signaling on the aNSC self-renewal. Thus, SIRT1 might play distinct different roles at different stages of neurogenesis. In non-committed NSCs, increased expression or higher activity of SIRT1 provides a permissive signal (via downregulating Notch signaling) for the NSCs to exit the pluripotent state. On the other hand, when the cells are committed to differentiation and ready for fate determination, SIRT1 might instruct the cells to differentiate into astrocytes rather than neurons (by suppressing pro-neuronal genes). We did not observe the latter effect of SIRT1 on the lineage specification of aNSCs/aNPCs in vivo (Fig. 5E,F). Presumably this is due to the fact that most aNSCs/aNPCs are committed to granule neurons in the adult DG (Bonaguidi et al., 2011; Ming and Song, 2005).

In summary, the current study showed that SIRT1 might serve as an important metabolic sensor for regulating adult hippocampal neurogenesis by cell-autonomously modulating the balance between aNSC/aNPC self-renewal and differentiation, in part through its suppression of Notch signaling. Our findings unveil the function of SIRT1 for regulating ‘stemness’ signaling in aNSCs and they underscore the potential function of metabolic sensors in dynamic regulation of adult neurogenesis.

**MATERIALS AND METHODS**

**Animals**

All mouse (*Mus musculus*) experiments were performed under the guidelines of the Animal Care and Administration Committee of the Institute of Neuroscience (NA-100410-1). Sirt1 KO and nestin-CreER<sup>22</sup>, R26R-IFP mice were generously provided by Dr Michael W. McBurney (University of Ottawa, Canada) and Dr A. J. Eisch (University of Texas Southwestern Medical Center, USA), respectively. Sirt1<sup>fl/h</sup>, nestin-Cre and Gfap-Gfp mice were purchased from Jackson Laboratory.

**BrdU and tamoxifen treatment**

Mice received intraperitoneal injections of BrdU (Sigma; 50 mg/kg body weight) according to the parameters indicated in the Results section. Tamoxifen (Sigma; 180 mg/kg body weight) was given for 3 days as previously described (Ables et al., 2010).

**Immunostaining**

Brains or cultured cells were fixed with 4% paraformaldehyde. Coronal sections of 40 µm were cut in six parallel sets. For BrdU staining, heat retrieval and HCl treatment were carried out before antibody incubation. Primary antibodies used are listed in supplementary material Table S1. Fluorescently conjugated secondary antibodies (Invitrogen; 1:500) were used. Nuclei were stained with 4’6-Diamidino-2-phenylindole (DAPI; Sigma).

**Confocal imaging and cell counting**

Confocal z-stack images were acquired on a FluoView1000 (Olympus) or Nikon A1 confocal laser microscope system (Nikon). Immune reactive cells at the SGZ in every sixth coronal section throughout the DG were counted. The density of cells was determined by dividing the total number of cells by the corresponding volume of DG.

**Analysis of SIRT1 fluorescence signals**

SIRT1 fluorescence intensity was assessed using ImageJ (NIH). The nuclei of each cell type were outlined according to DAPI staining, creating a mask of each cell type were outlined according to DAPI staining, creating a mask. Fluorescently conjugated secondary antibodies (Invitrogen; 1:500) were used. Nuclei were stained with 4’6-Diamidino-2-phenylindole (DAPI; Sigma).

**Neurosphere assays and differentiation assay**

Neurospheres were prepared as described previously (Brewer and Torricelli, 2007). aNSCs/aNPCs were seeded at a density of 6000, 4000 and 8000
cells/ml for the formation of primary, secondary and pharmacologically treated neurospheres, respectively. Resveratrol (Calbiochem) was added 4 days before assessment at day 7. DAPT (0.5 μM) was added 12 h after seeding of aNSCs/aNPCs. For 2-DG treatment, glucose (Sigma) and 2-DG (Inalco) were added to a glucose-free customized medium (Invitrogen) with a total concentration of 25 mM to keep the osmolarity constant. Each well was photographed for further analysis of neurosphere numbers and diameters after a 7- to 9-day incubation. Only neurospheres larger than 20 μm were assessed. Differentiation of dissociated aNSCs/aNPCs is induced by growth factor withdrawal and the addition of 1% fetal bovine serum (Gibco).

\[ \text{NAD}^+ / \text{NADH} \text{ quantification} \]
aNSCs/aNPCs were incubated with different concentrations of 2-DG (0, 0.5, 1, 2.5, 5 mM) for 24 h. The samples were collected and measured according to the manual that accompanies the \text{NAD}^+ / \text{NADH} \text{ Quantification Colorimetric Kit (BioVision).}

\[ \text{RNA extraction, reverse transcription and quantitative PCR} \]
Total RNA was isolated using the TRIzol reagent (Invitrogen). Reverse transcription was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). qPCRs were performed on a LightCycler 480 instrument (Roche Applied Science). The primers used are listed in supplementary material Table S2.

\[ \text{Microarray analysis} \]
Microarray hybridization was performed with oligonucleotide arrays (Mouse Genome 4×44 K array, Agilent Technologies). GO analysis was conducted using the Phenotype Analysis Tools from Mouse Genome Informatics. The results of a ‘Pathway to Gene’ analysis were provided by the SBC Analysis System at the Shanghai Biotechnology Corporation, China. Microarray data were deposited at the Gene Expression Omnibus (GEO) database under the accession number GSE39551.

\[ \text{Western blotting} \]
Western blotting was performed as described previously (Xue and Yuan, 2010). Primary antibodies used are listed in supplementary material Table S1.

\[ \text{Statistics} \]
Quantitative results are shown as mean±s.e.m. A paired t-test was carried out for the analysis of animal-related in vivo results and a Kolmogorov–Smirnov (K–S) test was used for neurosphere diameter analysis. Significance was set as follows: *P<0.05, **P<0.01, ***P<0.001.

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\[ \text{Competing interests} \]
The authors declare no competing financial interests.

\[ \text{Author contributions} \]
C.M., Q.Z., J.J., X.Y. and M.P. conceived and designed the experiments; C.M. performed all the experiments; C.M. and M.Y. analyzed the data; C.M., X.Y. and M.P. wrote the paper.

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\[ \text{Supplementary material} \]
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References


