Expression of DNMT1 in neural stem/precursor cells is critical for

survival of newly generated neurons in the adult hippocampus

Authors: Hirofumi Noguchi^{a,b}, Ayaka Kimura^a, Naoya Murao^{a,b}, Taito Matsuda^a,
Masakazu Namihira^c, and Kinichi Nakashima^a

a Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu
University, Fukuoka, Japan

b Laboratory of Gene Regulation Research, Graduate School of Biological Sciences,
Nara Institute of Science and Technology, Nara, Japan

c Neuroscience Research Institute, AIST, Ibaraki, Japan

Corresponding Author:

Kinichi Nakashima, Ph.D.

Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu
University, Fukuoka 812-8582, Japan, Phone: +81-92-642-6195, Fax: +81-92-642-6561,

16 Email: kin1@scb.med.kyushu-u.ac.jp

17 Number of pages: 46, figures: 6, and tables: 1

18

19 **Abstract**

20 Adult neurogenesis persists throughout life in the dentate gyrus (DG) of the
21 hippocampus, and its importance has been highlighted in hippocampus-dependent
22 learning and memory. Adult neurogenesis consists of multiple processes: maintenance
23 and neuronal differentiation of neural stem/precursor cells (NS/PCs), followed by
24 survival and maturation of newborn neurons and their integration into existing neuronal
25 circuitry. However, the mechanisms that govern these processes remain largely unclear.
26 Here we show that DNA methyltransferase 1 (DNMT1), an enzyme responsible for the
27 maintenance of DNA methylation, is highly expressed in proliferative cells in the adult
28 DG and plays an important role in the survival of newly generated neurons. Deletion of
29 *Dnmt1* in adult NS/PCs (aNS/PCs) did not affect the proliferation and differentiation of
30 aNS/PCs *per se*. However, it resulted in a decrease of newly generated mature neurons,
31 probably due to gradual cell death after aNS/PCs differentiated into neurons in the
32 hippocampus. Interestingly, loss of DNMT1 in post-mitotic neurons did not influence
33 their survival. Taken together, these findings suggest that the presence of DNMT1 in
34 aNS/PCs is crucial for the survival of newly generated neurons, but is dispensable once

35 they accomplish neuronal differentiation in the adult hippocampus.

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37 Keywords: DNA methyltransferase 1; Adult neurogenesis; DNA methylation; Neuronal

38 survival; Neural stem cells

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1. Introduction

Neural stem/precursor cells (NS/PCs) continue to exist in the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) as well as the subventricular zone (SVZ) in the adult mammalian brain, where neurogenesis persists throughout life (Altman and Das, 1965; Eriksson et al., 1998; Kuhn et al., 1996), and neurogenesis in the former region is known to be associated with learning and memory (Deng et al., 2010). Adult (a)NSCs in the DG divide infrequently and eventually become quiescent (Lugert et al., 2010; Mira et al., 2010; Morshead et al., 1994; Seri et al., 2001). Adult neurogenesis thus begins with the activation and proliferation of these quiescent aNSCs to become aNPCs, followed by neuronal differentiation and maturation. Although most adult newborn neurons are quickly eliminated, within four weeks after being generated (Dayer et al., 2003; Snyder et al., 2009), a few of them survive and integrate into pre-existing neuronal circuits in the hippocampus (Cameron et al., 1993; Dayer et al., 2003; Snyder et al., 2009). To ensure proper hippocampal functions, each step during adult neurogenesis (e.g., survival and maturation of newborn neurons) is precisely regulated by both cell extrinsic and intrinsic factors including extracellular signals from

the stem cell niche, transcription factors and epigenetic regulators (Covic et al., 2010; Ma et al., 2010; Ming and Song, 2011).

DNA methylation influences various biological processes, such as development, cell differentiation and genomic imprinting (Lewis et al., 2004; Meehan, 2003). DNA methylation in gene promoters is associated generally with gene repression (Klose and Bird, 2006), ensuring tissue-specific gene expression. In mammals, patterns of DNA methylation in the genome are established and maintained by the DNA methyltransferases DNMT1, DNMT3A and DNMT3B (Bestor, 2000; Robertson and Wolffe, 2000; Suzuki and Bird, 2008). Of these, DNMT1 is responsible for the maintenance of DNA methylation after each round of replication as cells divide. Accumulating studies of DNMT1 in several types of stem cells have indicated that DNMT1 contributes to tissue development and to stem cell maintenance and differentiation (Georgia et al., 2013; Sen et al., 2010; So et al., 2011; Tsai et al., 2012). For example, DNMT1 is essential to maintain the pool of pancreatic progenitors, its loss in murine pancreatic progenitors leading to their failure to survive, causing pancreatic atrophy (Georgia et al., 2013). In adult human epidermal progenitors, DNMT1 also

plays a critical role in maintaining the ability of progenitors to self-renew as well as in inhibiting their premature differentiation (Sen et al., 2010).

During central nervous system (CNS) development, deletion of *Dnmt1* in embryonic (e)NS/PCs also induces their premature differentiation (Fan et al., 2005; Hutnick et al., 2009). Neurons, astrocytes and oligodendrocytes are sequentially produced from eNS/PCs during cortical development (Gage, 2000; Kriegstein and Alvarez-Buylla, 2009; Temple, 2001), and epigenetic modifications including DNA methylation and histone modifications regulate these transitions (Adefuin et al., 2014; MuhChyi et al., 2013; Namihira and Nakashima, 2013). In mid-gestation, eNS/PCs predominantly differentiate into neurons while precocious astrocyte differentiation is inhibited through DNA methylation in astrocyte-specific gene promoters maintained by DNMT1 (Fan et al., 2005; Hutnick et al., 2009; Namihira et al., 2009; Takizawa et al., 2001). DNMT1-deficient eNS/PCs give rise precociously to astrocytes but also produce neurons having genome-wide hypomethylation that display abnormal dendritic morphology and neuronal function (Hutnick et al., 2009). It was further indicated that *Dnmt1* deletion in eNS/PCs leads to a significant decrease in the number of neurons,

89 resulting in an overall reduction of cortical and hippocampal volume. These findings
90 indicate that DNMT1 is required for the proper differentiation of eNS/PCs and
91 production of functional neurons during CNS development.

92 Although previous reports highlight the importance of DNMT1 in eNS/PCs
93 during CNS development, the function of DNMT1 in aNS/PCs and adult neurogenesis
94 has not been elucidated. Here, we investigated the function of DNMT1 in adult
95 neurogenesis using two types of Cre recombinase-expressing transgenic mice, which
96 allow specific deletion of *Dnmt1* in either aNS/PCs or post-mitotic neurons. Although
97 deletion of *Dnmt1* in aNS/PCs did not lead to defects in their proliferation or neuronal
98 differentiation, it did result in reduced numbers of newly generated mature neurons,
99 probably due to gradual cell death after the aNS/PCs differentiated into neurons.
100 Surprisingly, we found that *Dnmt1* deletion in post-mitotic neurons had no apparent
101 effect on neuronal survival, suggesting that the presence of DNMT1 in aNS/PCs but not
102 in neurons is required for neuronal survival and maturation, rather than for regulating
103 aNSCs, during adult neurogenesis.

2. Materials and Methods

2.1. Animals: generation of DNMT1 conditional mutant mice

For tamoxifen-inducible Cre-mediated *Dnmt1* deletion in aNS/PCs, *Dnmt1*^{2lox} (*Dnmt1*^{flox/flox}) mice (Fan et al., 2001) were crossed with Nestin-CreER^{T2} transgenic mice (Imayoshi et al., 2006). Tamoxifen administration in Nestin-CreER^{T2}; *Dnmt1* conditional mutant mice (DNMT1 cKO(Nes)) inactivates DNMT1 through deletion of exons 4 and 5 of *Dnmt1* in Nestin-expressing NS/PCs. Either Nestin-CreER^{T2}; *Dnmt1*^{flox/+} or CreER^{T2}-negative mice were used as controls. To investigate the function of DNMT1 in neurons, *Dnmt1*^{flox/flox} mice were crossed with Synapsin1-Cre transgenic mice (Zhu et al., 2001), obtained from the Jackson Laboratory), producing progeny in which Cre recombinase expression is restricted to synapsin1-expressing neurons (DNMT1 cKO(Syn)). Either synapsin1-Cre; *Dnmt1*^{flox/+} or Cre-negative mice were used as controls. All mice used in this study were maintained on a 12-h light/dark cycle with free access to food and water. All animal procedures were performed in accordance with the animal experimentation guidelines of National Institute of Science and Technology, which follow the National Institutes of Health

Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. *Tamoxifen and BrdU administration*

Tamoxifen (Sigma-Aldrich, T5648) was dissolved in sesame oil at 20 mg/ml. Eight-week-old mice were orally administered with 10 mg of tamoxifen with a feeding needle. To induce deletion of *Dnmt1* alleles through Cre-mediated recombination, the mice were treated with tamoxifen once a day for 4 days. For bromodeoxyuridine (BrdU) labeling, mice were intraperitoneally injected with BrdU (Sigma-Aldrich, B5002) dissolved in saline (0.9% NaCl) at a dose of 50 mg/kg once a day for 7 days.

2.3. *Tissue preparation*

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) before perfusion with 4% paraformaldehyde (PFA) in PBS. Brains were dissected and postfixed with 4% PFA in PBS overnight at 4°C. For cryoprotection, fixed brains were stored in 15% sucrose in PBS overnight at 4°C and then in 30% sucrose in PBS

overnight at 4°C. One side of the brain was embedded in optimal cutting temperature (OCT) compound (Tissue Tek, Sakura Finetek, 25608-930) and frozen at -80°C for cryosectioning. Frozen brains were serially sectioned in the coronal plane at 40-μm thickness, using a Leica CM 1900 cryostat (Leica Microsystems). Every sixth section was collected sequentially from anterior to posterior, and transferred to a well of a 6-well plate containing sterilized PBS and preserved at 4°C.

2.4. Immunohistochemistry

Cryosections were washed with PBS and blocked for 1 h at room temperature with blocking solution (3% FBS, 0.5% Triton X-100, 0.25% Tween-20), and incubated overnight at 4°C with primary antibodies diluted in blocking solution. The following primary antibodies were used in this study:

rabbit anti-DNMT1 (1:500; Cosmo Bio, BAM-70-203-EX); mouse anti-Ki67 (1:500; BD Biosciences, 550609); goat anti-Sox2 (1:100; Santa Cruz Biotechnology, sc-17320); mouse anti-Nestin (1:500; Millipore, MAB353); rabbit anti-DCX (1:500; Abcam, ab18723); mouse anti-NeuN (1:500; Millipore, MAB377); rat anti-Ctip2 (1:500; Abcam,

153 ab18465); chick anti-GFAP (1:500; Millipore, AB5541); goat anti-NeuroD (1:100;
154 Santa Cruz Biotechnology, sc-1084); mouse anti-S100 β (1:500; Sigma-Aldrich, S2532);
155 rabbit anti-active caspase-3 (1:500; R&D Systems, AF835); and rat anti-BrdU (1:500;
156 AbD Serotec, OBT0030).

157 For staining of Ki67, NeuroD and DNMT1, antigen retrieval was performed
158 by heating sections in target retrieval solution (DAKO) at 105°C for 15 min prior to
159 blocking. For detection of BrdU-labeled cells, free-floating sections were treated with 2
160 N HCl at 37°C for 15 min, and washed with PBS prior to blocking and incubation with
161 primary antibodies. After three washes in PBS, sections were incubated for 2 h with the
162 corresponding secondary antibodies: CF488 donkey anti-mouse IgG (H+L), highly
163 cross-adsorbed (1:500; Biotium, 20014); CF543 donkey anti-rabbit IgG (H+L), highly
164 cross-adsorbed (1:500; Biotium, 20038); CF647 donkey anti-goat IgG (H+L), highly
165 cross-adsorbed (1:500; Biotium, 20048); CF647 donkey anti-rabbit IgG (H+L), highly
166 cross-adsorbed (1:500; Biotium, 20047); and CF568 donkey anti-rat IgG (H+L), highly
167 cross-adsorbed (1:500; Biotium, 20092). Hoechst 33258 (1:500; Nacalai Tesque) was
168 used for nuclear staining. After a final rinse with PBS, sections were mounted, and

images were taken using a Zeiss LSM 780 confocal microscope.

2.5. Cell counting

Following previous reports (Kronenberg et al., 2003; Steiner et al., 2006), cell counting was performed on every sixth (for counting BrdU+ cells and active caspase-3+ cells) or twelfth (for counting Ki67+ cells) coronal hemisphere brain section containing the DG. Positive cells were counted in the series of collected sections throughout the entire DG. The total number of marker-positive cells in the DG was obtained by multiplying the resultant counts by 6 or 12 (according to the interval between sections), and then by 2 to estimate the total number in the DGs from both brain hemispheres. To calculate the percentage of marker-positive cells in BrdU-labeled cells, at least three sections from each mouse were counted. The total number of marker-positive cells in BrdU-labeled cells was estimated by multiplying the percentage of marker-positive cells among BrdU-labeled cells by the total number of BrdU-labeled cells. To estimate the survival ratio, the number of total BrdU-positive cells in the DG in each mouse at 4 weeks after the last BrdU injection was divided by the average number of total

BrdU-positive cells from three specimens at 1 day after the last BrdU injection and the mean value of them was calculated. In counting Sox2- and DCX-positive cells in the SGZ of Synapsin1-Cre; *Dnmt1* conditional mutant mice, at least three sections from each mouse were counted, and the number of marker-positive cells/mm in the SGZ was calculated by dividing the number of marker-positive cells in the SGZ by the length of the SGZ, which was measured using ImageJ software (NIH).

2.6. Cell culture

NSCs were collected and purified from the DGs of 8-week-old adult mice. NSCs were cultured on a poly-ornithine/fibronectin-coated 10-cm dish in N2 medium (N2-supplemented DMEM/F-12; Invitrogen, 11320-033), containing 10 ng/ml of basic fibroblast growth factor (bFGF) (PeproTech, 100-18B) and 10 ng/ml of epidermal growth factor (EGF) (PeproTech, AF100-15), under 5% CO₂ at 37°C. HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (heat inactivated, Biowest, 366 S1820) and gentamicin sulfate solution (100 mg/ml, Nacalai Tesque, 16672-04).

2.7. Lentiviral constructs, preparation of lentivirus and viral infection

The lentivirus vector used to express the short hairpin RNA (pLLX) was generously provided by Drs. Z. Zhou and M. E. Greenberg. pLLX is a dual-promoter lentivirus vector constructed by inserting the U6 promoter-driven shRNA cassette 5' to the ubiquitin-C promoter in the FUIGW plasmid (Lois et al., 2002; Zhou et al., 2006). pLLX was modified to express GFP together with a puromycin resistance gene under the ubiquitin C promoter. shRNA for *Dnmt1* lentivirus constructs were generated by inserting oligonucleotides into the *HpaI* and *XhoI* sites of pLLX. The oligonucleotide target sequence of mouse *Dnmt1* is ACCAAGCTGTGTAGTACTT, which locates in the 3'UTR of *Dnmt1* mRNA, and the control sequence is GCTTCAATTCGCGCACCTA, which does not exist in either mouse genomic DNA or mRNA. To prepare lentivirus, HEK293T cells were co-transfected with these constructs and lentiviral packaging vectors (pCAG-HIVgp and pCMV-VSV-G-RSV-Rev). The culture supernatants were collected 48 h after transfection, and virus was introduced into NSCs by adding the supernatants to the culture media. NSCs were infected with lentivirus and treated with puromycin (0.2 µg/ml; Sigma-Aldrich, P8833) 2 days after infection. For RNA

collection, infected NSCs were cultured for 1 week in N2 medium with bFGF and EGF.

2.8. Real-time PCR

Total RNA was isolated using Sepasol-RNA I Super G (Nacalai Tesque), according to the manufacturer's instructions, and each sample was reverse transcribed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative PCR reactions were performed using a KAPA SYBR Fast qPCR Kit (KAPA Biosystems) with ROX as reference dye. The primers used in this study are listed in Table S1. Expression levels of each gene were normalized to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and calculated relative to the control.

2.9. Statistical analysis

At least 3 mice per group were analyzed. Statistical analyses were performed using Student's *t*-test (for comparisons between two groups). All experiments were independently replicated at least three times. Differences were considered statistically significant at $P < 0.05$. Asterisks indicate significant differences (* < 0.05 ; ** < 0.01).

3. Results

3.1. DNMT1 is expressed broadly in the granule cell layer of the dentate gyrus but enriched in proliferating cells

We first examined the expression of DNMT1 in the adult mouse brain. Image from the Allen Brain Atlas (<http://www.brain-map.org/>) showed that *Dnmt1* is expressed broadly in the adult mouse brain, but much more highly in the hippocampus, especially in the granule cell layer (GCL) of the DG, than in other brain regions (Fig. 1A). We also confirmed DNMT1 protein expression in the GCL, consistent with the *in situ* hybridization image. Within the GCL, we noticed that cells highly expressing DNMT1 were located in the subgranular zone (SGZ), where aNS/PCs reside (Fig. 1B). aNS/PCs in the DG are categorized into three types (type-1, -2, and -3 cells) according to their marker expression, morphology and proliferative state (Imayoshi et al., 2011; Kempermann et al., 2004; Lugert et al., 2010). Type-1 cells, which exhibit a radial glial fiber extending toward the molecular layer (ML) through the GCL, are considered to be *bona fide* stem cells in the DG. Their nuclei and radial processes are labeled with antibodies for SRY (sex-determining region Y)-box 2 (Sox2) and for glial fibrillary

249 acidic protein (GFAP), respectively. The majority of type-1 NSCs stay in a quiescent
250 state and only infrequently divide (Mira et al., 2010; Morshead et al., 1994; Seri et al.,
251 2001). Once they start proliferating, they generate type-2 cells, which are highly
252 proliferative and become positive for both Sox2 and the proliferating cell marker Ki67.
253 Type-2 cells then give rise to neuroblasts (type-3 cells), which express the basic
254 helix-loop-helix proneuronal transcription factor neuronal differentiation (NeuroD) and
255 Ki67. Type-3 cells later lose Ki67 expression and differentiate into post-mitotic
256 neurons.

257 To identify DNMT1-expressing cell types in the adult DG, we performed
258 co-immunostaining of DNMT1 with various cell type-specific markers. We detected
259 weak DNMT1 expression in GFAP+/Sox2+ type-1 cells (Fig. 1C, white arrowheads)
260 and granule neurons (Fig. 1F, see below), but much stronger expression in
261 GFAP-/Sox2+ cells (Fig. 1C, white arrows). Co-immunostaining of DNMT1 with Sox2
262 and Ki67 demonstrated higher expression of DNMT in Sox2+/Ki67+ type-2 cells (Fig.
263 1D, white arrowheads) than in Ki67-/Sox2+ cells (white arrows). These results suggest
264 that DNMT1 is preferentially expressed in the proliferative cells and up-regulated as

265 type-1 cells turn into type-2 cells. Since type-3 cells are also proliferative, we further
266 investigated the expression of DNMT1 in these cells. We found that type-3 cells
267 (NeuroD+/Ki67+ cells) exhibit high expression of DNMT1 (Fig. 1E, white arrowheads)
268 and that its expression is weaker in Ki67-/NeuroD+ immature neurons (white arrows).
269 These results further emphasize that DNMT1 is expressed in proliferative cells in the
270 DG. Expression of B-cell CLL/lymphoma 11B (Bcl11b, also known as chicken
271 ovalbumin upstream promoter transcription factor-interacting protein 2 (Ctip2)), a zinc
272 finger transcription factor, is confined to mature granule neurons in the adult DG
273 (Simon et al., 2012). Consistent with the expression of DNMT1 in the immature neuron,
274 Ctip2+ mature neurons displayed weak expression of DNMT1 compared with Ki67+
275 proliferating cells (Fig. 1F, white arrowhead), suggesting that DNMT1 expression
276 declines as neuronal differentiation proceeds and the cells become post-mitotic. In this
277 context, GFAP+/S100 β + post-mitotic astrocytes in the ML also showed lower
278 expression of DNMT1 (Fig. 1G, white arrowhead). Taken together, these results
279 indicate, first, that type-1 cells increase their expression of DNMT1 as they exit the
280 quiescent state and generate type-2 cells; and, second, that higher DNMT1 expression

persists in proliferating cells (i.e., type-2 and type-3 cells) until they differentiate into post-mitotic neurons (Fig. 1H).

3.2. DNMT1 does not affect proliferation or maintenance of aNS/PCs

Since DNMT1 is highly expressed in proliferating cells in the SGZ, we investigated the function of DNMT1 in regulating the proliferation and maintenance of NS/PCs in the adult DG. To this end, we used Nestin-CreER^{T2} transgenic mice (Imayoshi et al., 2006), in which Cre recombinase is only expressed in Nestin-expressing NS/PCs (type-1 and -2 cells). *Dnmt1*^{fl^{ox}/fl^{ox}} mice were crossed with Nestin-CreER^{T2} transgenic mice to achieve tamoxifen-inducible deletion of *Dnmt1* in aNS/PCs (DNMT1 cKO(Nes)) (Fig. 2A). Eight-week-old mice were treated with tamoxifen once a day for 4 consecutive days, and we examined whether *Dnmt1* deletion affects the proliferation of NS/PCs at 8 and 36 days after the last tamoxifen injection (Fig. 2B). We confirmed *Dnmt1* deletion in proliferating cells and type-1 cells of DNMT1 cKO(Nes) DGs at 8 days after the last tamoxifen injection (Fig. 2C and D). The number of Ki67+ cells in control and DNMT1 cKO(Nes) DGs was comparable at 8

days and also at 36 days, suggesting that proliferation of aNS/PCs was not affected by the loss of DNMT1 (Fig. 2E and F).

NSCs in the adult DG are defined as type-1 cells and maintained in a quiescent state. NSCs express Sox2 and elaborate radial processes that are positive for GFAP and Nestin. To examine whether *Dnmt1* deletion impairs the maintenance of NSCs, we examined their morphology and number in the DG at 36 days after the last tamoxifen injection. However, we found no differences in either the morphology or the number of type-1 cells between control and DNMT1 cKO(Nes) DGs (Fig. 2G and H), suggesting that DNMT1 is dispensable for the maintenance of aNSCs in the DG.

3.3. DNMT1 in aNS/PCs is dispensable for their differentiation but important for the survival of newly generated neurons

To determine whether DNMT1 is required for differentiation of aNS/PCs, we performed a BrdU pulse-chase experiment. tamoxifen-treated mice were injected with BrdU once a day for 7 consecutive days, and the fate of BrdU-labeled cells was traced at 1 day and 4 weeks after the last BrdU injection (Fig. 3A). One day after the final

313 injection, the number of BrdU+ cells showed no significant difference between control
314 and DNMT1 cKO(Nes) mice (Fig. 3B and D), consistent with our above finding that
315 DNMT1 does not affect proliferation of aNS/PCs in the DG (Fig. 2C and D). In contrast,
316 we found a significant reduction in the number of remaining BrdU+ cells at 4 weeks
317 after the last BrdU injection in the DG of DNMT1 cKO(Nes) mice compared with the
318 control (Fig. 3C and D). We also calculated the ratio of the number of BrdU+ cells in
319 the DG at 4 weeks after the final BrdU injection to the number at 1 day and found that
320 the survival ratio of BrdU-labeled cells in DNMT1 cKO(Nes) (20.9%) was about half
321 that in the control (46.3%) (Fig. 3E).

322 We next investigated the type(s) of cells whose survival was reduced by the
323 deletion of *Dnmt1* in aNS/PCs. One day after the last BrdU injection, many BrdU+ cells
324 were also positive for the immature neuronal marker Doublecortin (DCX), and neither
325 the percentage of DCX+ cells in BrdU+ cells nor the total number of DCX+/BrdU+
326 cells in the DG differed between control and DNMT1 cKO(Nes) mice, indicating that
327 the onset of aNS/PC differentiation into the neuronal lineage and also the survival of
328 immature neurons were independent of DNMT1 (Fig. 3B, 3F and 3G). By contrast, the

number of cells doubly positive for the mature neuronal marker NeuN and BrdU was dramatically decreased at 4 weeks after the last BrdU injection in the DG of DNMT1 cKO(Nes) mice compared to the control (Fig. 3C and I). On the other hand, the numbers of cells that had differentiated at this time into astrocytes (S100 β +/BrdU+) in the DG of control and DNMT1 cKO(Nes) mice were comparable (Fig. 3H, I). These data indicate that deletion of *Dnmt1* in aNS/PCs does not impair differentiation, but depresses the survival of neurons during or after their maturation.

3.4. Deletion of Dnmt1 in post-mitotic neurons does not lead to a reduction of granule cells in the DG

The results presented above have shown that *Dnmt1* deletion at aNS/PC stages leads to decreased survival of cells after aNS/PCs differentiate into mature neurons. However, it is still unknown whether DNMT1 must be present continuously, from aNS/PCs to neurons, for the survival of mature neurons. To address this question, we deleted *Dnmt1* in post-mitotic neurons by crossing *Dnmt1*^{flox/flox} mice with Synapsin1-Cre transgenic mice (Zhu et al., 2001), producing progeny in which Cre

recombinase expression is restricted to synapsin1-expressing neurons (DNMT1 cKO(Syn)) (Fig. 4A). In the DG of DNMT1 cKO(Syn) mice, DNMT1 expression was detected only in Ki67+ proliferating cells located in the SGZ, but not in Ctip2+ granule neurons, indicating that deletion of *Dnmt1* in mature neurons in the DG of these transgenic mice was successful (Fig. 4B and C). However, although DNMT1 was almost completely lost, proper DG structure was retained in DNMT1 cKO(Syn), as assessed by the expression of Ctip2 and NeuN (Fig. 4B-D), indicating that deletion of *Dnmt1* after the cells have accomplished differentiation into mature neurons does not influence their survival.

We finally asked whether the deletion of *Dnmt1* in post-mitotic neurons influences neurogenesis and maintenance of aNS/PCs. The numbers of Sox2+ or DCX+ cells in the SGZ of DNMT1 KO(Syn) were comparable to those of the control (Fig. 4D-F), suggesting that neurogenesis and maintenance of aNS/PCs are unaffected by the loss of DNMT1 in post-mitotic neurons in the DG.

4. Discussion

We have shown in this study that DNMT1 has a key role in neuronal survival. DNMT1-deficient aNS/PCs differentiated normally into neurons. However, mature neurons generated from DNMT1-deficient aNS/PCs displayed poor survival, resulting in reduced numbers of mature newly generated neurons. Interestingly, using two transgenic mice that allowed lineage-specific *Dnmt1* deletion (Nestin-CreER^{T2} and Synapsin1-Cre), we found that DNMT1 in aNS/PCs, but not in post-mitotic neurons, is crucial for neuronal survival. Deleting *Dnmt1* in the post-mitotic neurons did not induce neuronal death in the DG. The GCL of DNMT1 cKO(Syn) mice was morphologically indistinguishable from that of control mice. These results suggest that DNMT1 in aNS/PCs is required for the survival of their descendants (mature neurons), rather than for differentiation and proliferation of aNS/PCs in the DG. We summarize these findings in Fig. 5.

DNMT1 is highly expressed in the proliferative cells, i.e., type-2 and type-3 cells, in the DG (Fig. 1D and E). On the other hand, although DNMT1 expression is still detectable, it becomes much weaker in post-mitotic neurons and in infrequently proliferating cells, including type-1 cells and astrocytes (Fig. 1C, F and G). This

381 expression profile of DNMT1 in the adult DG is reasonable as Dnmt1 expression is
382 known to be induced by a cell cycle regulator E2F and to be expressed maximally in S
383 phase (Kimura et al., 2003; McCabe et al., 2005), probably due to that the major role of
384 DNMT1 is to maintain DNA methylation after DNA replication. Nuclear protein 95
385 KDa (Np95, also known as Ubiquitin-like, containing PHD and RING finger domains 1
386 (UHRF1)), a key factor for recruiting DNMT1 to hemi-methylated CpG sites in the
387 genome, thus also plays an important role in the maintenance of DNA methylation by
388 cooperating with DNMT1 (Sharif et al., 2007). Expression of Np95 is restricted to
389 proliferating cells in both embryonic and adult brains (Murao et al., 2014). In the adult
390 DG, Np95 is highly expressed only in type-2 and -3 cells, and rarely in type-1 cells
391 when they start to proliferate. However, in contrast to the expression pattern of DNMT1,
392 Np95 expression is undetectable in either post-mitotic neurons or terminally
393 differentiated glial cells (astrocytes and oligodendrocytes) (Murao et al., 2014),
394 implying that DNMT1 has an as-yet-unknown function, other than maintenance of DNA
395 methylation, in these cells. Indeed, recent functional analyses have shown that DNMT1
396 can regulate gene expression without DNA methyltransferase activity through

interactions with histone modification enzymes and transcription factors (Clements et al., 2012; Espada, 2012; Espada et al., 2011). Therefore, in these post-mitotic and infrequently proliferating cells, DNMT1 may participate in regulating gene expression in a DNA methylation-independent manner, thereby contributing to the biological function of these cells.

Our results suggest that DNMT1 is not necessary for cell proliferation or differentiation of aNSCs in the DG (Figs. 2 and 3). Accumulated studies, however, have indicated a relationship of DNMT1 with cell proliferation and maintenance of stem cells (Georgia et al., 2013; Sen et al., 2010; So et al., 2011; Tsai et al., 2012). Inactivation of DNMT1 reportedly leads to demethylation of the promoters of cyclin-dependent kinase (CDK) inhibitor genes (*p16* and *p21*) and increases their expression, resulting in cell cycle arrest and/or cell senescence (So et al., 2011). In mesenchymal stem cells (MSCs), Oct4 and Nanog directly up-regulate the expression of DNMT1, thereby repressing *p16* and *p21* expression (Tsai et al., 2012). Moreover, DNMT1 also inhibits the expression of genes associated with lineage differentiation in MSCs. This contributes to the maintenance of undifferentiated state of MSCs. Likewise, in somatic tissue, *DNMT1*

413 deletion results in enhanced cell cycle arrest and premature differentiation of epidermal
414 progenitor cells, attributable to increased expression of CDK inhibitors and epidermal
415 differentiation-related genes (Sen et al., 2010). These findings highlight that DNMT1 is
416 required for cell proliferation and proper differentiation of certain types of stem cells. In
417 contrast to these reports, our data showed that NSCs in adult DGs did not exhibit
418 defects in cell proliferation or differentiation in response to DNMT1 loss. In support of
419 this finding, in *in vitro* culture of aNS/PCs, we also observed no difference in the
420 expression of CDK inhibitor genes (*p16* and *p21*) or *cyclin D1* after *Dnmt1* knockdown
421 (Fig. S1A). Such inconsistent outcomes of *Dnmt1* deletion in different cell types have
422 also been reported in the developing pancreas, in which pancreatic progenitors show no
423 evidence of premature differentiation or of defects in proliferation (Georgia et al., 2013).
424 The variable effects of DNMT1 deficiency in distinct cell types imply the existence of
425 mechanisms by which DNMT1 regulates proliferation and maintenance of stem cells in
426 a cell context-dependent fashion. A further possible explanation for the minor impact of
427 *Dnmt1* deletion on aNS/PC regulation may relate to the unique features of these cells,
428 which proliferate rarely and stay in a quiescent state, thus reducing the need for

429 maintenance DNA methylation.

430 In previous studies, DNMT1 function was investigated in eNS/PCs in the
431 context of neuronal survival during mouse CNS development (Fan et al., 2001; Hutnick
432 et al., 2009). *Dnmt1* deletion in eNS/PCs of the developing brain triggered DNA
433 hypomethylation and induced neuronal cell death, resulting in postnatal lethality of the
434 mice due to defective neuronal respiratory control (Fan et al., 2001). On the other hand,
435 deletion of *Dnmt1* in mature neurons caused neither DNA hypomethylation nor defects
436 in neuronal outgrowth or survival (Fan et al., 2001; Feng et al., 2010). Telencephalic
437 eNS/PC-specific *Dnmt1* deletion led to the production of DNA-hypomethylated neurons
438 with abnormalities in morphology and function (Hutnick et al., 2009): hypomethylated
439 cortical neurons exhibited defects in neuronal maturation, including aberrant dendritic
440 arborization and attenuated neuronal excitability, which are likely to have caused
441 increased neuronal death. Likewise, deletion of *Dnmt1* in retinal progenitors also
442 triggered DNA hypomethylation and led to impaired maturation and survival of retinal
443 neurons (Rhee et al., 2012). These findings suggest that DNMT1-dependent methylation
444 in eNS/PCs plays an essential role in the proper maturation and survival of neurons. In

445 the absence of DNMT1, proliferation could trigger passive DNA demethylation as cells
446 divide. As shown in the present study, type-2 and -3 cells in the DG are proliferative and
447 express DNMT1 at a high level during neuronal differentiation (Fig. 1D and E). Thus,
448 loss of DNMT1 in the aNS/PCs should lead to DNA demethylation, which would result
449 in the production of hypomethylated granule neurons. This might in turn impair
450 neuronal functions and induce poor survival of granule neurons in the DG of DNMT1
451 cKO(Nes). Telencephalic eNS/PC-specific *Dnmt1* deletion showed aberrant up- and
452 down-regulated expression of apoptosis- and neuronal function-related genes,
453 respectively (Hutnick et al., 2009). DNMT1 may therefore also regulate the expression
454 of such genes in the course of neuronal differentiation in the adult DG, which would
455 ensure the proper function and survival of differentiated neurons. Using *in vitro* culture
456 of aNS/PCs, we examined the expression of apoptosis- and neuronal function-related
457 genes after *Dnmt1* knockdown, but could find no difference in the expression of the
458 genes examined, such as *Ngfr* and *Kcnh5*, which were reported to be down-regulated by
459 telencephalic eNS/PC-specific *Dnmt1* deletion (Hutnick et al., 2009). Instead, we found
460 that *Dnmt1* knockdown in aNS/PCs up-regulated huntingtin interacting protein 1 (Hip1),

a known apoptosis-related gene (Fig. S1B). Hip1 induces apoptosis via the caspase pathway by directly binding and activating caspase-9 (Choi et al., 2006; Hackam et al., 2000). Neuronal cell death after NMDA-induced excitotoxicity is caused by a caspase-3 mediated-apoptotic mechanism (Okamoto et al., 2002; Zeron et al., 2004). Hip1 also interacts with NMDA receptors and modulates NMDA-induced excitotoxicity (Metzler et al., 2007), and a reduction of Hip1 decreased NMDA-induced neuronal apoptosis in both cortical and hippocampal neurons. Collectively, these findings suggest that increased expression of Hip1 might account for the reduced survival of granule neurons in DNMT1 cKO(Nes) mice that we observed in this study, and further investigation is clearly warranted to determine the exact mechanism by which DNMT1 in aNS/PCs contributes to the survival of granule neurons.

In summary, the results presented here provide substantial evidence that DNMT1 is required for the survival of neurons in the adult DG. In particular, DNMT1 in aNS/PCs, but not in post-mitotic neurons, plays a key role in neuronal survival. DNMT1 is highly expressed in proliferative cells in the adult DG, where it may regulate the expression of genes that induce neuronal cell death and disturb neuronal function

and maturation. Although we cannot yet determine the exact mechanism by which *Dnmt1* deletion leads to poor survival, the fact that DNMT1 is necessary for the maintenance of DNA methylation implies the importance of DNA methylation in regulating neuronal survival. We believe that the present findings will lead to a deeper understanding of the importance of epigenetic modifications in neurogenesis in the adult DG.

Acknowledgements

We thank Y. Bessho, T. Matsui, Y. Nakahata, T. Imamura and S. Katada for valuable discussions and M.E. Greenberg and Z. Zhou for sharing reagents. We are grateful to R. Jaenisch for *Dnmt1*^{flox} mice, R. Kageyama for Nestin-CreER^{T2} mice. We also appreciate the members of our laboratories, in particular T. Sanosaka and B. Juliandi for technical help and suggestions, A.M.D. Adefuin and M.C. Sanosaka for writing this manuscript and experimental support and C. Soldati for establishment of adult NS/PCs. We would like to thank I. Smith for helpful comments and grammatical corrections on the manuscript. Furthermore, we are very grateful to M. Tano and Y. Nakagawa for their

excellent secretarial assistance. We appreciate the technical assistance from The Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences. This work was funded by Core Research for Evolutional Science and Technology (CREST) from Japan Science and Technology Agency. H.N. received funding from a Grant-in-Aid for JSPS Fellows (no. 13J09870).

Author Contributions

H.N. contributed to the concept, design, execution and analysis of the experiments, and wrote the manuscript. A.K. contributed to the design and analysis of the experiments for DNMT1 cKO(Syn) mice. N.M. and T.M. contributed to analysis of the experiments and provided advice and technical expertise. M.N. provided advice and technical expertise and supervised the project. K.N. supervised the project and contributed to the concept and design of the experiments, provided funding, and wrote the manuscript.

Corresponding author

Correspondence to Kinichi Nakashima

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Figure Legends

Fig. 1. DNMT1 is highly expressed in proliferating cells in the adult DG. (A) *In situ* hybridization image of *Dnmt1* in a sagittal adult mouse brain section obtained from the Allen Brain Atlas. Red and yellow indicate high expression levels of *Dnmt1*. Note that *Dnmt1* is particularly highly expressed in the DG. TH, thalamus. HY, hypothalamus. (B) Representative immunofluorescence images of DNMT1 staining (red) in the DG. The inset shows Hoechst staining. Higher magnification of the white box in the left image (right) showed that DNMT1 is enriched in cells located in the SGZ of the DG. Scale bars: 200 μ m (left), 50 μ m (right). (C) Representative immunofluorescence images for DNMT1 (red), GFAP (green) and Sox2 (cyan) in the DG. Arrowheads designate GFAP⁺/Sox2⁺ type-1 cells. White arrows indicate GFAP⁻/Sox2⁺ cells, which show higher expression of DNMT1. (D) Representative immunofluorescence images for DNMT1 (red), Ki67 (green) and Sox2 (cyan) in the DG. White arrowheads indicate Ki67⁺/Sox2⁺ type-2 cells, which show high expression of DNMT1 compared with Ki67⁻/Sox2⁺ cells (white arrows). (E) Representative immunofluorescence images for

DNMT1 (red), Ki67 (green) and NeuroD (cyan) in the DG. The white arrowhead indicates a Ki67⁺/NeuroD⁺ type-3 cell. The white arrow represents a Ki67⁻/NeuroD⁺ immature neuron. Note that DNMT1 expression is strong in type-3 cells but weak in immature neurons. (F) Representative immunofluorescence images for DNMT1 (red), Ki67 (green) and Ctip2 (cyan) in the DG. Ctip2⁺ mature neurons show weaker expression of DNMT1 than Ki67⁺ proliferating cells (white arrowhead). (G) Representative immunofluorescence images for DNMT1 (red), GFAP (green) and S100 β (cyan) in the DG. The white arrowhead indicates a GFAP⁺/S100 β ⁺ astrocyte. Scale bar: 20 μ m for (C-G). (H) A schematic illustration of the expression of DNMT1 and other markers in each cell type in the DG.

Fig. 2. Loss of DNMT1 does not impair cell proliferation or maintenance in aNS/PCs.

(A) Diagram of the *Dnmt1* conditional knockout strategy in aNS/PCs. *Dnmt1*^{flox/flox} mice were crossed with Nestin-CreER^{T2} mice, yielding progeny in which tamoxifen administration results in the deletion of *Dnmt1* (exon 4 and 5) in Nestin-expressing aNS/PCs. (B) Nestin-CreER^{T2};*Dnmt1*^{flox/flox} (DNMT1 cKO(Nes)) and their littermate

control mice were injected with tamoxifen once a day for 4 consecutive days and fixed at 8 days or 36 days after the last tamoxifen injection. (C) Representative immunofluorescence images for DNMT1 (red) and Ki67 (green) in the DG of control and DNMT1 cKO(Nes) mice at 8 days after the last tamoxifen injection. Scale bar: 100 μ m. (D) Representative immunofluorescence images for DNMT1 (red), GFAP (green) and Sox2 (cyan) in the DG of control and DNMT1 cKO(Nes) mice at 8 days after the last tamoxifen injection. Scale bar: 20 μ m. (E) Representative immunofluorescence images for Ki67 (green) in the DG of control and DNMT1 cKO(Nes) mice at 8 and 36 days after the last tamoxifen injection. DNA was stained with Hoechst (blue). Scale bar: 200 μ m. (F) Quantification of Ki67⁺ cells in (E). There is no difference between control and DNMT1 cKO(Nes) mice. (G) Representative immunofluorescence images for Nestin (red), GFAP (green) and Sox2 (cyan) in the DG of control and DNMT1 cKO(Nes) mice. Nestin⁺/GFAP⁺/Sox2⁺ cells (white arrowheads) indicate type-1 cells, showing the comparable morphology of type-1 cells between control and DNMT1 cKO(Nes) mice. Scale bar: 50 μ m. (H) The bar graph indicates the number of type-1 cells in the DG. There is no difference between control and DNMT1 cKO(Nes) mice.

748 In (F) and (H), values represent mean $\pm$ SEM; n.s., not significant with $P > 0.05$.

749 Student's *t*-test.

750

751 **Fig. 3.** DNMT1 deletion in aNS/PCs does not affect their differentiation but results in

752 reduced numbers of mature neurons. (A) Experimental scheme of tamoxifen and BrdU

753 injection for BrdU-positive cell tracing. Tamoxifen-treated mice were injected with

754 BrdU once a day for 7 days and fixed 1 day or 4 weeks after the last BrdU injection. (B)

755 Representative immunofluorescence images for BrdU (red) and DCX (green) in the DG

756 of control and DNMT1 cKO(Nes) mice at 1 day after the last BrdU injection. DNA is

757 stained with Hoechst (blue). Scale bar: 200 μ m. (C) Representative

758 immunofluorescence images for BrdU (red) and NeuN (green) in the DG of control and

759 DNMT1 cKO(Nes) mice at 4 weeks after the last BrdU injection. DNA is stained with

760 Hoechst (blue). Scale bar: 200 μ m. (D) Quantification of BrdU+ cells in (B) and (C),

761 showing a significant reduction of BrdU+ cells in the DGs of DNMT1 cKO(Nes) mice

762 4 weeks after the last BrdU injection. (E) Survival ratio of BrdU+ cells, calculated by

763 dividing the number of BrdU+ cells at 4 weeks after by the number at 1 day after the

last BrdU injection. DNMT1 cKO(Nes) mice showed a significantly reduced survival ratio. (F and G) Quantification of the percentage of DCX+ cells among BrdU+ cells (F) and the total number of BrdU+/DCX+ cells (G) in the DG. (H) Representative immunofluorescence images for BrdU (red) and S100 β (green) in the ML of control and DNMT1 cKO(Nes) DGs at 4 weeks after the last BrdU injection. Arrowheads indicate representative BrdU-labeled S100 β + cells. Scale bar: 50 μ m. (I) The bar graph indicates the number of BrdU+/NeuN+ or BrdU+/S100 β + cells in the DG of control and DNMT1 cKO(Nes) mice at 4 weeks after the last BrdU injection. DNMT1 cKO(Nes) mice show a significant reduction of BrdU+/NeuN+ cells. Values represent mean $\pm$ SEM; n.s., not significant with $P > 0.05$. * $P < 0.05$. Student's *t*-test.

Fig. 4. Deletion of *Dnmt1* in post-mitotic neurons does not cause neuronal loss or defective neurogenesis. (A) Diagram of the *Dnmt1* conditional knockout strategy in aNS/PCs. *Dnmt1*^{flox/flox} mice were crossed with Synapsin1-Cre mice, yielding progeny in which *Dnmt1* in *synapsin1*-expressing post-mitotic neurons is deleted (DNMT1 cKO(Syn)). (B) Representative immunofluorescence images for DNMT1 (red), Ki67

(green) and Ctip2 (cyan) in the DG of control and DNMT1 cKO(Syn) mice. Scale bar: 200 μ m. (C) Higher-magnification images of the white boxes in (B). Scale bar: 100 μ m. (D) Representative immunofluorescence images for DCX (red), NeuN (green) and Sox2 (cyan) in the DG of control and DNMT1 cKO(Syn) mice, showing indistinguishable structure of the GCL between control and DNMT1 cKO(Syn) mice. Scale bar: 200 μ m. (E and F) Quantification of the number of Sox2+ (E) and DCX+ cells (F) in the SGZ of the DG. There are no significant differences between control and DNMT1 cKO(Syn) mice. Values represent mean $\pm$ SEM; n.s., not significant with $P > 0.05$. Student's *t*-test.

Fig. 5. Summary of DNMT1 function in adult neurogenesis. DNMT1 is strongly expressed in proliferating type-2 and -3 cells during neurogenesis in the adult DG. Loss of DNMT1 in aNS/PCs does not lead to defects in cell proliferation or maintenance of aNSCs. Instead, the effect of *Dnmt1* deletion (i.e., poor survival) emerges after aNS/PCs accomplish their differentiation to neurons (middle). On the other hand, deletion of *Dnmt1* in post-mitotic neurons does not affect neuronal survival (bottom).