**Supplementary Method**

**glucMS-qPCR assay.**

Genomic DNA were purified using AllPrep DNA/RNA Mini Kit (QIAGEN) from BJF1 mouse cerebrum and liver. 200 ng of genomic DNA from BJF1 mouse cerebrum and liver were treated with or without T4-BGT (New England Biolabs) and UDP-glucose for the glucosylation of hmC according to the instruction manual accompanied with the enzyme, followed by the purification of DNA using AMPure XP. Then the DNA was digested by the restriction endonuclease *Msp* I (New England Biolabs) that is sensitive to glucosyl-5-hydroxymethylcytosine and digested DNA was purified using AMPure XP, resuspended in 10 mM Tris-HCl (pH 8.0). Finally, the DNA amount was quantified by 260 nm absorbance by Nanodrop One (Thermo Scientific) and the 20 ng of digested genomic DNA was amplified and quantified using qPCR with specific primers for *Arhgap27*, *Nhlrc1* and *Peg3* (no digestion control). qPCR was conducted using LightCycler 480 system with 384 well block and LightCycler 480 SYBR Green I Master Mix (Roche Life Science). Reaction volume was 15 µl and PCR cycles was done as the following condition. After incubate at 95 °C 5 minutes, 40 cycles of denaturing at 95 °C 10 seconds, annealing at 60 °C 15 seconds and extension at 72 °C 10 seconds. All samples were analyzed in triplicate and qPCR results were quantified as mean percentage of the amount of *Arhgap27* or *Nhlrc1* per the mean amount of *Peg3*. The following primers were used for qPCR amplification.

*Arhgap27*-F (chr11:103333805-103333823); GTGAGGCGCTGCCTTGTCT

*Arhgap27*-R (chr11:103333942-103333923); CCAGACCAGGTGTGGATG

*Nhlrc1*-F (chr13:47014069-47014088); CTCTGCATCAGTCACCAGGA

*Nhlrc1*-R (chr13:47014335-47014316); GGGAAAGAGACGGGTCAAGAT

*Peg3*-F (chr7:6704106-6704124); TAAGCAATACGGGCAGCCT

*Peg3*-R (chr7:6704315-6704295); CCAACAAACTTCTGGTAACGC
Hairpin TAB sequencing.

200 ng of genomic DNA were digested with the appropriate restriction endonuclease (i.e. Bfa I (New England Biolabs) for Arhgap27 and Taq I (Takara) for Nhlc1), end-repaired and A-tailed as in the EnIGMA method. Then the DNA was ligated with hairpin-shaped adaptor DNA. The resulting DNA was further digested with appropriate second restriction enzymes (i.e. Hae III (Takara) for Arhgap27 and Nhlc1). The DNA was next cleaned up with AMPure XP and treated with the T4-BGT and the TET1 enzyme (WiseGene) following the manufacturer’s instructions.
Supplementary Figure 1

(A) Schematics of the procedure for preparing the model template DNA. First, four DNA fragments were mixed and annealed. Then, fragments A-C were ligated with T4 DNA ligase. Finally, the opposite strand was synthesized using the 3’ end of fragment C as the primer. CpG positions of fragment B are indicated by red arrows.

(B) Bisulfite treated model template DNA. The cytosine (except that of CpG) of model template DNA sequence is converted to thymine. The Taq I restriction enzyme recognition site used for COBRA experiment is indicated.
Supplementary Figure 2
The estimation of the hmC ratio by T4-BGT and GSRE.

The hmC modification existed in the Arhgap27 and Nhlrc1 locus was estimated by glucosylation of hmC by T4-BGT, Msp I digestion and qPCR. qPCR with specific primers for Arhgap27, Nhlrc1 and Peg3 (no digestion control) were performed and the fraction of hmC are presented as percentage of the Msp I resistant DNA amount of Arhgap27 or Nhlrc1 per the amount of Peg3. White bar represents T4-BGT+ and black bar represents T4-BGT- experiment (background). Error bar represents the standard error of the qPCR quantification. The Msp I recognition sites (CCGG) are located 10 bp downstream of the last CpG of the Arhgap27 region and the first CpG of the Nhlrc1 region, shown in figure 3.
The Arhgap27 regions of the genome (chr11:103333922-103333851) from the cerebrum of the BJF1 mouse was analyzed using the EnIGMA method. 50 reads were randomly picked up and the cytosine modification were summarized for each molecule. The black circles designate methylated cytosine, the red circles hydroxymethylated cytosine and the white circles unmodified cytosine. The grey circle a cytosine for which the modification status could not be determined because the cytosine in the original strand was unmethylated, while the cytosine in the indicator strand was methylated. (A) Top strand of Arhgap27 in the BJF1 cerebrum. (B) Bottom strand of Arhgap27 in the BJF1 cerebrum.
Supplementary Figure 4

A schematic of the hairpin bisulfite and hairpin TAB sequence procedures is shown. The black circles designate methylated cytosine, the red circles hydroxymethylated cytosine, yellow circles carboxy cytosine and the white circles unmodified cytosine. The top strand is shown with an orange line and bottom strand a green line. The DNA was digested with the appropriate restriction endonuclease, then the hairpin DNA was ligated and digested with the second appropriate restriction enzyme. The resulting DNA was directly treated with bisulfite solution for hairpin bisulfite or oxidized by the TET1 enzyme followed by the bisulfite conversion. Finally, the resulting PCR was amplified with the appropriate primers and sequenced.
Hairpin bisulfite and hairpin TAB sequencing. The calculated CpG cytosine modifications in both strands is presented as a bar graph. The full or hemi-methylated/hydroxymethylated percentage is shown for hairpin bisulfite and the full or hemi-hydroxymethylated cytosine percentage is shown for the hairpin TAB sequencing. (A) Hairpin bisulfite sequencing of Arhgap27 in the BJF1 mouse cerebrum. (B) Hairpin TAB sequencing of Arhgap27 in the BJF1 mouse cerebrum. (C) Hairpin bisulfite sequencing of Nhlrc1 in the BJF1 mouse cerebrum. (D) Hairpin TAB sequencing of Nhlrc1 in the BJF1 mouse cerebrum.