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Transient Accumulation of 5-Carboxylcytosine Indicates Involvement of Active Demethylation in Lineage Specification of Neural Stem Cells

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SUMMARY

5-Methylcytosine (5mC) is an epigenetic modification involved in regulation of gene activity during differentiation. Tet dioxygenases oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Both 5fC and 5caC can be excised from DNA by thymine-DNA glycosylase (TDG) followed by regeneration of unmodified cytosine via the base excision repair pathway. Despite evidence that this mechanism is operative in embryonic stem cells, the role of TDG-dependent demethylation in differentiation and development is currently unclear. Here, we demonstrate that widespread oxidation of 5hmC to 5caC occurs in postimplantation mouse embryos. We show that 5fC and 5caC are transiently accumulated during lineage specification of neural stem cells (NSCs) in culture and in vivo. Moreover, 5caC is enriched at the cell-type-specific promoters during differentiation of NSCs, and TDG knockdown leads to increased 5fC/5caC levels in differentiating NSCs. Our data suggest that active demethylation contributes to epigenetic reprogramming determining lineage specification in embryonic brain.

INTRODUCTION

5-Methylcytosine (5mC) is a DNA modification contributing to the regulation of gene activity during development and differentiation (Reik et al., 2001; Bird, 2002). Tet (Ten-eleven translocation) dioxygenases (TET1/2/3) can oxidize 5mC, generating 5-hydroxymethylcytosine (5hmC), which, according to a growing body of evidence, plays specific biological roles in embryonic stem cells (ESCs) and the mammalian brain (Tahiliani et al., 2009; Ficz et al., 2011; Lister et al., 2013). 5hmC can be enzymatically oxidized further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al., 2011; He et al., 2011). Both 5fC and 5caC can be recognized and excised from DNA by thymine-DNA glycosylase (TDG) followed by subsequent regeneration of unmodified cytosine by the components of base excision repair (BER) pathway (He et al., 2011; Maiti and Drohat, 2011). Correspondingly, recent genome-wide analyses of 5fC/5caC distribution in mouse ESCs (mESCs) revealed a TDG-dependent accumulation of these marks in gene regulatory elements, suggesting that this mechanism of active demethylation is operative in mESCs (Shen et al., 2013; Song et al., 2013).

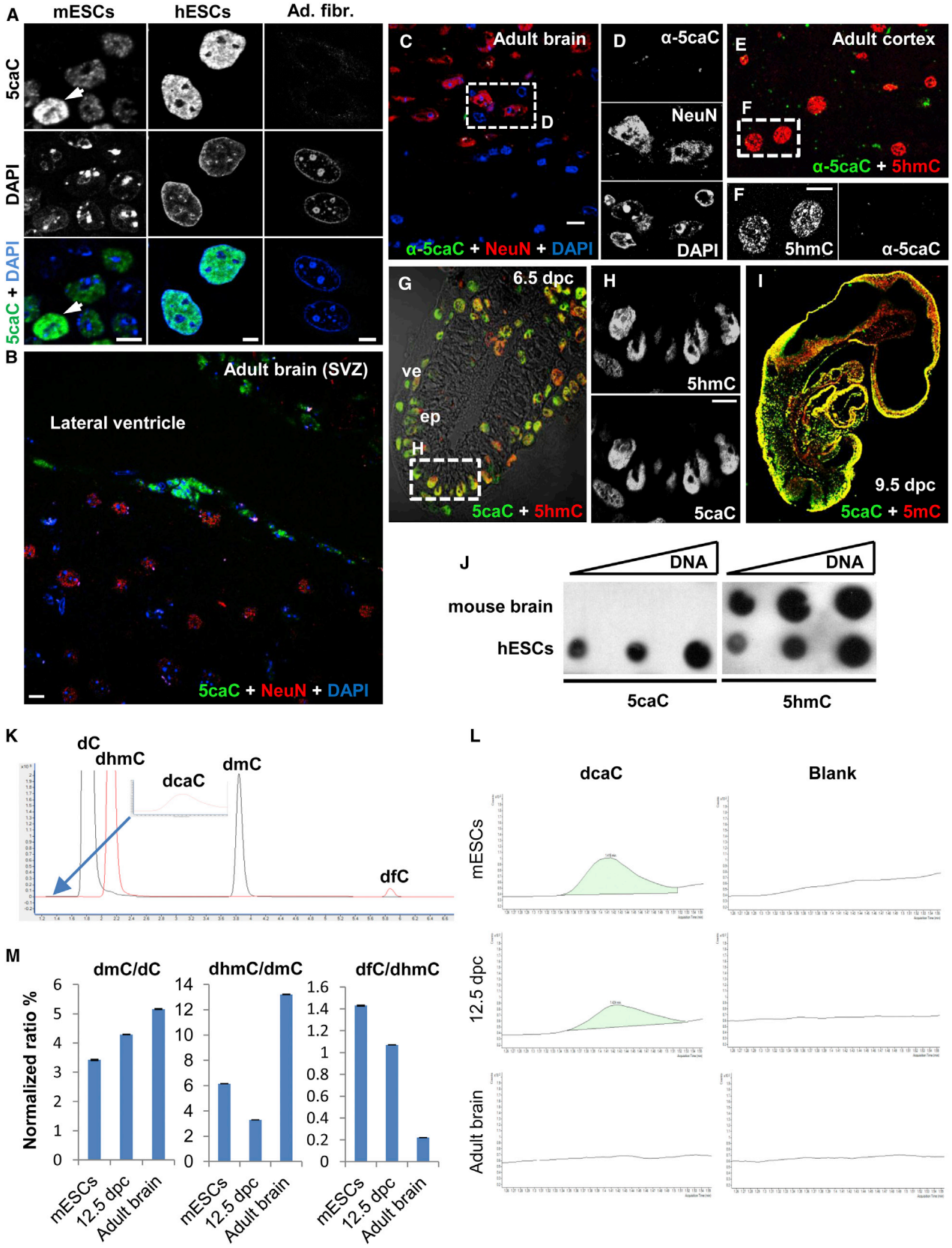
Paradoxically, contrary to the mESC-based results, recent studies imply that a passive replication-dependent demethylation mechanism is involved in epigenetic reprogramming during development of primordial germ cells (PGCs) (Seisenberger et al., 2012; Hackett et al., 2013). Likewise, although 5fC and 5caC are detectable in mouse zygotes, these marks are diluted in a replication-dependent manner during preimplantation development instead of being actively removed from DNA (Inoue et al., 2011). Thus, the role of BER-dependent demethylation in cellular differentiation and development is currently unclear.

In this study, we aimed to examine if a TDG/BER-dependent active DNA demethylation pathway is employed during mammalian embryogenesis and differentiation.

RESULTS

Widespread Oxidation of 5hmC to 5caC Occurs in ESCs and in Mouse Postimplantation Embryos

Since 5fC and 5caC can serve as intermediates in active demethylation, we checked whether these marks are detectable in ESCs and postimplantation embryos employing a sensitive immunostaining method based on the use of peroxidase-conjugated secondary antibody and tyramide amplification reagent, which we have previously applied to 5hmC detection (Ruzov



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et al., 2011). Using a 5caC antibody (Active Motif), which specifically discriminates this mark from other forms of modified cytosine (Inoue et al., 2011), we performed 5caC immunostaining on mESCs, human ESCs (hESCs), and mouse embryonic sections using human adult-derived fibroblasts and adult mouse brain tissue as controls (Figures 1A–1I). Whereas hESCs and a subpopulation of mESCs were highly positive for 5caC, it was undetectable in the fibroblast cultures (Figure 1A). Likewise, although some cells in the subventricular zone (SVZ) of the brain displayed a nonnegligible 5caC staining (Figure 1B), we could not detect it in the hippocampus or cortex of adult brain (Figures 1C–1F; Figure S1A). In contrast, 5caC was present in both epiblast and visceral endoderm of embryos at 6.5 days postcoitum (dpc) and in a range of embryonic tissues at 9.5 and 12.5 dpc (Figures 1G–1I; Figure S1B). We confirmed the specificity of this staining in competition experiments and in dot blots (Figure 1J; Figures S1C and S1D).

Although we observed elevated levels of 5fC signal in the same contexts as 5caC enrichment, the intensity of 5fC staining was always considerably weaker than that of 5caC (Figures S1E and S1F). Since 5fC antibody was at least ten times less sensitive than 5caC in dot blots with modified DNA standards (Figure S1G), the weak intensity of 5fC staining was likely due to the relatively low sensitivity of the corresponding antibody.

To confirm our results, we performed mass spectrometry (MS) detection of 5caC and 5fC in mESCs, embryonic head tissue at 12.5 dpc, and the adult brain samples. Although 5caC signal was not strong enough for the accurate MS quantification, in agreement with our antibody-based results, we could detect this mark by MS in ESCs and in 12.5-dpc embryonic head samples but not in the DNA isolated from the adult mouse brain (Figures 1K and 1L; Figure S1H). MS quantification of 5fC in the same samples revealed that the 5fC/5hmC ratio was also significantly lower in the adult brain DNA compared with that derived from 12.5 dpc embryonic tissue or ESCs (Figure 1M).

Whereas the intensity of 5caC immunostaining signal was fairly uniform in hESCs, it varied widely between different cells in a mESC colony, ranging from undetectable (54.2% cells) to intensities comparable to that observed in hESCs (12.5% cells) (Figures S2A–S2D). Correspondingly, the levels of 5caC differed several fold between mouse and human ESCs in DNA dot blots (Figure S2E). Although the percentage of 5caC highly positive mESCs appeared higher in 2i medium than in serum-containing medium (Figure S2F), patterns of 5caC distribution were similar for both culturing conditions. Likewise, mESCs synchronized in early S-phase retained the heterogeneity of 5caC signal (Figure S2G). We also did not detect any correlation between the intensity of 5caC staining and the expression levels of Oct4 or Nanog in mESCs (Figure S2H). Notably, in both hESCs and mESCs, 5caC was predominantly euchromatic, displaying a significantly higher degree of colocalization with 5hmC than with 5mC (Figures S3A–S3J).

Dynamics of the Spatial Distribution of 5caC and 5hmC in Mouse Embryonic Brain

Because 5caC was detectable in embryonic brain, but not in most of the adult brain cells (Figures 1B–1F; Figure S1B), we assessed the distribution of both 5hmC and 5caC during several stages of embryonic brain development. In the adult brain, 5hmC was considerably more enriched in NeuN-expressing postmitotic neurons than in glial cells (Figures S4A and S4B). In contrast, most of the brain cells 12.5 dpc exhibited 5hmC staining of fairly equal strength (Figures S4C and S4D). This pattern of 5hmC distribution changed at 13.5 dpc where, similar to adult brain, 5hmC staining intensity was variable between different cellular populations (Figures S4C–S4F). This alteration in the patterns of 5hmC distribution corresponds to a period when active specification of neuronal and glial precursors is underway (Liu et al., 2002; Delaunay et al., 2008) and correlates with a gradual increase in mRNA levels of *Tet2*, de novo methyltransferase *Dnmt3a*, and neuronal markers (*β-III tubulin*) and with a concomitant reduction of stem cell markers (e. g. *Sox2*) and *Dnmt3b* mRNAs (Figures S4G–S4I). Notably, the expression of BER pathway components, *Tdg* and *PARP1*, peaks at the same time point, between 9.5 and 13.5 dpc (Figure S4J).

5caC staining intensity in brain cells was relatively low at 11.5 dpc, peaking at 12.5 dpc, and then dropping by 70% at 13.5 dpc (Figures 2A and 2B). This corresponded to a decrease of 5caC:5hmC colocalization (from 40% to 3%–5%) during developmental progression (Figure 2C). DNA dot blots confirmed the immunostaining results (Figures 2D and S2E). 5caC and 5hmC were distributed in a semi-inverse manner in brain cells 12.5–13.5 dpc, with 5caC staining becoming more restricted by 13.5 dpc (Figures 2E–2I). Notably, whereas the majority of

Figure 1. 5caC Is Detectable in ESCs and Postimplantation Embryos

(A) 5caC signal in mESCs, hESCs, and human adult-derived fibroblasts (Ad. fibr.). Samples were processed using identical experimental conditions and imaged at the same settings. Single channels and merge views are shown.

(B–D) 5caC costaining with NeuN in the SVZ (B) and in a region of adult brain localized at the border of gray and white matter, shown in (C) and (D).

(E and F) 5caC costaining with 5hmC in a representative region of adult brain cortex.

(G and H) 5caC and 5hmC signals in mouse embryo 6.5 dpc. Ep, epiblast; ve, visceral endoderm.

(I) 5caC/5mC immunostaining of a sagittal section of mouse embryo 9.5 dpc.

Merge views are shown in (B), (C), (E), (G), and (I); single channels for the areas marked with dotted squares are shown in (D, (F), and (H). The slides shown in (E), (F), and (G) through (I) were processed in parallel and imaged at the same settings. Scale bars, 10 μm.

(J) DNA dot blot of 5caC and 5hmC in hESCs and adult mouse brain tissue.

(K) Overlaid of total ion chromatogram of global dC, dmC, dhmC, dfC, and dcaC content recorded in dynamic multiple reaction monitoring mode. Red line profile represents approximately 250 times more concentrated DNA than the black line profile. The insert shows a 1,000× expansion of the mass count axis in the dcaC region.

(L) dcaC peaks in mESCs, head tissue 12.5 dpc, and adult brain DNA samples accompanied with blank runs shown in the same scale. The amount of DNA analyzed in mESC samples were about three times higher than in the other samples.

(M) dmC/dC, dhmC/dmC, and dfC/dhmC ratios obtained from the quantification of MS peaks in mESCs, head tissue 12.5 dpc, and adult brain DNA samples. See also Figures S1–S3.

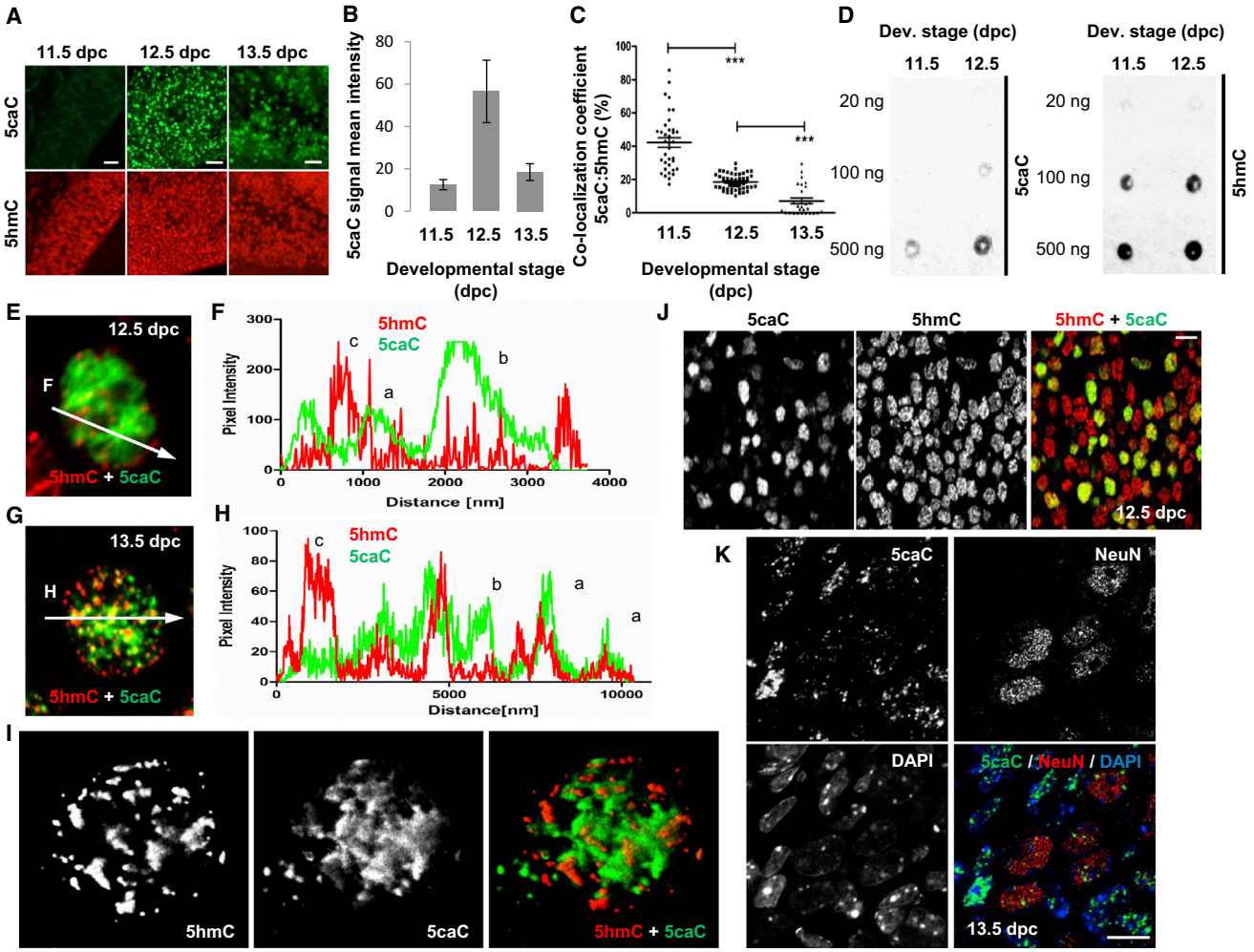


Figure 2. 5caC Is Transiently Accumulated in the Embryonic Brain between 11.5 and 13.5 dpc

(A) 5caC/5hmC immunostaining of equivalent forebrain regions analogous to the ones presented in Figure S1C at 11.5, 12.5, and 13.5 dpc. Sections were processed using identical experimental conditions and imaged at the same settings.

(B) 5caC signal intensity quantification in the sections shown in (A). Data are mean signal intensities ± SEM.

(C) 5caC:5hmC colocalization coefficient values for the equivalent region of embryonic forebrain at 11.5, 12.5, and 13.5 dpc. ***$p < 0.0001$.

(D) DNA dot blot of 5caC and 5hmC in embryonic head tissue 11.5 and 12.5 dpc. The amounts of DNA loaded on to membranes are indicated.

(E–H) Merge views in (E) and (G) and the profiles of 5hmC and 5caC signal intensities in (F) and (H) demonstrate the pattern of 5hmC and 5caC nuclear distribution in brain cells 12.5 and 13.5 dpc. 5caC intensity follows that of 5hmC in some regions (a), whereas in other areas, high 5caC signal corresponds to low 5hmC intensity (b) or strong 5hmC staining parallels weak 5caC signal (c).

(I) 3D reconstruction of 5hmC and 5caC signals distribution in a nucleus of a forebrain cell 13.5 dpc.

(J) 5caC and 5hmC staining in forebrain 12.5 dpc.

(K) 5caC codetection with NeuN in brain 13.5 dpc.

Single channels and merge views are shown in (I) through (K).

Scale bars, 25 μm in (A) and 10 μm in (J) and (K).

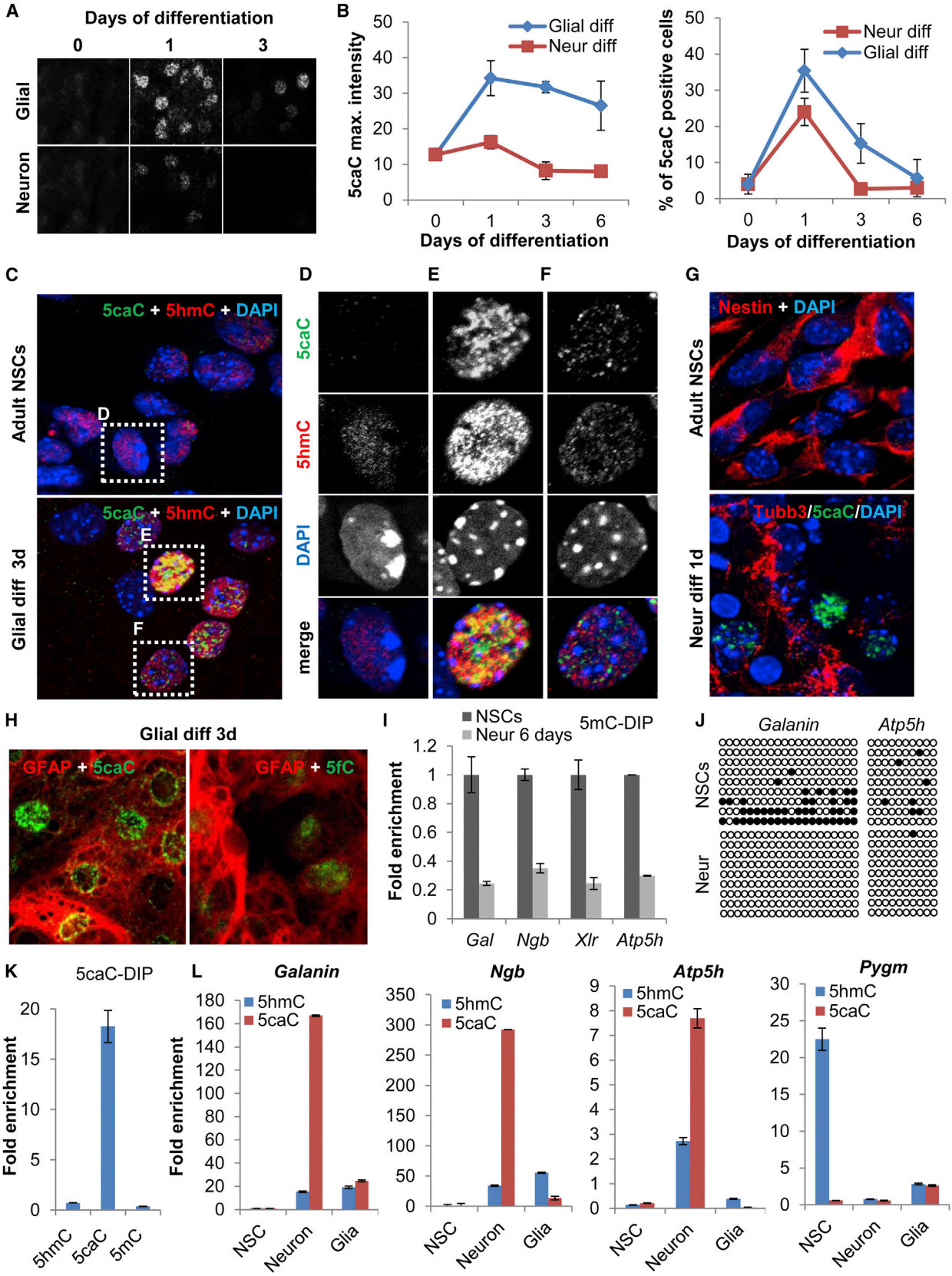
See also Figure S1C.

brain cells 12.5 dpc exhibited 5hmC signal of similar strength, the 5caC staining intensity varied between them from strong to virtually undetectable (Figure 2J). Moreover, levels of 5caC signal were lower in NeuN-expressing cells at 13.5 dpc, indicating that the degree of 5hmC oxidation in embryonic brain may be cell type specific (Figure 2K).

Thus, the cell populations of embryonic brain start to differ in their 5hmC content between 12.5 and 13.5 dpc, which parallels with temporary accumulation of 5caC at certain regions of their genome.

5fC and 5caC Are Transiently Accumulated during Neuronal and Glial Differentiation of Neural Stem Cells

To identify the 5caC-enriched cell types, we examined the levels of this mark during differentiation of adult neural stem cells (NSCs) to neuronal and glial lineages in vitro. Although 5caC



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was undetectable in the majority of undifferentiated NSCs, strongly 5caC-positive cells started to appear in NSC cultures at initial stages of glial and neuronal differentiation simultaneously, with cells expressing early neuronal (β-III tubulin) and glial (glial fibrillary acidic protein; GFAP) markers (Figures 3A–3H). This 5caC accumulation was transient, with highest numbers of 5caC-positive cells at days 1–3 after induction of differentiation (Figures 3A and 3B). The 5caC dynamics and staining intensity differed between the two differentiation regimes, with markedly higher levels of 5caC signal observed during glial differentiation (Figures 3A and 3B). Similar to embryonic brain (Figures 2D–2H), 5caC and 5hmC exhibited semi-inverse patterns of nuclear distribution in differentiating NSCs (Figures 3A and 3C–3F). Although 5fC was also detectable at the same stages of glial differentiation, its staining intensity was considerably weaker than that of 5caC (Figure 3H).

A number of promoters of genes involved in adult neurogenesis were reported to be hypermethylated in *Tet1* knockout (KO) mice (Zhang et al., 2013). Our 5mC DNA immunoprecipitation (DIP) and bisulfite sequencing experiments revealed that several of these promoters exhibited decreases in 5mC levels during neural differentiation of NSCs (Figures 3I and 3J). To test if this demethylation involved oxidation of 5hmC to 5caC, we used 5hmC/5caC DIP. In agreement with a previous study (Shen et al., 2013), the 5caC antibody specifically recognized this mark in DIP with synthetically modified DNA fragments (Figure 3K). While 5caC was effectively undetectable at tested promoters in undifferentiated NSCs, it was enriched at the promoters of *Galanin*, *Neuroglobin* (*Ngb*), and *Atp5h* in the cells differentiating into neuronal, but not into glial, lineages (Figure 3L).

Collectively, these results indicate that 5caC transiently accumulates at cell-type-specific genomic regions during the initial stages of the differentiation of NSCs.

TDG Knockdown Leads to an Increase in 5fC/5caC during Glial Differentiation

Since the 5caC accumulation was transient, to determine whether TDG is involved in elimination of this mark from DNA, we performed RNA-interference-mediated TDG knockdown (siTDG) in fetal NSCs, followed by their differentiation and subsequent analysis of 5caC distribution (Figure 4A). siTDG treatment resulted in approximately 75% downregulation of TDG expression compared to NSCs transfected with nontargeting small interfering RNA (siControl) (Figure 4B). Neither siTDG nor siControl undifferentiated NSCs exhibited any significant 5caC (Figures 4C and 4D) or 5fC (data not shown) staining. In line with previous observations that *Tdg* KO neuronal progenitor cells failed to differentiate to neurons due to rapid loss of cell viability (Cortázar et al., 2011), siTDG resulted in massive cell death during the initial 2 days of neuronal differentiation, complicating any analysis of 5caC distribution. However, although most siControl cells exhibited weak or absent 5fC/5caC staining at day 3 of glial differentiation, we observed a significant increase in the number of highly 5fC/5caC-positive cells following siTDG, with a high proportion of 5caC-positive GFAP-expressing cells, suggesting that TDG is involved in elimination of this mark from DNA during differentiation (Figures 4E and 4F).

Given that, using DIP, we detected the accumulation of 5caC at CpG-rich promoter regions of a number of key glial markers (*Olig1*, *Olig2*, *Fgfr1*, *Fgfr4*, *Pdgfr*, and *GFAP*) during glial differentiation (Figure 4G), which corresponded to a demethylation of specific CpGs in differentiated cells (Figure 4H), we examined expression of these genes in siTDG glial cells. Remarkably, some of the corresponding mRNAs were significantly (four to five times for *GFAP* and *Pdgfr)* upregulated on siTDG treatment (Figure 4I), which may indicate a functional link between 5hmC oxidation and transcription akin to that previously reported for mESCs (Raiber et al., 2012).

DISCUSSION

The profiles of 5mC and 5hmC distribution are dynamic during brain development and vary between different brain cell types (Lister et al., 2013; Kriaucionis and Heintz, 2009). Moreover, in fetal brain, 5hmC is enriched at putative regulatory elements that are hypomethylated in the adult brain, suggesting Tet-dependent demethylation of these sequences during development (Lister et al., 2013). In line with this, TET1 has been reported to promote BER-dependent active DNA demethylation in the adult brain via conversion of 5mC to 5hmC (Guo et al., 2011). Furthermore, *Tet1* KO mice are characterized by altered neurogenesis, poor learning, and impaired memory extinction, associated with hypermethylation of promoters of neurogenesis-related genes (Zhang et al., 2013; Rudenko et al., 2013). The demethylation of specific promoters has also been

Figure 3. 5caC Is Transiently Accumulated during Differentiation of Adult NSCs

(A) 5caC staining in adult NSCs differentiating toward glial and neuronal lineages at indicated days postinduction. Samples were processed in parallel and imaged at the same settings.

(B) Results of quantification of 5caC signal maximal intensity and percentages of 5caC-positive cells during initial days of adult NSC differentiation. Data are means ± SEM.

(C–F) Codetection of 5caC with 5hmC in undifferentiated adult NSCs (upper panel) and at day 3 of their glial differentiation (lower panel). Merged views are shown in (C) and single channels for the nuclei marked with dotted rectangles in (D) through (F).

(G) Immunostaining of undifferentiated adult NSCs for nestin and NSCs at day 1 of neuronal differentiation for 5caC and β-III tubulin (Tubb3).

(H) Coimmunostaining of 5caC (left panel) and 5fC (right panel) with GFAP in NSCs at day 3 of glial differentiation. Identical exposure settings were used.

(I) 5mC DIP of indicated promoters in undifferentiated NSCs and in NSCs at day 6 of neuronal differentiation.

(J) Bisulfite sequencing of indicated promoter regions in undifferentiated NSCs and NSCs at day 6 of neuronal differentiation (Neur diff). Filled circles represent protected (5mC or 5hmC), and empty circles represent unprotected (unmodified C) CpGs.

(K) 5caC DIP of 5hmC-, 5caC-, or 5mC-containing oligonucleotides spiked with carrier DNA.

(L) 5hmC and 5caC DIP on indicated promoters in undifferentiated NSCs and in NSCs differentiating toward neuronal (Neuron) and glial (Glia) lineages. Experimental error is expressed as ±SEM.

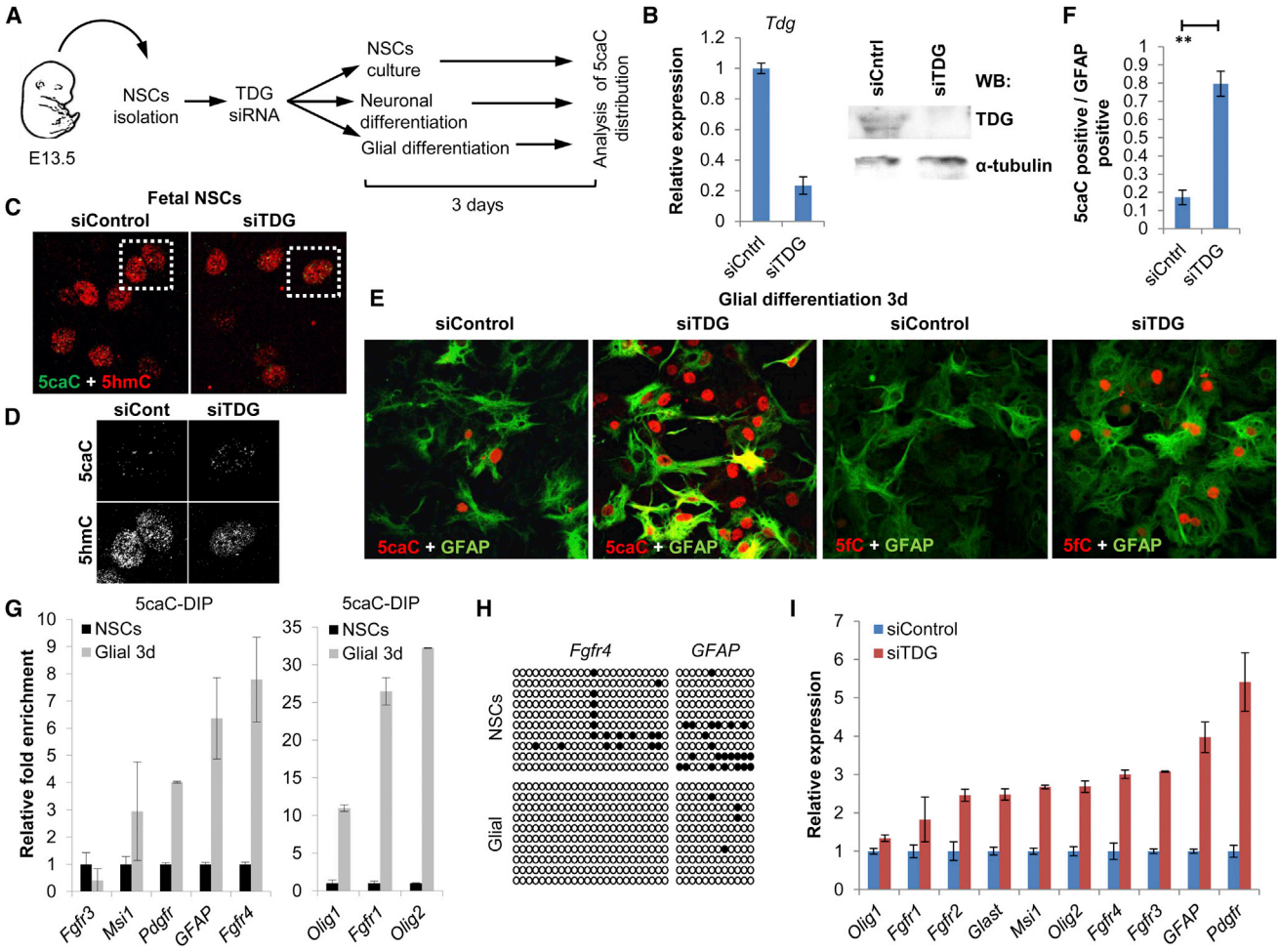

Figure 4. TDG Depletion Leads to Increase in 5fC/5caC Content during Glial Differentiation

(A) Schematic illustrating the design of experiment on TDG depletion in differentiating NSCs.

(B) Expression of TDG in siControl and siTDG NSCs.

(C and D) 5caC/5hmC immunostaining of siControl and siTDG undifferentiated NSCs. Merged views are shown in (C), and single channels for insets are shown in (D).

(E) Coimmunostaining of 5caC (left panels) and 5fC (right panels) with GFAP in siControl and siTDG NSCs at day 3 of glial differentiation. Merged views are shown.

(F) Numbers of 5caC-positive cells normalized to the numbers of GFAP-expressing cells in siControl and siTDG NSCs at day 3 of glial differentiation. Data are means ± SEM. **$p < 0.001$.

(G) Bisulfite sequencing of indicated CpG-rich promoter regions in NSCs and glial cells. Filled circles represent protected (5mC or 5hmC) CpGs; empty circles represent unprotected (unmodified C) CpGs.

(H) 5caC DIP on indicated promoters in undifferentiated NSCs and in NSCs at day 3 of glial differentiation.

(I) Relative expression levels of indicated mRNAs in siTDG and siControl NSCs at day 3 of glial differentiation.

implicated in the mechanisms of neuronal to glial switch as a determinant of astrocyte (Fan et al., 2005; Namihira et al., 2009) and neuronal differentiation (Martinowich et al., 2003). Correspondingly, in the present study, we show that the initial stages of glial and neuronal differentiation are characterized by global accumulation of 5caC, suggesting an involvement of a 5caC/TDG/BER-dependent demethylation in lineage specification of NSCs. In agreement with our results, *Tdg* KO leads to aberrant patterns of DNA methylation and embryonic lethality at 11.5 dpc (Cortellino et al., 2011; Cortázar et al., 2011), the stage immediately preceding the peak of 5caC enrichment in embryonic brain that we observed. Likewise, demethylation of *GFAP* promoter, where we detected 5caC accumulation during glial differentiation, has been reported as a requirement for GFAP expression and astrocyte differentiation (Namihira et al., 2009).

Consistent with the *Tdg* KO study in which epigenetic abnormalities were evident only upon cell lineage commitment (Cortázar et al., 2011), we witnessed an increase in 5caC-positive cells during differentiation but not in undifferentiated siTDG NSCs. This may indicate that 5caC/TDG/BER-dependent demethylation is utilized as a general mechanism for rearrangement of the 5hmC/5mC profiles during terminal differentiation of postmitotic somatic cell types in mammals. In contrast, analogous to

PGCs and preimplantation embryos (Hackett et al., 2013; Inoue et al., 2011), a potentially less mutagenic replication-dependent demethylation mechanism may be operative in actively dividing embryonic cellular populations.

In this respect, the elevated levels of 5caC we observed in a subpopulation of mESCs may indicate the existence of a dynamic stem cell state associated with epigenetic priming of differentiation. Contrasting with mESCs, we observed strong uniform 5caC staining in hESCs. Given that unlike mESC media the KnockOut Serum Replacement used for our hESCs culture contains ascorbic acid (Chung et al., 2010), promoting Tet-mediated 5caC generation (Yin et al., 2013), the high levels of this mark in hESCs may be due to the ascorbic-acid-dependent activation of Tet enzymatic activity occurring in these cells.

EXPERIMENTAL PROCEDURES

ESCs Culture

All the animal-involved procedures were performed in accordance with the University of Nottingham review board. HM1 mESCs were cultured either in serum-containing media or under 2i medium conditions. HUES-7 hESCs were maintained without feeders in conditioned medium containing KnockOut Serum Replacement (Life Technologies).

Derivation, Culture, and Differentiation of NSCs and RNA Interference Knockdown of TDG

The derivation, culture, and differentiation of NSCs were performed as described elsewhere (Alcock and Sottile, 2009). For TDG depletion, fetal NSCs were transfected with 50 pmol of Dharmacon siGENOME siRNA duplexes (Thermo Fisher Scientific) against mouse TDG (Thermo Fisher Scientific, catalog number M-040666-01) and nontargeting siRNA #2 (Thermo Fisher Scientific, catalog number D-001210-02) using Lipofectamine 2000 in antibiotic-free medium. The transfections were repeated after 48 hr. Differentiation was induced 24 hr after the second transfection. Control NSCs were kept in NSC medium. Cells were collected for analysis 72 hr after induction of differentiation.

Immunocytochemistry, Immunohistochemistry, and Dot Blots

These procedures were performed as described elsewhere (Ruzov et al., 2011). Antibodies are listed in the Supplemental Information.

Mass Spectrometry

This was carried out according to the procedures of Hashimoto et al. (2014).

Confocal Microscopy

Images (400–600 nm optical sections) were acquired with a Zeiss LSM 700 AxioObserver confocal microscope using a Plan-Apochromat 63×/1.40 Oil DIC M27 objective and processed using ImageJ, Adobe Photoshop, and ZEN Zeiss LSM 700 imaging software.

5mC, 5hmC, and 5caC DIP

These were carried out according to the procedures of Ficz et al. (2011) and Shen et al. (2013) using mouse monoclonal 5mC antibody (clone 33D3, Diagenode), rabbit polyclonal 5hmC and 5caC antibodies (Active Motif), and magnetic Dynabeads (Invitrogen).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.05.003.

AUTHOR CONTRIBUTIONS

L.M.W. and A.A. performed immunostaining, microscopy, and image analysis; Z.F. contributed to DIP, siRNA analysis, and real-time PCR; N.D., S.G., J.M.F., and I.R.C. performed MS; T.D., D.C., J.E.D., S.S., and V.S. contributed cell lines and reagents; A.R., L.M.W., A.A., Z.F., M.L., V.S., and A.D.J. analyzed the data; A.R. conceived the project and wrote the paper.

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