Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

5-Carboxylcytosine is resistant towards phosphodiesterase I digestion: implications for epigenetic modification quantification by mass spectrometry

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Experimental Procedures

Oligonucleotides.

DNA oligo with 5fC was synthesized by the C-tailing method. DNA oligos 5'-GTCGACCGGATC-3' and 5'-TTGGATCCGGTCGACTT-3' were annealed and then incubated with 5-formyl-2'-dCTP (Trilink Biotech) and Klenow Fragment 3'→5' exo- (New England Biolabs) in NEBuffer 2 for 2 hrs at 37 °C. The DNA oligo with 5caC was synthesized using Expedite 8900 Nucleic Acid Synthesis System with 5-Carboxyl-dC-CE Phosphoramidite (Glen Research). Purified oligonucleotides were characterized by Voyager-DE MALDI-TOF (matrix assisted laser desorption ionization time-of-flight) Biospectrometry Workstation.

Cell culture and DNA isolation.

HEK 293T cells were maintained in DMEM (Gibco), supplemented with 10 % fetal serum (Sigma), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) at 37 °C under 5 % CO_2 atmosphere. The coding sequence of mouse Tet1 catalytic domain (amino acid 1367-2039) was cloned into pcDNA3 vector and transfect into 293T cells. Cells were harvested 40 hrs after transfection. Genomic DNA (gDNA) was extracted from the cultured cells using TRIzol Reagent (Invitrogen). After measuring the concentration and checking the quality by NanoDrop™ 2000/2000c Spectrophotometers (ThermoFisher), the gDNA was stored at - 80 °C or used directly in the next step.

Wild-type ($Tdg^{flox/flox}$) and Tdg knockout ($Tdg^{-/-}$) mouse embryonic stem cells (mESCs) were maintained on gelatin-coated plates in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15 % FBS, 2 mM L-glutamine, 1 × non-essential amino acids, 1 × penicillin/streptavidin, 0.1 mM β-mercaptoethanol, 10 ng/ml LIF, 1 μM PD0325901, and 3 μM CHIR99021 at 37 ˚C under 5 % CO_2 atmosphere. Genomic DNA was extracted with Blood & Cell Culture DNA Maxi Kit (Qiagen).

Enzymatic hydrolysis of DNA.

Different DNA digestion methods were used for the digestion of oligonucleotides or gDNA samples as below:

PDE1 or Benzonase/PDE1 protocol. DNA samples were incubated in hydrolysis solution containing 45 mM NaCl (Invitrogen), 9 mM $MgCl_2$ (Ambion), 9 mM Tris-HCl (pH 7.9, Gibco), 5 mU Phosphodiesterase I (Sigma-Aldrich), 0.5 μg Alkaline phosphatase (APL) (Sigma-Aldrich), 9.36 ng/μL EHNA hydrochloride (Sigma-Aldrich) and 3.52 μM Deferoxamine (Sigma-Aldrich) at 37 °C for 2 hrs. The digested samples were filtered with Amicon Ultra-0.5 mL 10K centrifugal filters (Merck Millipore) to remove the proteins. An equal volume of HPLC buffer A (H_2O containing 10 mM ammonium acetate, pH 6.0) was added to the filtered samples, and then subjected to HPLC-MS/MS analysis. For the Benzonase/PDE1 digestion method, 25 U Benzonase Nuclease (Sigma-Aldrich) was added to the solution described above.

DNase I/PDE1 protocol. For general DNA digestion, 2.5 μL of the DNase I (1U/L), 2 μL 1mU/mL PDE1, 0.5 μL ALP and 2 μL of the 10 X DNase I Buffer were added to 10 μL DNA sample (about

1 μg). The reaction mixture was incubated at 37 °C for 2 hrs. After that, 60 μL of HPLC buffer A (H_2O containing 10 mM ammonium acetate, pH 6.0) was added to the samples, and the solution was filtered with Amicon Ultra-0.5 mL 10K centrifugal filters (Merck Millipore) to remove the proteins before HPLC-MS/MS analysis.

DNA Degradase Plus protocol. For general DNA digestion, 1 μL of the DNA Degradase Plus (5 U/mL, Zymo Research (US)) and 2 μL of the 10 X DNA Degradase Reaction Buffer were added to 17 μL DNA sample (about 1 μg). The reaction mixture was incubated at 37 °C for 2 hrs. After that, 60 μL of HPLC buffer A was added to the samples, and the solution was filtered with Amicon Ultra-0.5 mL 10K centrifugal filters to remove the proteins before HPLC-MS/MS analysis.

Nucleoside digestion Mix protocol. Nucleoside Digestion Mix was purchased from New England Biolabs (US). For general DNA digestion, 1 μL of the Nucleoside Digestion Mix and 2 μL of the 10 X Nucleoside Digestion Mix Reaction Buffer were added to 17 μL DNA sample (about 1 μg). The reaction mixture was incubated at 37 °C for 2 hrs. After that, 60 μL of HPLC buffer A was added to the samples, and the solution was filtered with Amicon Ultra-0.5 mL 10K centrifugal filters to remove the proteins before HPLC-MS/MS analysis.

P1 Nuclease protocol. P1 Nuclease hydrolysis protocol contains two steps of digestion. About 1 μg DNA sample was first denatured at 100 °C for 3 min and quickly transferred to ice. 5 μL 100 mM ammonium acetate buffer (pH 4.6) and 2 U P1 nuclease was then added to the DNA sample (the total volume was 20 μL). The reaction mixture was incubated at 37 °C overnight. Then, 16 μL of H_2O, 4.3 μL of 10 × alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 3 μL of 1 mU/mL PDE1 and 0.6 μL of ALP were added into the DNA digestion solution. The incubation was continued at 37 °C for an additional 3 hrs. After that, 40 μL of HPLC buffer A was added to the samples, and the solution was filtered with Amicon Ultra-0.5 mL 10K centrifugal filters to remove the proteins before HPLC-MS/MS analysis.

HPLC-MS/MS analysis.

The HPLC-MS/MS analysis was carried out with 1290 Infinity LC Systems (Agilent) coupled with a 6495B Triple Quadrupole Mass Spectrometer (Agilent). A ZORBAX Eclipse Plus C18 column (2.1 x 150 mm, 1.8-Micron, Agilent) was used. The column temperature was maintained at 40 °C, and the solvent system was water containing 10mM ammonium acetate (pH 6.0, solvent A) and methanol (solvent B) with 0.4 mL/min flow rate. The gradient was: 0-5 min; 0 % solvent B; 5-8 min; 0-5.63 % solvent B; 8-9 min; 5.63 % solvent B; 9-16 min; 5.63-13.66 % solvent B; 16-17 min; 13.66-100 % solvent B; 17-21 min; 100 % solvent B; 21-24.3 min; 100-0 % solvent B; 24.3-25 min; 0 % solvent B. The dynamic mul-tiple reaction monitoring mode (dMRM) of the MS was used for quantification. The source-dependent parameters were as follows: gas temperature 230 °C, gas flow 14 L/min, nebulizer 40 psi, sheath gas temperature 400 °C, sheath gas flow 11 L/min, capillary voltage 1500 V in the positive ion mode, nozzle voltage 0 V, high pressure RF 110 V and low pressure RF 80 V, both in the positive ion mode. The fragmentor voltage was 380 V for all compounds, while other compound-dependent parameters were summarized in Table S1.

Supplementary Table 1. Compound-dependent HPLC-MS/MS parameters used for nucleosides quantification.

RT: retention time, CE: collision energy; CAE: cell accelerator voltage. All the nucleosides were analyzed in the positive mode.

Compound	Precursor Ion (m/z)	Product Ion (m/z)	RT (min)	Delta RT(min)	CE (V)	CAE (V)
dA+H	252	136	13.78	2	10	4
dT+H	243	127	11.07	2	10	4
dG+H	268	152	9.64	2	10	4
dC+H	228	112	3.71	1.5	10	4
5mC+H	242	126	9.05	1.5	12	4
5hmC+H	258	142	4.34	2	12	4
5fC+H	256	140	10.69	2	8	4
5caC+H	272	156	1.75	3	12	4

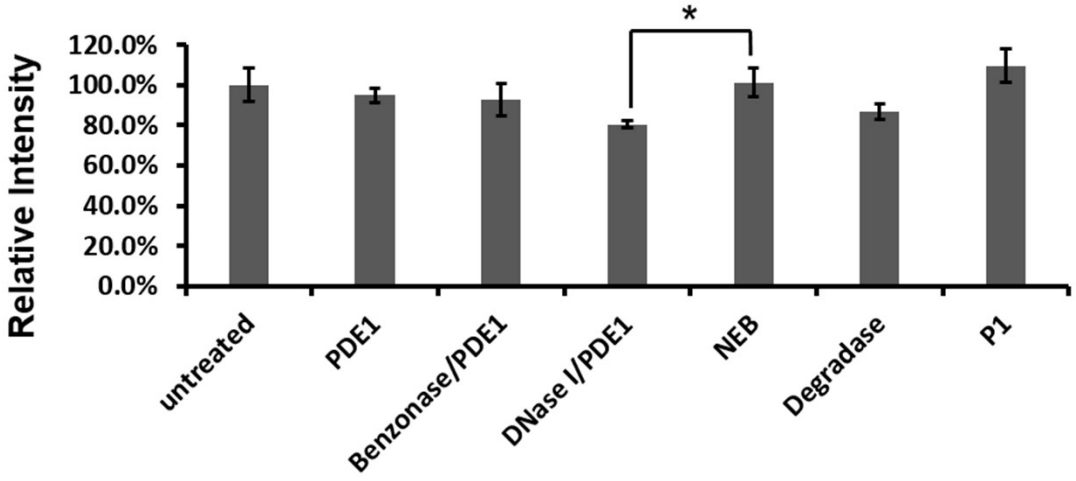

Figure S1. HPLC-MS/MS results of single 5caC 2'-deoxynucleoside treated with different hydrolysis methods. 10 nM of 5caC standard was used in each reaction. Error bars show SD （n = 3）; P value of Dunnett's multiple comparison test using NEB Digestion Mix as control. *P < 0.05.

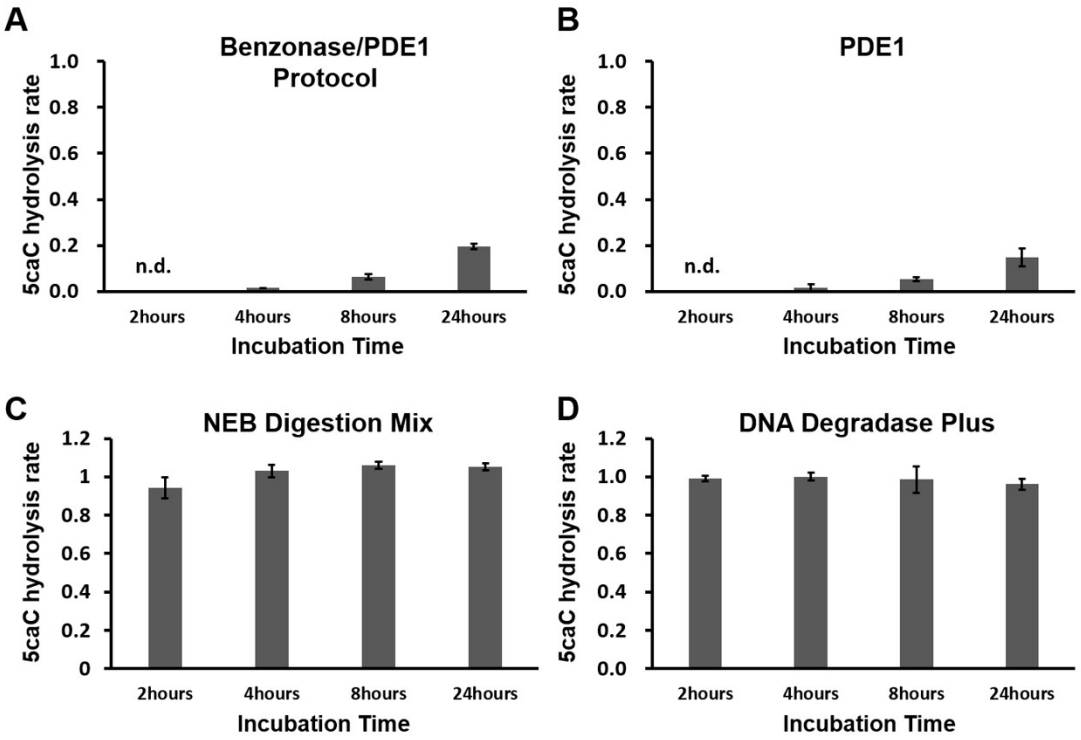

Figure S2. Time-dependent hydrolysis results of 5caC-containing ODN with different hydrolysis protocols. Three independent samples were prepared for each condition. (A) Benzonase, (B) PDE1, (C) NEB Nucleoside Digestion Mix and (D) DNA Degradase Plus.