Metabolically Generated Stable Isotope-Labeled Deoxynucleoside Code for Tracing DNA N⁶-Methyladenine in Human CellsBaodong Liu,^{†,‡} Xiaoling Liu,^{†,‡} Weiyei Lai,^{†,‡} and Hailin Wang^{*,†,‡,§}[†]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China[‡]University of Chinese Academy of Sciences, Beijing 100049, China

Supporting Information

ABSTRACT: DNA N⁶-methyl-2'-deoxyadenosine (6mdA) is an epigenetic modification in both eukaryotes and bacteria. Here we exploited stable isotope-labeled deoxynucleoside [¹⁵N₅]-2'-deoxyadenosine ([¹⁵N₅]-dA) as an initiation tracer and for the first time developed a metabolically differential tracing code for monitoring DNA 6mdA in human cells. We demonstrate that the initiation tracer [¹⁵N₅]-dA undergoes a specific and efficient adenine deamination reaction leading to the loss of the exocyclic amine ¹⁵N, and further utilizes the purine salvage pathway to generate mainly both [¹⁵N₄]-dA and [¹⁵N₄]-2'-deoxyguanosine ([¹⁵N₄]-dG) in mammalian genomes. However, [¹⁵N₅]-dA is largely retained in the genomes of mycoplasmas, which are often found in cultured cells and experimental animals. Consequently, the methylation of dA generates 6mdA with a consistent coding pattern, with a predominance of [¹⁵N₄]-6mdA. Therefore, mammalian DNA 6mdA can be potentially discriminated from that generated by infecting mycoplasmas. Collectively, we show a promising approach for identification of authentic DNA 6mdA in human cells and determine if the human cells are contaminated with mycoplasmas.

DNA 6mdA has been found widely existing in prokaryotes and functioning in bacterial restriction–modification systems,^{1–3} cell division,⁴ transcriptional regulation,⁵ and DNA replication and repair.^{6–9} However, DNA 6mdA has only directly been found in a few eukaryotes for a long time.^{10–15} Until recently, we showed that 6mdA is abundantly present in *Drosophila melanogaster* and tightly regulates early embryonic development and control transposon's expression.¹⁶ Together with the study of *Caenorhabditis elegans*¹⁷ and *Chlamydomonas algae*,¹⁸ it showed that 6mdA is a new potential epigenetic mark in the eukaryotic genome.^{16–18} Late work suggested that DNA 6mdA was detectable in *Xenopus laevis*, zebrafish, pig, and mouse.^{19–21} The content of 6mdA is about 2% (6mdA/dA) in *E. coli*, and about 0.001–0.07% in *Drosophila*.¹⁶ In reality, animals always live with various bacteria together. Noticeably, mycoplasmas, a genus of the smallest bacteria known, lack a cell wall around their cell membranes and are unaffected by many common antibiotics, and thus are often found in experimental cells and animals.^{22–24} Due to the possible coexistence of bacteria and mammals, this brings a great challenge to rationally interpret the experiment results and to identify 6mdA-related gene activities. To unlock the 6mdA mystery, an analytical technology is highly demanding to identify and quantify DNA 6mdA of different origins and to further the study of diverse gene activities in mammalian cells.

A few methods have been developed for detection of 6mdA in the eukaryotic genome, including liquid chromatography–

tandem mass spectrometry (LC-MS/MS),^{16,25,26} immunoassays,^{21,27} and DNA sequencing.^{16–19,21,28} Both LC-MS and antibody-based assays cannot tell the origins of DNA 6mdA, for example, from bacteria or mammals.²⁴ DNA sequencing-based assays might be used to discriminate endogenous DNA 6mdA from that generated by bacteria through genome-wide sequence mapping. A disadvantage is that these sequencing methods are not amenable to rapid identification of 6mdA origins and thus cannot be used routinely for monitoring 6mdA-related cellular activities, for example, writing (by methyltransferases), erasing (by demethylases), and reading (by reader proteins). Therefore, a rapid and simple approach is emergently required for a sensitive and robust identification of the origins of DNA 6mdA.

In this study, we reported the development and application of stable isotope-labeled deoxynucleoside [¹⁵N₅]-2'-deoxyadenosine ([¹⁵N₅]-dA) as an initiation tracer, combined with UHPLC-MS/MS analysis, for accurate and rapid identification and detection of target DNA 6mdA. This unique tracing approach enables us to specifically and quantitatively evaluate the origins and metabolism of DNA 6mdA. Typically we found in this work that the initiation tracer [¹⁵N₅]-dA could be converted into [¹⁵N₄]-dA and [¹⁵N₄]-dG in mammalian cells, thus explaining the formation of [¹⁵N₄]-6mdA. In contrast, the

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Figure 1. Characterization of stable isotope ^{15}N -labeled DNA adenine in the genome of $^{15}\text{N}_5$ -dA-treated 293T cells. The 293T cells were treated with $20\ \mu\text{M}$ [$^{15}\text{N}_5$]-dA for 24 h. (A) Schematic illustration of LC-MS analysis of ^{15}N -labeled dA. (B) UHPLC-Q-TOF-MS analysis of ^{15}N -labeled dA. (C) UHPLC-QQQ-MS/MS analysis of ^{15}N -labeled dA. nd = not detectable.

isotopic labeling of [$^{15}\text{N}_5$]-dA is largely retained in mycoplasma, thus leading to the generation of [$^{15}\text{N}_5$]-6mdA. All these stable isotope-labeled 2'-deoxynucleosides can be used as unique code marking the DNA 6mdA origins. Essentially, here we provide an excellent platform for development of new applications which would enable sensitive and robust analysis of authentic 6mdA modification in diverse mammalian cells.

EXPERIMENTAL SECTION

Chemicals and Materials. [$^{15}\text{N}_5$]-dA (96–98%) was purchased from Cambridge Isotope Laboratories (Andover, MA). 2'-Deoxyadenosine monohydrate ($\text{dA}\cdot\text{H}_2\text{O}$, $\geq 99\%$), 2'-deoxyguanosine monohydrate ($\text{dG}\cdot\text{H}_2\text{O}$, $\geq 99\%$), EHNA hydrochloride ($\geq 98\%$), LC-MS grade methanol (Fluka, St. Louis, MO), and formic acid (for mass spectrometry, $\approx 98\%$) were ordered from Sigma-Aldrich (St. Louis, MO). Deoxyribonuclease I (DNase I), *Bam*HI, *Eco*RI, and calf intestinal alkaline phosphatase (CIP) were obtained from New England Biolabs (Ipswich, MA). Crotalus adamanteus venom phosphodiesterase I (SVP) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Ultrapure water was prepared using an ELGA PureLab-Ultra purifier (High Wycombe, UK).

Cell Culture and Genomic DNA Extraction and Digestion. Human kidney 293T cells were seeded in plastic dishes (Corning, Corning Inc., NY) and grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Thermo Fisher Scientific Inc., MA), supplemented with 4 mM L-glutamine, 4.5 g/L glucose, 100 $\mu\text{g}/\text{mL}$ penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10% fetal bovine serum (FBS; Biological Industries Ltd., Haemek, Israel). The dishes were incubated at $37\ ^\circ\text{C}$ in a humidified incubator with 5% CO_2 .

Cells were harvested, and genomic DNA of 293T was extracted using a Genomic DNA Purification Kit (Promega, Madison, WI) following the manufacturer's instructions. The concentration of DNA was evaluated by NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) at 260 nm. Per 5 μg of DNA was digested into single 2'-deoxyribonucleosides using 0.5 U DNase I, 0.002 U SVP, and 1.5 U CIP as previously described.^{29,30} Prior to DNA extraction and digestion, all the used buffers were first filtrated through a $0.2\ \mu\text{m}$ membrane and then sterilized by high temperature and pressure. The enzymatic digestion products were filtered by ultrafiltration tubes with a molecular weight cutoff of 3 kDa (Pall Corporation, Port Washington, NY) to remove the enzymes and then subjected to UHPLC ESI-MS/MS analysis.

Cultured Cells Treatment with [$^{15}\text{N}_5$]-dA. 293T cells of 9×10^5 were seeded in a 6-well cell culture cluster (Corning, Corning Inc., NY). [$^{15}\text{N}_5$]-dA of $20\ \mu\text{M}$ (final concentration) was added into the culture medium (2 mL/well). The control cells grew in the medium without adding any [$^{15}\text{N}_5$]-dA. Unless otherwise specified, 2 mL of medium was used for each well of cultured plates. After 24-h treatment, the cells were harvested and genomic DNA was extracted for UHPLC-MS analysis.

Inhibition of Human Adenosine Deaminase Activity. RNA interference oligos were ordered from Genepharma (Suzhou, China). Ada siRNA: sense 5'-GAUGAGACCAUCCCAGGAATT-3', antisense 5'-UUCUGGGAUGGUCUCAUCTT-3'; Negative Control siRNA: sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3'. 293T cells of 2×10^5 were seeded in a 6-well plate, and medium was replaced with fresh medium 24 h later. Then the preparation Ada siRNA mixture was transfected using Lipofectamine RNAiMAX (Life Tech-

Figure 2. Identification of the ¹⁵N loss position in the [¹⁵N₄]-dA converted from [¹⁵N₅]-dA in 293T cells. The 293T cells were treated with 20 μM [¹⁵N₅]-dA for 24 h. (A, B) Fragmentation analysis of [¹⁵N₄]-dA by Q-TOF target MS/MS with a collision energy of 20 eV (A) or 55 eV (B). (C, D) The dissociative pattern of [¹⁵N₄]-dA obtained at a collision energy of 55 eV. Asterisk labeling indicates the fragments generated from the dissociated 2-deoxyribose (B).

nologies) following the manufacture's instruction. After 24 h, the medium was supplemented with 20 μM [¹⁵N₅]-dA. Then, after 24 h-treatment, the cells were harvested for DNA extraction and analysis. For the inhibitor-involved Ada experiments, 293T cells of 3 × 10⁵ were seeded in a 6-well plate and EHNA of 0, 1, 5, or 20 μM was added to the medium containing 20 μM [¹⁵N₅]-dA. After 24 h, the cells were then harvested for DNA extraction and analysis.

UHPLC-ESI-MS/MS Analysis. *UHPLC Separation.* The UHPLC separation was performed on an Agilent 1290 series equipped with an autosampler (temperature set at 4 °C), a chromatographic column thermostat (temperature set at 30 °C), and a Zorbax Eclipse Plus C18 column (50 mm × 2.1 mm i.d., 1.8 μm particle size, Agilent Technologies, Palo Alto, CA) with a C18 cartridge guard-column. The flow-rate was 0.3 mL/min, and each sample was injected with a volume of 5.0 μL.

For the separation of dA and dG, the mobile phase consisted of 95% solvent A (0.1% FA aqueous solution) and 5% solvent B (pure methanol) and isocratic elution for 4 min. For the separation of 6mdA and other DNA component, the optimized mobile phase consisted of two solvents: 95% solvent A (water) and 5% solvent B (pure methanol). An optimized gradient elution was as follows: 0.0–2.0 min, 5% B; 2.1–4.3 min, 5%–

20% B; 4.3–6.0 min, 20% B; 6.1–9.0 min, 100% B; 9.1–13.0 min, 5% B.

UHPLC-Q-TOF MS Analysis. The MS characterization was performed on an Agilent 1290 UHPLC system coupled with 6530 Q-TOF-MS (Agilent Technologies, Palo Alto, CA). The ion source Dual AJS ESI was operated in the positive ion mode. Detector parameters were set as drying gas heater, 300 °C; drying gas flow, 9 L/min; nebulizer pressure, 15 psi; sheath gas temperature, 300 °C; sheath gas flow, 12 L/min; capillary voltage, 3,500 V; fragmentation voltage, 135 V; and nozzle voltage, 0 eV. Every calibration (range from 100 to 1,700 *m/z*) should be performed when the instrument is turned on. Mass error within 10 ppm is considered to be accurate and acceptable.

UHPLC-Triple Quadrupole MS Analysis. The triple quadrupole mass spectrometer (6410B, Agilent) was operated in the positive ion mode. A multiple reaction monitoring (MRM) mode was adopted for selective detection: 266.1 → 150.1 for 6mdA, 270.1 → 154.1 for [¹⁵N₄]-6mdA, and 271.1 → 155.1 for [¹⁵N₅]-6mdA. The collision energy was set at 15 eV. Nitrogen was used for nebulization and desolvation. The nebulization gas was set at 40 psi, the flow rate of desolvation gas was 9 L/min, and the source temperature was set at 300 °C. Capillary voltage was set at 3,500 V. High purity nitrogen (99.999%) was used as

the collision gas. Each sample was analyzed three times. The 6mda frequency was calibrated by the external standard method.

RESULTS AND DISCUSSION

Predominant Incorporation of Ultimate Product [$^{15}\text{N}_4$]-dA into Genomic DNA for [$^{15}\text{N}_5$]-dA Tracing. It is known that the deoxynucleoside [$^{15}\text{N}_5$]-dA can be weakly incorporated into bacterial and plant genomes,^{31,32} but the process is not known for mammalian cells. We first treated human 293T cells with [$^{15}\text{N}_5$]-dA. Next, the treated 293T cells were harvested, and then genomic DNA was extracted and enzymatically digested into the mixture of single nucleosides (Figure 1A). At last, we exploited UHPLC (ultrahigh performance LC) separation coupled with either high mass resolution Q-TOF-MS or highly sensitive triple quadrupole MS (QQQ-MS) to characterize the potential tracing products (Figure 1A). By high mass resolution Q-TOF-MS analysis, in addition to the known unlabeled-dA (measured m/z : 252.1093 amu, deviation: +0.8 ppm; Figure 1B), unexpectedly, we observed a new peak corresponding to [$^{15}\text{N}_4$]-dA (measured m/z : 256.0969 amu, deviation: -1.6 ppm) rather than [$^{15}\text{N}_5$]-dA (theoretical m/z : 257.0943 amu). We did detect a minor peak at m/z = 257.1007, which displays a high mass deviation (+25 ppm) from [$^{15}\text{N}_5$]-dA (theoretical m/z : 257.0943 amu) but very low mass deviation (+0.8 ppm) from that of natural plus one isotope of [$^{15}\text{N}_4$]-dA ([$^{15}\text{N}_4$]-dA + 1, theoretical m/z : 257.1005) (Figure 1B). Thus, the observed peak at m/z = 257.1007 is assigned as the natural plus one isotope of [$^{15}\text{N}_4$]-dA. Essentially, the tracer [$^{15}\text{N}_5$]-dA itself is undetectable in the genome of the [$^{15}\text{N}_5$]-dA-treated 293T cells. Of note, after the treatment of the unlabeled dA, we did not observe any cytotoxicity to 293T cells (0–100 μM , Supporting Information Figure S1). The observation also indicates that [$^{15}\text{N}_5$]-dA, which is chemically the same as the unlabeled dA, will not change the cell growth and viability.

By exploiting highly sensitive UHPLC-QQQ-MS analysis, we also observed [$^{15}\text{N}_4$]-dA (product ion of m/z 256.1 $\rightarrow$ 140.1), but failed to observe [$^{15}\text{N}_5$]-dA (m/z 257.1 $\rightarrow$ 141.1) after subtracting the contribution from the natural plus one isotope of [$^{15}\text{N}_4$]-dA ([$^{15}\text{N}_4$]-dA + 1) (Figure 1C and Supporting Information Table S1). By a 24 h-treatment of the growing 293T cells with [$^{15}\text{N}_5$]-dA, the observed intensity of [$^{15}\text{N}_4$]-dA (m/z 256.1 $\rightarrow$ 140.1) is about 25.5% of the unlabeled dA (m/z 252.1 $\rightarrow$ 136.1, Supporting Information Table S1). This is the first report on the observation of genomic [$^{15}\text{N}_4$]-dA upon treating the cells with [$^{15}\text{N}_5$]-dA.

Identification of the ^{15}N -Lost Position of [$^{15}\text{N}_5$]-dA $\rightarrow$ [$^{15}\text{N}_4$]-dA by High Resolution Mass Spectrometry. As described above, we observed the preferential incorporation of [$^{15}\text{N}_4$]-dA into genomic DNA when the 293T cells were treated with [$^{15}\text{N}_5$]-dA. Now the question is which ^{15}N is lost. For this purpose, we collected the dA-pertinent fraction from HPLC separation of the enzymatically digested genomic DNA (a mixture of single 2'-deoxynucleosides, e.g., dA, dG, dC, T) extracted from [$^{15}\text{N}_5$]-dA-treated cells (Supporting Information Figure S2). Of note, the unlabeled dA and the isotope-labeled dA ([$^{15}\text{N}_4$]-dA) overlapped and could not be separated by HPLC, and thus were collected together. However, these molecular-weight-different dA species could be separated for fragmentation analysis using the target MS/MS mode of Q-TOF MS because of its high mass resolution.

With a given collision energy of 20 eV, most of the aimed precursor ion [$^{15}\text{N}_4$]-dA was fully dissociated into the labeled adenine bases (measured m/z : 140.0501, deviation: +1.4 ppm) upon the loss of the 2'-deoxyribose moiety (Figure 2A). We also observed a fragment of measured m/z = 117.0551, assigned as the protonated 2-deoxyribose (theoretical m/z : 117.0546, deviation: +4.3 ppm) (Figure 2A).

Given a 55 eV collision energy, we observed 12 product ions from the fragmentation of the unlabeled dA. Consistent with a previous report,³³ we assigned characteristic product ions of the unlabeled dA as 136.0622 (A, a loss of 2'-deoxyribose, deviation: +2.9 ppm), 119.0352 (A - NH_3 , no deviation), 109.0501 (A - HCN, deviation: -7.3 ppm), 94.0399 (A - NH_2CN , deviation: -1.1 ppm), 92.0244 (A - NH_3 - HCN, deviation: +1.1 ppm), 67.0292 (A - $\text{C}_2\text{H}_3\text{N}_3$, deviation: +1.5 ppm), 65.0139 (A - $\text{C}_2\text{H}_3\text{N}_3$, deviation: +7.7 ppm). Interestingly, we observed 16 different product ions from the fragmentation of [$^{15}\text{N}_4$]-dA (Figure 2B). This will assist us to identify the ^{15}N -lost position of [$^{15}\text{N}_4$]-dA. Essentially, ammonia (NH_3) is expelled in approximately equal amounts from the ring nitrogen N-1 and the exocyclic nitrogen N-6, with little or no involvement of N-3, N-7, and N-9.³³ For the fragmentation of [$^{15}\text{N}_4$]-dA at the collision energy of 55 eV, we observed that a further loss of ammonia from [$^{15}\text{N}_4$]-A (formed by the loss of the 2-deoxyribose) would generate two product ions of 123.0233 ([$^{15}\text{N}_4$]-A - NH_3 , lost one amine group without any ^{15}N atom; deviation: -0.8 ppm) and 122.0260 ([$^{15}\text{N}_4$]-A - $^{15}\text{NH}_3$, lost one amine group with one ^{15}N atom; deviation: -2.5 ppm) with equal contribution. This suggests one ^{15}N loss: either from the ring nitrogen N-1 or the exocyclic nitrogen N-6. In other words, only one ^{15}N is retained among the ring nitrogen N-1 and the exocyclic nitrogen N-6 of [$^{15}\text{N}_4$]-A (Figure 2C). By direct loss of HCN, we only observed one fragmentation of m/z 112.0412 ([$^{15}\text{N}_4$]-A - HC^{15}N ; deviation: -7.1 ppm). Since the loss of HCN occurs at the ring N-1 position³³ (Figure 2D), the observation suggests that the ring N-1 position of [$^{15}\text{N}_4$]-A retains the stable isotope ^{15}N . Otherwise, there should be a peak at m/z 113.039 with a similar signal instead of the peak at m/z 112.0412.

All the fragmentation analysis (Supporting Information Figure S3 and Table S2) consistently supports a loss of ^{15}N occurring at the position of exocyclic N6-amino- ^{15}N by the conversion of [$^{15}\text{N}_5$]-dA into [$^{15}\text{N}_4$]-dA in the treated cells.

Exocyclic N6-Deamination of [$^{15}\text{N}_5$]-dA by the Purine Salvage Pathway. As revealed by high resolution Q-TOF MS-based fragmentation analysis, [$^{15}\text{N}_5$]-dA undergoes a certain biological process involving the loss of the exocyclic N⁶ nitrogen. As reported previously, purine nucleosides can be incorporated into genomic DNA through a classic purine salvage pathway^{34–37} (Supporting Information Scheme S1). This pathway occurs with or without adenine deaminase (Ada)-mediated deamination.^{34–36} If purine nucleoside [$^{15}\text{N}_5$]-dA is incorporated into DNA through the salvage pathway but not undergoing Ada-mediated deamination, the major product of DNA incorporation must be [$^{15}\text{N}_5$]-dA. However, as we observed, [$^{15}\text{N}_4$]-dA predominated in the [$^{15}\text{N}_5$]-dA-treated cellular DNA. Therefore, following the 293T cells treated with [$^{15}\text{N}_5$]-dA, [$^{15}\text{N}_5$]-dA must undergo the exocyclic deamination reaction. Essentially, in human cells, adenine can be deaminated at the exocyclic amino group to form hypoxanthine, to which rapidly is added an amino group from aspartate to the N⁶ position for replacing the keto group to return back to adenine or to the N² position to generate guanine by certain enzymes³⁷

Figure 3. Observation of $[^{15}\text{N}_4]\text{-dG}$ in genomic DNA of the $[^{15}\text{N}_5]\text{-dA}$ -treated 293T cells. The 293T cells were treated with $20\ \mu\text{M}$ $[^{15}\text{N}_5]\text{-dA}$ for 24 h. (A) UHPLC-Q-TOF-MS analysis of ^{15}N -labeled dG. (B) HPLC-QQQ-MS/MS analysis of ^{15}N -labeled dG.

Figure 4. Human adenosine deaminase (Ada) involved with the exocyclic deamination of $[^{15}\text{N}_5]\text{-dA}$ in the $[^{15}\text{N}_5]\text{-dA}$ -treated 293T cells. The 293T cells were treated with $20\ \mu\text{M}$ $[^{15}\text{N}_5]\text{-dA}$ for 24 h. (A, B) UHPLC-QQQ-MS/MS analysis of ^{15}N -labeled dA and ^{15}N -labeled dG in the genomic DNA of the $[^{15}\text{N}_5]\text{-dA}$ -treated 293T cells, in which the Ada gene was knocked down with siRNA (A) or the Ada activity was inhibited by EHNA (B). nd = not detectable. Error bar (A(e), B(d)) indicates the standard deviation ($n = 3$).

(Supporting Information Scheme S1). Based on this inference, $[^{15}\text{N}_4]\text{-dG}$ could be generated after the exocyclic deamination of $[^{15}\text{N}_5]\text{-dA}$ (see Supporting Information Scheme S1). Indeed, by high mass resolution Q-TOF MS analysis, we observed abundant $[^{15}\text{N}_4]\text{-dG}$ (measured m/z : 272.0920, deviation: -0.7 ppm) (Figure 3A). By further UHPLC-MS/MS analysis, we also observed the peak of $[^{15}\text{N}_4]\text{-dG}$ at the product ion of $272.1 \rightarrow 156.1$ (Figure 3B). Of note, its labeling ratio ($[^{15}\text{N}_4]\text{-dG/dG}$) was slightly higher than $[^{15}\text{N}_4]\text{-dA}$ ($[^{15}\text{N}_4]\text{-dA/dA}$) (Supporting Information Table S1).

To investigate whether Ada is involved with the exocyclic deamination of $[^{15}\text{N}_5]\text{-dA}$, we further knocked down Ada gene expression using siRNA. Interestingly, we observed that the signals of $[^{15}\text{N}_4]\text{-dA}$ and $[^{15}\text{N}_4]\text{-dG}$ in genomic DNA decreased by 37.2% and 31.8% (Figure 4A), respectively, confirming the involvement of Ada. Of note, despite the decrease in both $[^{15}\text{N}_4]\text{-dA}$ and $[^{15}\text{N}_4]\text{-dG}$, we did not observe any $[^{15}\text{N}_5]\text{-dA}$ incorporated into genomic DNA. This suggests that the Ada-involved purine salvage pathway is responsible for the

Figure 5. Metabolism of [¹⁵N₅]-dA in mycoplasma-infected 293T cells. The 293T cells were treated with 20 μM [¹⁵N₅]-dA for 24 h. (A) The chromatograms were obtained from the UHPLC-QQQ-MS/MS analysis of the isotope ¹⁵N-labeled dA, dG, and 6mdA to the corresponding unlabeled 2'-deoxynucleosides. (B) The peak area ratio of the three forms of 6mdA in Mycoplasma+ 293T cells. (C) The distribution of the three forms of 6mdA in Mycoplasma+ 293T cells. (D) The peak area ratio of [¹⁵N₅]-labeled 6mdA to [¹⁵N₄]-labeled 6mdA in mycoplasma+ 293T cells. Note: The natural plus one isotopes of [¹⁵N₄]-dA and [¹⁵N₄]-dG cannot be discriminated from respective [¹⁵N₅]-dA and [¹⁵N₅]-dG by triple quadrupole MS; however, their amount can be derived from the amount of respective [¹⁵N₄]-dA and [¹⁵N₄]-dG with a percentage of 5.88% and 5.85%. The amounts of the natural plus one isotopes of [¹⁵N₄]-dA and [¹⁵N₄]-dG were deducted from the signal of 257.1 → 141.1 for dA, 273.1 → 157.1 for dG; and the residues are equal to that of [¹⁵N₅]-dA and [¹⁵N₅]-dG. The natural plus one isotopes of [¹⁵N₄]-dA and [¹⁵N₄]-dG were calculated by the Isotope Distribution Calculator of the Agilent Qualitative Analysis B.04.00 workstation. nd = not detectable. Error bar (B, D) indicates the standard deviation (n = 3).

incorporation of exogenous dA into genomic DNA of the tested cells.

To corroborate this conclusion, we next examined the DNA incorporation of [¹⁵N₅]-dA using the Ada inhibitor. By treating 293T cells with an Ada inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA),^{38,39} the signals of [¹⁵N₄]-dA and [¹⁵N₄]-dG in genomic DNA decreased by 75.0%–96.4% and 75.4%–96.8% (Figure 4B), respectively. Meanwhile, we observed the presence of [¹⁵N₅]-dA in genomic DNA, but the signal of [¹⁵N₅]-dA is less than 5% of that for dA.

All these data consistently support that the DNA incorporation of [¹⁵N₅]-dA predominantly involves the adenine deamination-dominated purine salvage pathway.

Genomic [¹⁵N₅]-dA and [¹⁵N₅]-6mdA Mark the Mycoplasma Origins. Most of the infecting mycoplasmas that have very active DNA adenine N6-methyltransferases may generate indiscriminable DNA 6mdA to the experimental cells. To this purpose, we examined one type of cultured 293T cells infected with *Mycoplasma hyorhinis* SK76. The identity of the *Mycoplasma hyorhinis* SK76 was confirmed using universal PCR, species-specific PCR,⁴⁰ and DNA sequencing (Supporting Information Figure S4 and Tables S3–S5). In these mycoplasma-infected 293T cells, we observed abundant 6mdA with a level of about 560 6mdA per million dA (data not shown). In contrast, in the cells that were not infected by any mycoplasma, the 6mdA was undetectable (lower than 4.0 6mdA per 10 million dA) (Figure 5A and 5B).

We then explored the metabolic difference of stable isotope ¹⁵N-labeled dA between mycoplasma-positive and -negative

293T cells. By treating 293T cells with [¹⁵N₅]-dA, we consistently observed [¹⁵N₄]-dA and [¹⁵N₄]-dG in the extracted DNA. The ratios of [¹⁵N₄]-dA and [¹⁵N₄]-dG of mycoplasma-positive DNA to the respective unlabeled nucleoside (dA or dG) are 1.28- and 0.96-fold as high as that of mycoplasma-negative DNA (Figure 5B). Interestingly, by subtracting the natural plus one isotope of [¹⁵N₄]-dA, [¹⁵N₅]-dA remains undetectable in mycoplasma-negative DNA, but it becomes detectable in mycoplasma-positive DNA. More importantly, we surprisingly found that all three types of 6mdA could be detected in the cells infected with mycoplasma (Figure 5A and 5B), including [¹⁵N₅]-6mdA (44%), [¹⁵N₄]-6mdA (24%), and unlabeled 6mdA (32%) (Figure 5C). It may be pointed out that [¹⁵N₅]-6mdA predominates among three detected 6mdA nucleosides.

Consistent with the above observations, there is no detectable adenine deamination in *Mycoplasma hyorhinis*.⁴¹ Moreover, mycoplasma has a capacity of intaking nucleosides from both the host nucleotide pool and the medium.²²

As we found in normal 293T cells, [¹⁵N₅]-dA is exclusively converted into [¹⁵N₄]-dA and [¹⁵N₄]-dG in the host nucleotide pool, but [¹⁵N₅]-dA in the medium cannot be directly utilized by the cultured 293T cells themselves (Figures 1–3). When they infected the cultured human cells, the mycoplasma (distributed along cell membrane) uptake both [¹⁵N₅]-dA in the culturing medium and [¹⁵N₄]-dA and [¹⁵N₄]-dG from the host nucleotide pool. To this end, if 6mdA is generated by cultured human cells themselves, [¹⁵N₄]-6mdA predominates; otherwise, as caused by the infected mycoplasma, [¹⁵N₅]-6mdA is overwhelming. Therefore, as shown in Figure 5D, it is more

sensitive to evaluate mycoplasma infection by measuring the ratio of [$^{15}\text{N}_5$]-6mdA to [$^{15}\text{N}_4$]-6mdA.

In this study, we utilized stable isotope-labeled deoxynucleoside [$^{15}\text{N}_5$]-dA as an initiation tracer for monitoring the metabolism of endogenous 2'-deoxyadenine and its modification (6mdA) in DNA. Combined with UHPLC-MS analysis, we found that [$^{15}\text{N}_5$]-dA was converted into [$^{15}\text{N}_4$]-dA and [$^{15}\text{N}_4$]-dG, and then efficiently incorporated into genomic DNA through a purine salvage pathway.

Mycoplasma grows even faster (1–9 h/cycle²²) than cultured human cells (~24 h/cycle), and generates abundant 6mdA in its genome (~2%⁴²). Since mycoplasma DNA and the genomic DNA of the tested cells cannot be separated in the DNA extraction process, the 6mdA abundantly generated by mycoplasma will mix with the rare but important part of 6mdA contributed by the tested or host cells themselves. Despite their different origins, the chemical identities are the same. Therefore, the mixture of 6mdA from different origins cannot be discriminated by known LC-MS and other methods except time-consuming and labor-intensive genome-wide DNA sequencing. Here, we found a significant metabolic difference of [$^{15}\text{N}_5$]-dA between the mycoplasma and the cultured human cells. Mycoplasma that coinhabited in the cultured human cells can uptake [$^{15}\text{N}_5$]-dA in the medium accompanied by [$^{15}\text{N}_4$]-dA and [$^{15}\text{N}_4$]-dG in the nucleotide pool of the host cells. However, the cultured human 293T cells (host cells) mainly uptake [$^{15}\text{N}_4$]-dA and [$^{15}\text{N}_4$]-dG in the nucleotide pool. By taking advantage of this metabolic difference in deamination of dA between the mycoplasmas and the host human cells, we could discriminate the mycoplasma DNA and the genomic DNA of host cells.

It is also possible that mycoplasma methyltransferases may relocate into the host nucleus and methylate the host genome at GATC sites,⁴³ since when DNA extraction is performed, both mycoplasma and host cells are lysed rapidly. However, the observed predominance of [$^{15}\text{N}_5$]-6mdA over [$^{15}\text{N}_4$]-6mdA suggests that the methylation of host genomic DNA (containing [$^{15}\text{N}_4$]-dA but no [$^{15}\text{N}_5$]-dA) by mycoplasma methyltransferases should be very minor if it did happen.

Essentially, the generation of the stable isotope-labeled deoxynucleotide code by the initiation tracer [$^{15}\text{N}_5$]-dA is mainly dependent on the Ada-dictated purine salvage pathway. By this pathway, a number of tracing products are generated, including [$^{15}\text{N}_4$]-dA, [$^{15}\text{N}_5$]-6mdA, [$^{15}\text{N}_4$]-6mdA, and [$^{15}\text{N}_4$]-dG. The purine salvage pathway is common in many cells^{44,45} and involves adenine deamination.^{46–48} The adenine deamination involves adenosine deaminase, expressed in all human tissues, and catalyzes the conversion of adenosine and dA to inosine and 2'-deoxyinosine, respectively.^{37,46,49} Since it exploits the Ada deamination activity, the [$^{15}\text{N}_5$]-dA initiated tracing approach can be applied for many human cells, and shows general and promising applications.

CONCLUSIONS

We demonstrate a metabolically differential deamination of stable isotope-labeled [$^{15}\text{N}_5$]-dA tracer between cultured human cells and mycoplasma, and show the tracing of genomic dA and its modification 6mdA in cellular DNA. By this approach, combined with UHPLC-MS analysis, we show that DNA 6mdA generated by mycoplasma predominates in the form of [$^{15}\text{N}_5$]-6mdA. The DNA 6mdA of both origins can be discriminated from the DNA 6mdA species attributed to the cultured human cells, which predominantly exists as [$^{15}\text{N}_4$]-

6mdA. We also prove that the exocyclic deamination of [$^{15}\text{N}_5$]-dA is dependent on the Ada-dictated purine salvage pathway, and prove that this pathway is very critical for the incorporation of exogenous dA into genomic DNA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01152.

Extended experimental procedures, possible purine salvage pathways, effect of dA treatment on the cell viability of 293T cells, HPLC fractionation of dA and dG, collision induced dissociation pathways for protonated [$^{15}\text{N}_4$]-dA, identification of mycoplasma, labeling efficiency of 2'-deoxyadenine and 2'-deoxyguanine, accuracy and relative abundance of the fragments generated from collision-induced dissociation of [$^{15}\text{N}_4$]-dA, PCR primers, PCR procedure, and sequences PDF)

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Notes

The authors declare no competing financial interest.

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