JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
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2′-*O*-Methyl-5-hydroxymethylcytidine: A Second Oxidative Derivative of 5-Methylcytidine in RNA

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Supporting Information

ABSTRACT: 5-Hydroxymethylcytidine (hm^5C) was recently identified as a direct metabolite of m^5C in RNA. We investigated the stability of hm^5C in human cells using bio-isotopologues and LC-MS/HRMS. This has led to the discovery of a second oxidative metabolite of m^5C in RNA, namely 2′-*O*-methyl-5-hydroxymethylcytidine (hm^5Cm). Subsequent quantitative analysis of total RNA from higher organisms revealed varying levels and TET-independent formation of this new RNA modification.

There is a wide chemical diversity of ribonucleoside modifications in RNA.[1,2] While epigenetic events such as DNA methylation and histone modifications are understood to be dynamic and reversible processes, RNA modifications, have long been considered relatively static and stable marks. However, it was recently shown that the enzyme FTO mediates the oxidative demethylation of m^6A via N6-hydroxy- and N6-formylcytidine in mRNA.[3,4] This first example of reversible RNA methylation has opened up the possibility that RNA modifications may also be dynamic, with potential regulatory roles analogous to reversible epigenetic modifications. In support of this, we recently showed that m^5C undergoes similar oxidative metabolism in RNA to produce hm^5C and that the latter modification is conserved across Archaea, Bacteria and Eukarya.[5] Furthermore, Fu et al. reported the ability of TET enzymes to oxidize m^5C to hm^5C in synthetic RNA strands in vitro and showed the dependency of hm^5C on the TET3 enzyme in an in vivo knockout mouse model.[6] Together, these studies established hm^5C as a new RNA modification that is introduced through active, enzyme-catalyzed oxidation, rather than passive, reactive oxygen species-mediated oxidation of m^5C.

In contrast with m^6A, which is predominantly an mRNA modification, we had determined by quantitative LC-MS/HRMS that hm^5C is enriched in tRNA fractions (Figure S1). The turnover of m^5C into hm^5C is of particular interest as the extent of m^5C modification at specific tRNA sites plays a key role in regulating the cellular stress response. For example, the absence of m^5C triggers increased stress-induced cleavage of tRNAs and sensitizes organisms to oxidative stress.[7,8] Furthermore, tRNA wobble modifications can change as a result of exposure to toxic agents and thereby trigger stress-specific enhancement of translation of proteins critical to the cell stress response.[9,10] These response mechanisms require a dynamic control of tRNA modifications which can either be achieved through their reversible introduction or specific tRNA turnover/degradation.

We investigated whether hm^5C is indeed subject to dynamic turnover and looked for the existence of additional, novel oxidative metabolites of m^5C to examine the presence of an active cytidine-C5 demethylation pathway in RNA.

As a means to study the relative stabilities of m^5C and hm^5C in tRNA-enriched fractions, as compared to tRNA turnover, we selected stable isotope tracing monitored by mass spectrometry (Figure 1). We adapted methods previously reported by us in which we studied both RNA and DNA methylation and their

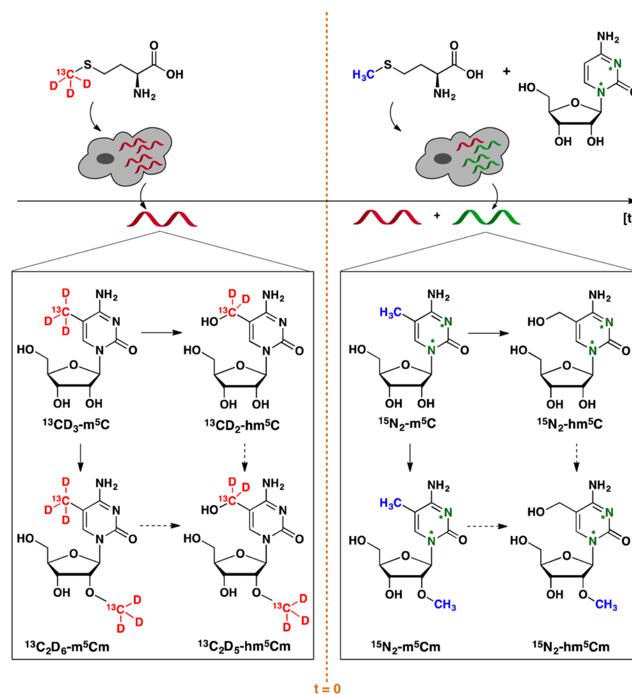

Figure 1. Overview of the stable isotope, dual-labeling approach. Cells were grown in the presence of [methyl-$^{13}CD_3$]-L-methionine until near quantitative isotope incorporation for m^5C was observed by LC-MS/HRMS. Heavy methionine (red) was then removed and a mixture of unlabeled L-methionine (blue) and 1,3-$^{15}N_2$-cytidine (green) was added.

Received: November 25, 2016
Published: January 20, 2017

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DOI: 10.1021/jacs.6b12180
J. Am. Chem. Soc. 2017, 139, 1766−1769

oxidative pathways.[5,11,12] Briefly, human HEK293T cells were cultured in the presence of stable isotope labeled (SIL) methionine, $^{13}CD_3$-L-methionine, to metabolically $^{13}CD_3$ label the methyl group of m^5C in RNA (Figure 1). The medium was then replaced with medium containing unlabeled methionine and SIL labeled 1,3-$^{15}N_2$-cytidine (Figure 1, $t = 0$) and cells were collected at hourly intervals and subjected to total RNA isolation, over the course of 15 h, approximately a complete cell cycle of a HEK293T cell. The total RNA fractions were each subsequently enriched for tRNAs by fractional precipitation, enzymatically digested into nucleosides and subjected to mass spectrometric analysis to quantify the SIL forms of both m^5C ($^{13}CD_3$-m^5C, $^{15}N_2$-m^5C) and hm^5C ($^{13}CD_2$-hm^5C, $^{15}N_2$-hm^5C). 1,3-$^{15}N_2$-cytidine was included to ensure the differential labeling of RNA synthesized during (Figure 1, before $t = 0$) and after (Figure 1, from $t = 0$ onward) the $^{13}CD_3$-L-methionine labeling. This distinguishes the apparent $^{13}CD_n$ decay as a result of isotope dilution due to cell proliferation or tRNA turnover, rather than modification turnover.

Thus, we measured the abundances of the different isotopologues of C, m^5C and hm^5C in the tRNA-enriched digests and calculated the amount of their $^{13}CD_n$ labeled (m^5C and hm^5C) and unlabeled (C) fractions relative to the sum of the total amounts of any given modification (Figure 2).

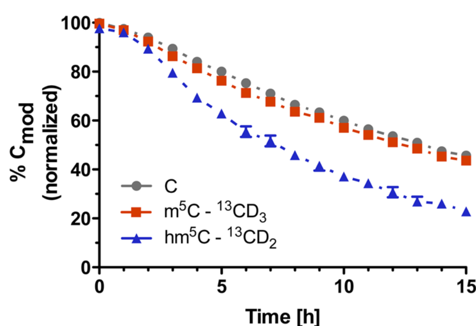

Figure 2. Amounts of the $^{13}CD_n$-labeled fractions of a modification relative to the sum of the total amounts of the same modification in small RNAs from $^{13}CD_n$-labeled HEK293T cells grown in the presence of 1,3-$^{15}N_2$-cytidine and absence of $^{13}CD_3$-L-methionine as a function of time (i.e., for m^5C: % $^{13}CD_3$-m^5C = {[$^{13}CD_3$-m^5C]/([$^{13}CD_3$-m^5C] + [$^{15}N_2$-m^5C] + [m^5C])} × 100).

We observed a 50% decrease of unlabeled cytidines (Figure 2, gray trace), consistent with the cell population and total RNA doubling in this time, which dilutes the $^{15}N_2$ label by 50% due to the addition of 1,3-$^{15}N_2$-cytidine at $t = 0$. We observed 5-methylcytidine (Figure 2, red trace) had comparable stability to cytidine, indicating the bulk of m^5C residues was not subject to active turnover. Because only a small fraction of m^5C residues is converted to hm^5C (~0.1%), this turnover was not sufficient to detect by our approach. However, when we considered the turnover of hm^5C (Figure 2, blue trace), we observed a strikingly steeper initial slope for its decay and a much lower relative abundance of the $^{13}CD_2$ isotopologue after 15 h as compared to m^5C. Consequently, hm^5C-containing RNA transcripts could either be unstable and subject to accelerated degradation, or hm^5C itself could be actively metabolized within its RNA transcript. To explore the latter hypothesis, we screened the dual SIL RNA samples for other oxidative derivatives of m^5C. Recently, it was shown that the Fe(II)-dependent oxygenase ALKBH1/ABH1 oxidizes m^5C at position 34 in human mitochondrial $tRNA^{Met}$ to f^5C and hm^5C was not observed as an intermediate in this study.[13] We therefore considered the hitherto unknown 2′-OH methylated derivative of hm^5C, 2′-*O*-methyl-5-hydroxymethylcytidine (hm^5Cm), as a potential downstream product of hm^5C metabolism in subsequent LC-MS/HRMS analyses. 2′-*O*-Methylation has been observed in several RNA classes[14] and close examination of previous extracted ion counts and fragmentation patterns of hm^5C led us to hypothesize the presence of hm^5Cm in RNA. Thus, using the tRNA-enriched digests from the 8 h time point, we targeted the mass spectrometry for 2′-*O*-[$^{13}CD_3$-methyl]-5-[$^{13}CD_2$-hydroxymethyl]-cytidine, 2′-*O*-methyl-1,3-[$^{15}N_2$]-5-hydroxymethylcytidine and the minor, completely unlabeled hm^5Cm isotopologues. As depicted in Figure 3 (left), we could extract all the corresponding product ions. This, together with the observed coelution of all the isotopologues during liquid chromatography, provided the first evidence for the presence of hm^5Cm in RNA. The slightly earlier elution of deuteriated compounds, as observed for $^{13}C_2D_5$ labeled hm^5Cm (Figure 3, left, red trace), is commonly observed in liquid chromatography of deuterium labeled compounds due to their different polarity, polarizability and molecular volume compared to their lighter isotopologues.[15]

To assess the stability of this novel RNA modification in relation to C, m^5C and hm^5C we determined the levels of $^{13}C_xD_y$-labeled modifications after 15 h, from our previous time decay study. As shown in Figure 3 (right), around 50% of all hm^5Cm are still $^{13}C_2D_5$-labeled after a complete HEK293T cell cycle. This is comparable to that observed for rC (45%) and m^5C (50%), two residues that we identified as stable. In contrast, $^{13}CD_2$-hm^5C accounts for only 29% of all hm^5C residues after 15 h. These data demonstrate that hm^5Cm is a stable modification. To establish unequivocally hm^5Cm as a novel RNA modification, we synthesized a reference standard for hm^5Cm by sodium persulfate-mediated oxidation of commercially obtained m^5Cm[16] and performed quantitative LC-MS/HRMS analysis of total RNA samples form a variety of organisms. Thereby, we measured the abundance of m^5C, hm^5C, m^5Cm and hm^5Cm (Figure 4). We selected HEK293T cells and murine brain tissue as human and mammalian examples, respectively. Furthermore, we chose models with previously reported low (*Caenorhabditis elegans*), high (*Arabidopsis thaliana*) and undetermined (*Drosophila melanogaster*) absolute levels of hm^5C.[5,17] As shown in Figure 5, we measured hm^5C levels that agreed with those previously described by us and others.[5,6] *A. thaliana* RNA exhibited the highest hm^5C levels (130 ppm), whereas *C. elegans* RNA showed the lowest (<10 ppm). Although the presence of hm^5C in *D. melanogaster* total RNA was previously demonstrated by dot blot experiments, we could not verify these results by LC-MS/HRMS.[17] On the other hand, we could readily observe the 2′-OH methylated form of hm^5C, hm^5Cm, in the latter organism. This may indicate that currently used antibodies cannot discriminate between hm^5C and hm^5Cm.[17] In general, organisms exhibiting a very low or undetectable level of hm^5C, actually showed a relatively high, detectable level of the 2′-OH methylated form, hm^5Cm. In addition to *D. melanogaster*, this is exemplified by *C. elegans*, which contains 30 ppm hm^5Cm in total RNA. For the human cells and mouse brain the abundances of methylated and unmethylated hm^5C are comparable. These results suggest that oxidation of C5-methylated cytidines (m^5C and/or m^5Cm) is a widespread process and eukaryotes seem to select largely for either the 2′-

DOI: 10.1021/jacs.6b12180
J. Am. Chem. Soc. 2017, 139, 1766−1769

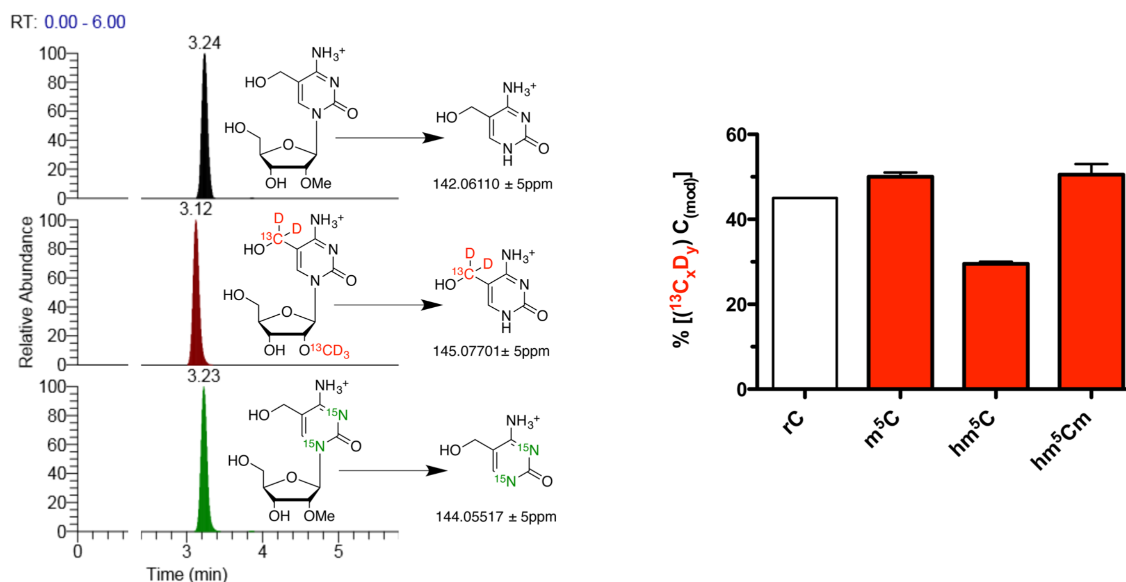

Figure 3. (Left) Differential labeling of hm^5*Cm*. LC-MS/HRMS analysis of RNA obtained from HEK293T cells grown in regular (top), [methyl-$^{13}CD_3$]-L-methionine- (middle) or 1,3-$^{15}N_2$-cytidine-supplemented (bottom) medium. Extracted ion counts are shown for hm^{5}C, $^{13}CD_2$-hm^{5}C and $^{15}N_2$-hm^{5}C. Right) Analysis of the levels of $^{13}C_xD_y$ labeled modifications after 15 h.

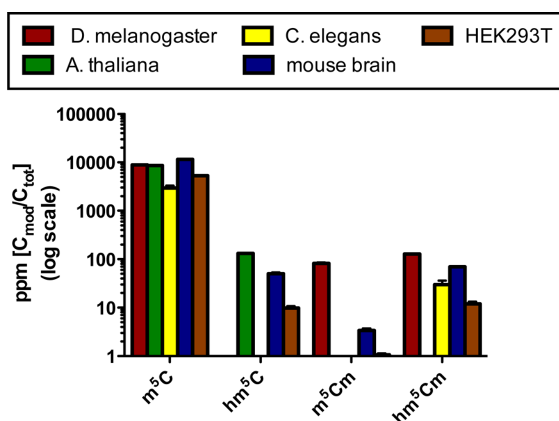

Figure 4. Abundance of m^{5}C and its derivatives as determined by quantitative LC-MS/HRMS.

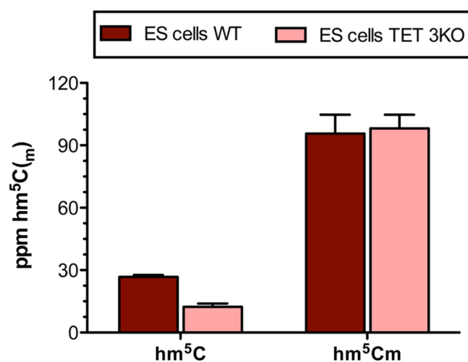

Figure 5. Abundance of hm^{5}C and hm^5*Cm* as determined by quantitative LC-MS/HRMS in mouse embryonic WT and TET triple KO stem cells.

OH methylated or unmetylated derivative with only mammalian RNA containing both forms of C5-hydroxymethylation (hm^{5}C and hm^5*Cm*).

Although further studies are required to fully discern the function of hm^5*Cm*, it should be noted that methylation of 2′-hydroxyl groups in tRNA molecules has been previously observed to occur at the first position of the anticodon to promote codon–anticodon interaction.[18] Furthermore, 2′-*O*-methylation can block the ability of the 2′-position of the nucleoside to serve as a proton donor and therefore prevents RNA hydrolysis, increasing the lifetime of the RNA.[19] We therefore propose that hm^5*Cm* may promote the stability of tRNAs themselves and the stability of duplex formation with complementary RNA molecules. It is noteworthy that the oxidative derivative of hm^5*Cm*, 2′-*O*-methyl-5-formylcytidine is already known to be present at the wobble position of cytoplasmic tRNAs.[20]

The TET family of enzymes was previously reported to be capable of oxidizing m^{5}C to hm^{5}C in RNA both in vitro and in vivo.[6,17] To shed light on whether hm^5*Cm* is also TET-dependent, we measured its levels in TET triple knockout (TKO) mouse embryonic stem cells that have been mutated in the catalytic domain of all three TET enzymes and therefore have no residual TET activity (Figure 5).[21] Interestingly, as we show here, hm^5*Cm* is not TET-dependent. RNA obtained from TET wild type and TET TKO cells show equal amounts of the 2′-*O*-methylated version of hm^{5}C. This shows that hm^5*Cm* is generated by an enzyme other than TET, which is in accordance with the findings that hm^5*Cm* is highest in organisms that do not express TET (*C. elegans*) or express TETs at a reduced level (*D. melanogaster*).

In conclusion, we have identified a novel derivative of C5-methylated ribonucleosides in RNA from mammalian cells, tissue and several organisms. The exact functional roles of hm^{5}C and hm^5*Cm* and the relationship between them will be the subject of future studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b12180.

DOI: 10.1021/jacs.6b12180
J. Am. Chem. Soc. 2017, 139, 1766−1769

Detailed experimental procedures, supporting figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a Wellcome Trust Senior Investigator award to SB (grant no. 099232/z/12/z). The Balasubramanian group is supported by core funding from Cancer Research U.K. (C14303/A17197). Prof. Daniel St. Johnston is thanked for the provision of *D. melanogaster* for total RNA isolation.

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