Supporting Information

DNA-friendly Cu(II)/TEMPO-catalyzed 5-hydroxymethylcytosine-specific oxidation

Taku Matsushita, Yuya Moriyama, Genta Nagae, Hiroyuki Aburatani and Akimitsu Okamoto*

Contents

Experimental

Copper-catalyzed 5hmC oxidation, piperidine cleavage and PAGE analysis. A solution of DNA in water (100 μM, 2 μL) was added to a mixture of each solution of copper(II) perchlorate in water (50 mM, 10 μL), TEMPO in acetonitrile (50 mM, 10 μL), bipyridine in acetonitrile (50 mM, 10 μL), sodium hydroxide in water (50 mM, 10 μL) and water (55 μL). The mixture was incubated at 25 °C for 45 h. For the PAGE analysis of the products, the reaction mixture is treated with hot piperidine. After the reaction mixture was desalted through a filter, piperidine (9 μL) was added to cleave DNA at the reacted nucleotide. After incubation of the mixture at 90 °C for 2 h, the mixture was concentrated *in vacuo*. The reaction products were analyzed with 20% polyacrylamide gel electrophoresis.

Biotin labeling and recovery of 5hmC DNA. A mixture of the solutions of DNA strands with different strand length, DNA2 5'-FAM-GTTGTGAGG5hmCGCTGCCCCCA-3' (100 μM, 4 μL) and DNA3 5'-FAM-AAAAAAG5mCGAAAAAA-3' (50 μM, 6 μL), was added into a mixture of each solution of 4,4'-dimethyl-2,2'-bipyridyl in acetonitrile (50 mM, 15 μL), copper(II) perchlorate in water (50 mM, 10 μL), TEMPO in acetonitrile (50 mM, 10 μL), sodium hydroxide in water (50 mM, 10 μL), and water (55 μL). The mixture was incubated at 50 °C for 24 h. After filtration for desalt, solutions of biotin-AC5-hydrazide in DMSO (100 mM, 3 μL) and anisidine in acetonitrile (500 mM, 25 μL) were added into the filtrated mixture and the solution was incubated at 25 °C for 55 h. The reaction mixture was passed through a filter. A solution (140 μL) of streptavidin-coated magnetic beads (Dynabeads M-280) was added into one solution and stirred at 25 °C for 44 h. The magnetic beads were collected by a magnet. A half amount of collected beads was put into 95% formamide containing 10 mM EDTA (100 μL) and heated at 95 °C for 10 min. After the supernatant was collected, the magnet beads as a residue was treated with hot formamide four times. The collected supernatant was dialyzed. The solution was concentrated *in vacuo*. The reaction products were analyzed with 20% polyacrylamide gel electrophoresis (7M urea).

Bisulfite treatment after oxidation. To 20 μL of target DNA solution (3.85 μM), 107 μL of 4 M bisulfite solution, 6 μL of 10 mM hydroquinone solution, and 7 μL of 6N sodium hydroxide were added, heated by thermal cycler. The PCR cycle is as follows: 95 °C for 0.5 min, and then 60 °C for 15 min. The cycle was repeated 20 times. The mixture was purified through Micro Bio-Spin P-6 Gel Columns (BioRad) by centrifuging at 1,000G for 4 min. To the solution, 300 μL of ethanol, 5 μL of 3 M sodium acetate, and 4 μL of Dr. GenTLE Precipitation Carrier (TakaraBio) were added and centrifuged at 12000G for 30 min. Water (10 μL) was added to the precipitate after removal the supernatant. The solution was used for sequencing without further purification.

Quantitative PCR after oxidation. (1) Cu-catalyzed oxidation. A solution of DNA in water (100 μM, 2 μL) was added to a mixture of each solution of copper(II) perchlorate in water (50 mM, 10 μL), TEMPO in acetonitrile (50 mM, 10 μL), bipyridine in acetonitrile (50 mM, 10 μL), sodium hydroxide in water

(50 mM, 10 μL) and water (55 μL). The mixture was incubated at 25 °C for 24 h. (2) Ru-catalyzed oxidation. A solution of DNA in water (100 μM, 2 μL) was added to a mixture of each solution of potassium perruthenate in water (3 mM, 3 μL) and sodium hydroxide in water (50 mM, 97 μL). The mixture was incubated at 0 °C for 1 h. (3) Control. A solution of DNA in water (100 μM, 2 μL) was added to water (100 μL). The mixture was incubated at 0 °C for 1 h. (4) qPCR. The mixture was diluted to 1/1000 and added into the PCR mixture containing reverse and forward primers (5'-AGTAGTATTTTTTAAGTTTTGGGGG-3' and 5'-CAAATCCCTTTATAAAAAAAAACAACAATAACAACAT-3', respectively; 100 μM, 2 μL), KOD Dash DNA polymerase (TOYOBO, 0.2 μL) and 1/1000 SYBR Green I (2 μL). The PCR cycle is as follows: 95 °C for 15 sec, 55 °C for 15 sec, 75 °C for 15 sec. The cycle was repeated 50 times. The concentration of the undamaged DNA was calculated from the sigmoid curves given from the fluorescent intensity of DNA-binding SYBR green I and the linear calibration line give from standard DNA concentrations.

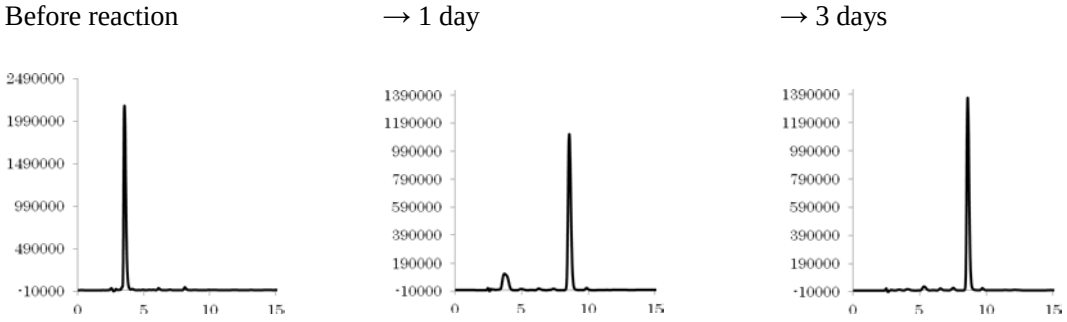
High-resolution microarray. For conventional BS assay, 1μg of genomic DNA was treated using EZ DNA methylation Kit (Zymo Research). For TAB assay, 1 μg of DNA was treated using 5hmC TAB-seq Kit (Wisegene) before BS treatment. The following Infinium step was performed according to Illumina's standard protocol of the Infinium MethylationEPIC BeadChip (Illumina). For each CpG site, the β-value was calculated by using the following equation: {the intensity of the methylated allele (M)} / (M+{the intensity of the unmethylated allele (U)}+α) (α: a constant (100)). This β-value, which ranged from 0 (unmethylated) to 1 (fully methylated), reflects the methylation level of the individual CpG site represented by the probe.

Table S1. Detection of cytosine derivatives in the Infinium microarray after chemical conversions.[a]

	C	5mC	5hmC	Detection as β values
BS	U	M	M	5hmC+5mC
CuTe-oxBS	U	M	U	5mC
TAB	U	U	M	5hmC

[a] U, unmethylated alleles; M, methylated alleles

(a)



(b)

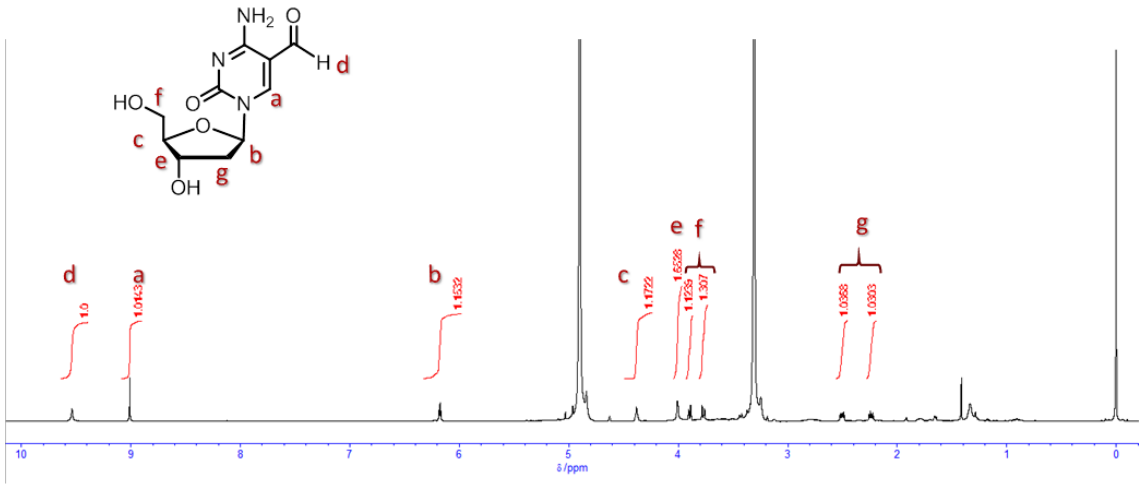
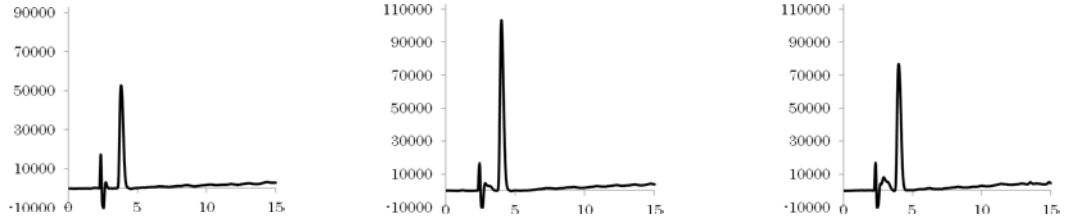


Figure S1. Cu(II)/TEMPO oxidation of d5hmC. (a) HPLC analysis before/after the reaction for d5hmC. The X-axis shows the retention time (min). The Y-axis shows the absorption at 254 nm. The nucleotides were analyzed using reverse phase HPLC on a 5-ODS-H column (10 × 150 mm, elution carried out using a solvent mixture of 0.1 M triethylammonium acetate (pH = 7.0), and a linear gradient over 15 min from 2% to 8% acetonitrile). (b) ^{1}H-NMR chart (600 MHz, methanol-d_4) of the reaction product, d5fC.

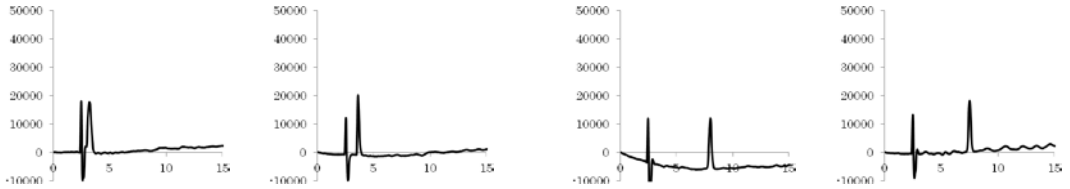
d5mC

Before reaction → 1 day → 3 days



dC → 3 days

dT → 3 days



dG → 3 days

dA → 3 days

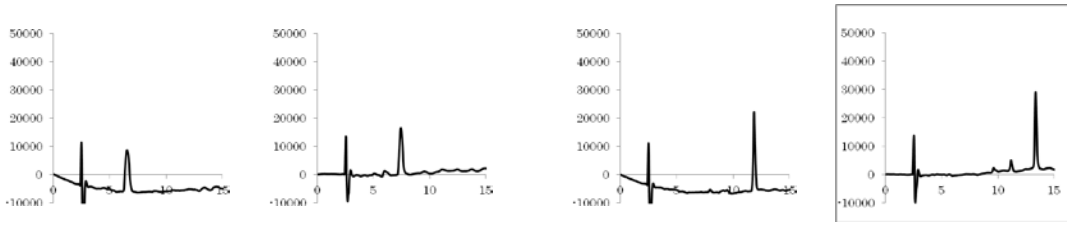


Figure S2. HPLC analysis before/after the reaction for nucleosides, d5mC, dC, dT, dA and dG. The X-axis shows the retention time (min). The Y-axis shows the absorption at 254 nm. The nucleotides were analyzed using reverse phase HPLC on a 5-ODS-H column (10 × 150 mm, elution carried out using a solvent mixture of 0.1 M triethylammonium acetate (pH = 7.0), and a linear gradient over 15 min from 2% to 8% acetonitrile).

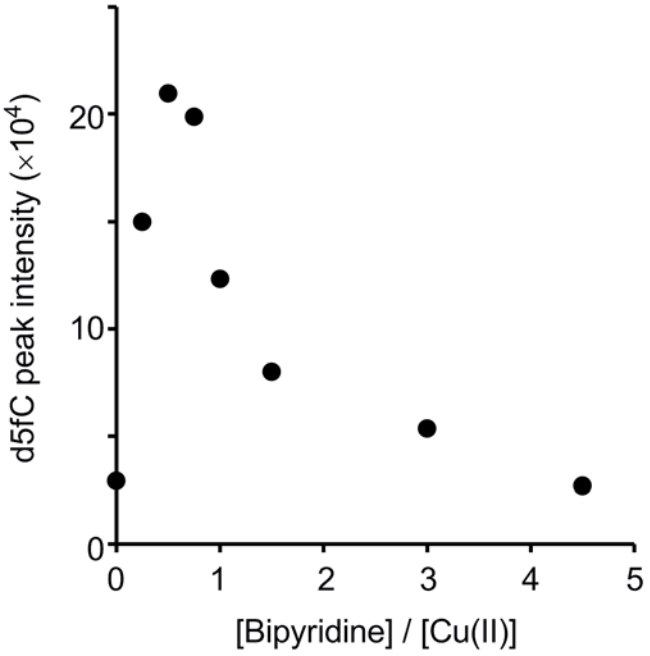


Figure S3. Effective oxidation of d5hmC to d5fC in the presence of bipyridine.

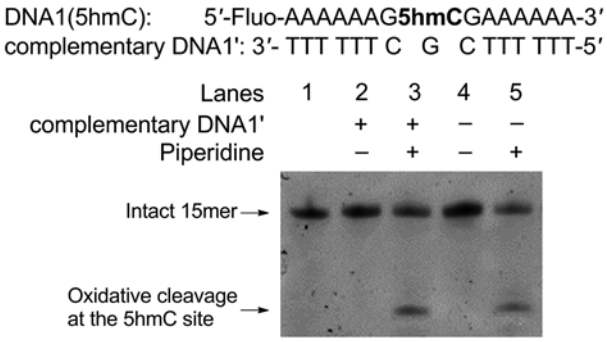


Figure S4. Piperidine cleavage at the oxidized 5hmC site after treatment of single-stranded / double stranded DNA1(5hmC) with the mixture of copper(II) perchlorate and TEMPO. Lane 1: Intact DNA1(5hmC). Lanes2−4: The reaction products of DNA1(5hmC) after piperidine treatment.

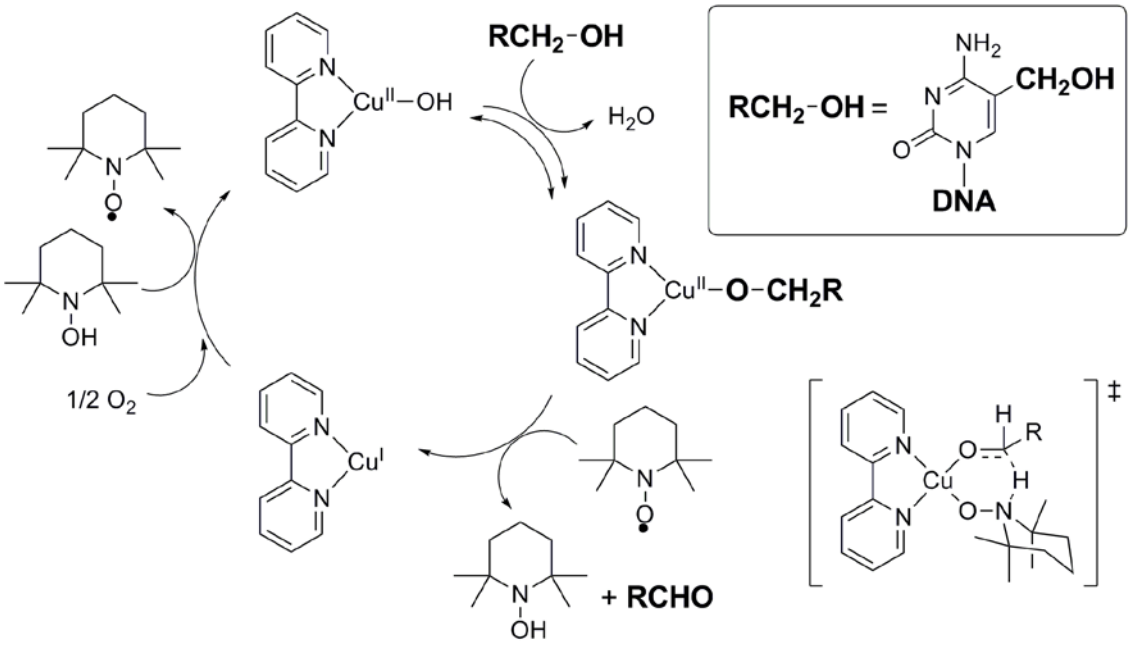


Figure S5. Catalytic mechanism for copper ion-catalyzed oxidation of 5hmC and its transition state.

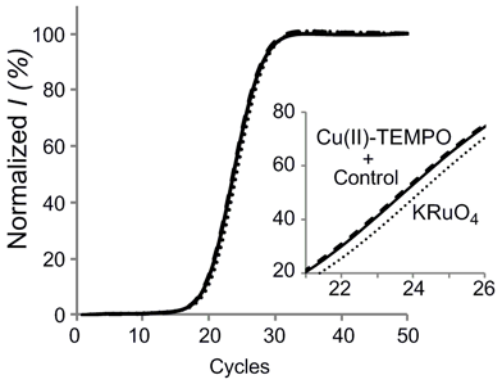


Figure S6. The sigmoid curves observed in the quantitative PCR for the DNA treated with Cu(II)/TEMPO oxidation overlapped that given from intact DNA. Solid line, control; dashed line, Cu(II)/TEMPO system; dotted line, potassium perruthenate system. Inset: An expanded figure in the region of PCR cycles 21−26.

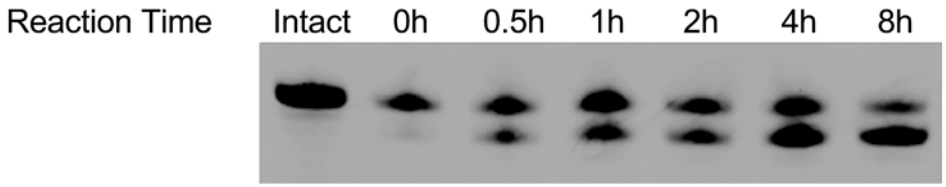


Figure S7. Degradation of DNA1(5hmC) with potassium perruthenate. The reaction product after the reaction at 0 °C was filtered for desalting and analyzed by PAGE.

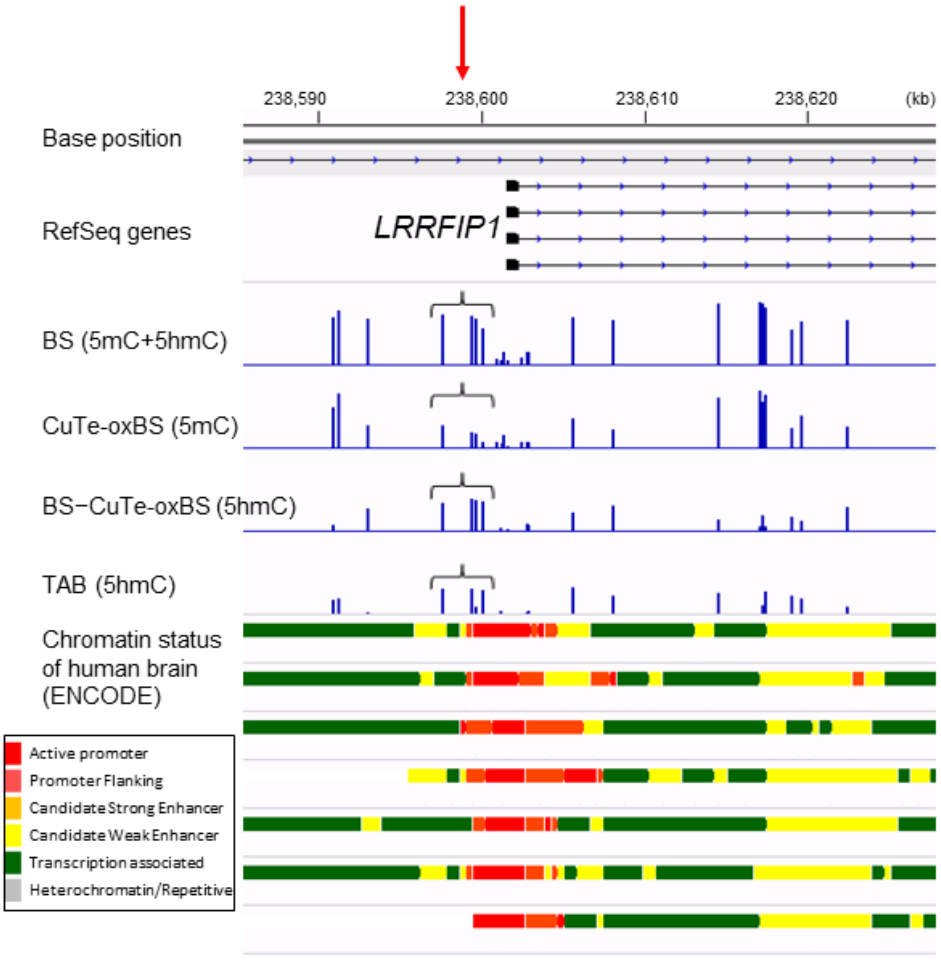


Figure S8. An example of the 5mC, 5hmC, and total cytosine modification (5mC + 5hmC) profiles. This is shown for the LRRFIP1 promoter region (four of five types of transcription products) as a bar chart (Upper part). The red arrow shows the 5hmC-rich region detected in this study. Color bars in the lower part represent seven typical examples of the chromatin statuses of human brains from anatomically different regions around this promoter.

Scheme S1. Possible strand-end degradation with potassium perruthenate.