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Letters

Chemical Method to Sequence 5-Formylcytosine on RNA

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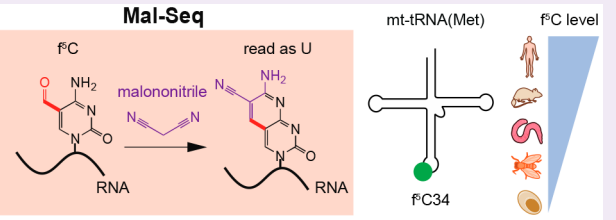
ABSTRACT: Epitranscriptomic RNA modifications can regulate biological processes, but there remains a major gap in our ability to identify and measure individual modifications at nucleotide resolution. Here we present Mal-Seq, a chemical method for sequencing 5-formylcytosine (f^{5}C) modifications on RNA based on the selective and efficient malononitrile-mediated labeling of f^{5}C residues to generate adducts that are read as C-to-T mutations upon reverse transcription and polymerase chain reaction amplification. We apply Mal-Seq to characterize the prevalence of f^{5}C at the wobble position of mt-tRNA(Met) in different organisms and tissue types and find that high-level f^{5}C modification is present in mammals but lacking in lower eukaryotes. Our work sheds light on mitochondrial tRNA modifications throughout eukaryotic evolution and provides a general platform for characterizing the f^{5}C epitranscriptome.



The function of cellular RNA can be modulated by chemical modifications installed post-transcriptionally. Known as the epitranscriptome, over 150 distinct modifications have been reported to exist on RNA.[1,2] A number of well-studied modifications have important roles in RNA metabolism, protein translation, and RNA trafficking;[3] however, we lack information on the function and distribution of most modifications. Furthermore, RNA modification levels and their associated writer, eraser, and reader proteins can be dysregulated in certain disease states,[4] underscoring the need for a comprehensive understanding of epitranscriptomic mechanisms in biological systems.

A major challenge in the study of RNA modifications is the ability to map modifications at single-nucleotide resolution and measure their stoichiometry.[5] Whereas next-generation sequencing (NGS) has revolutionized transcriptomic studies, many modifications are "silent" upon RNA-seq analysis because they are reverse-transcribed like the parent unmodified base, necessitating the development of alternative approaches for modification-specific sequencing. Approaches for modification mapping compatible with Illumina sequencing[6] (the most commonly utilized NGS platform) generally fall into two categories: (1) antibody enrichment of modified RNAs[7,8] or (2) chemical/enzymatic conversion of the modified base to an adduct that can be identified based on a distinctive reverse-transcription (RT) signature.[9−11] The second approach, in particular, when the signature is a sequence mutation as opposed to an RT stop, is often preferred because it can provide higher resolution, less sequence bias, and modification stoichiometry; however, the current strategies for RNA modification mapping mediated by chemical or enzymatic conversion are applicable to only a small number of modified bases, and there is a great need for the development of new approaches to characterize the epitranscriptome.

Herein we develop a chemical approach to sequence 5-formylcytosine (f^{5}C) on RNA at single-nucleotide resolution. 5-Formylcytosine has been found on isolated tRNA isoacceptors,[12−14] but we lack robust approaches to quantitatively sequence this modification and characterize its distribution across the transcriptome. Our strategy, which we name Mal-Seq, is based on selective malononitrile-mediated labeling (Figure 1a) and C-to-T conversion upon RT, amplification, and sequencing. Chemical labeling with malononitrile is mild, efficient, and quantitative, and we exploit these properties to measure the levels of f^{5}C at C34 on mt-tRNA(Met) in diverse organisms and tissue types.

To sequence RNA f^{5}C, we surveyed the literature for chemical transformations that would be selective for the modified base and generate a mutational signature. Notably, Yi and coworkers previously demonstrated that malononitrile[15] and 1,3-indandione[16] react with f^{5}C in DNA to form adducts that induce C-to-T mutations upon DNA polymerase read-through (Figure 1a and Figure S1a). Therefore, we investigated the suitability of these reactions for sequencing f^{5}C on RNA using total RNA and an artificial f^{5}C-containing RNA transcript generated by *in vitro* transcription as model substrates. We started by quantifying the depletion of f^{5}C in RNA upon treatment with malononitrile or 1,3-indandione

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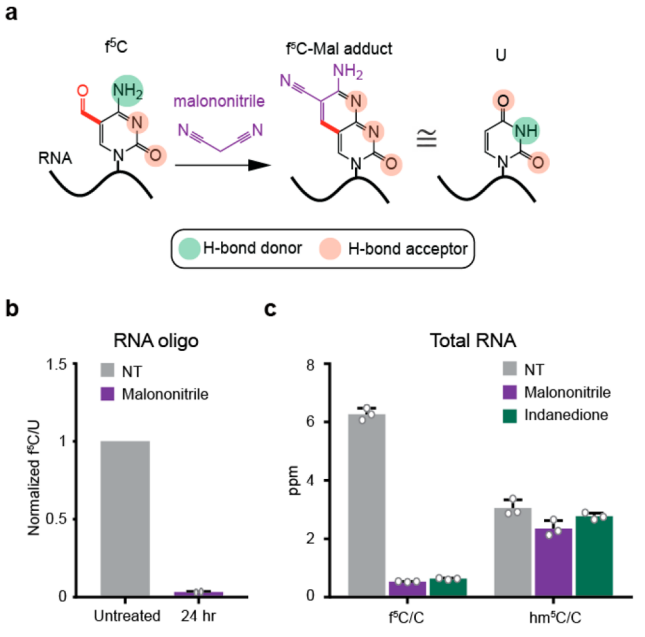

Figure 1. Malononitrile labeling of f^{5}C on RNA. (a) Structure of malononitrile-f^{5}C adduct and effects on Watson−Crick base pairing. (b) LC-QQQ-MS analysis of f^{5}C levels in RNA oligo1 after treatment with malononitrile. Data are the mean ± s.d. (n = 2). (c) LC-QQQ-MS analysis of f^{5}C and hm^{5}C levels in total RNA before and after treatment with malononitrile or 1,3-indandione. Data are the mean ± s.d. (n = 3).

using nucleoside LC-MS/MS (Table S1). Gratifyingly, we measured a 96.9 ± 0.004% reduction in f^{5}C levels upon the treatment of our model f^{5}C RNA with malononitrile (Figure 1b). In addition, the treatment of total cellular RNA with malononitrile or 1,3-indandione resulted in a 90−92% (malononitrile: 91.5 ± 0.002%; 1,3-indandione: 90.0 ± 0.004%) reduction of f^{5}C levels (Figure 1c). Importantly, the levels of related modifications in total RNA such as 5-methylcytidine (m^{5}C) or 5-hydroxymethylcytidine (hm^{5}C) remained unchanged (Figure 1c and Figure S1c), and analysis of the RNA integrity using gel electrophoresis or a Bioanalyzer assay demonstrated minimal RNA degradation (Figure S1d,e), indicating that these transformations are selective for f^{5}C and sufficiently mild for RNA sequencing.

Next, we tested whether the generated f^{5}C adducts would produce a sequence mutation upon reverse-transcription polymerase chain reaction (RT-PCR). Given the comparable reaction efficiency between f^{5}C and malononitrile or 1,3-indandione, we chose to work with malononitrile due to its enhanced solubility. We treated an *in vitro* transcribed RNA containing a single f^{5}C site at 100% stoichiometry with malononitrile and performed RT-PCR. The f^{5}C site was positioned in a Taqα1 digestion site such that the mutation of C to another base could be monitored by restriction enzyme digestion[17] (Figure 2a). As shown, the RT-PCR products generated from untreated f^{5}C RNA or an unmodified RNA were quantitatively digested by Taqα1, whereas malononitrile treatment of the f^{5}C RNA inhibited digestion by ∼50−60%

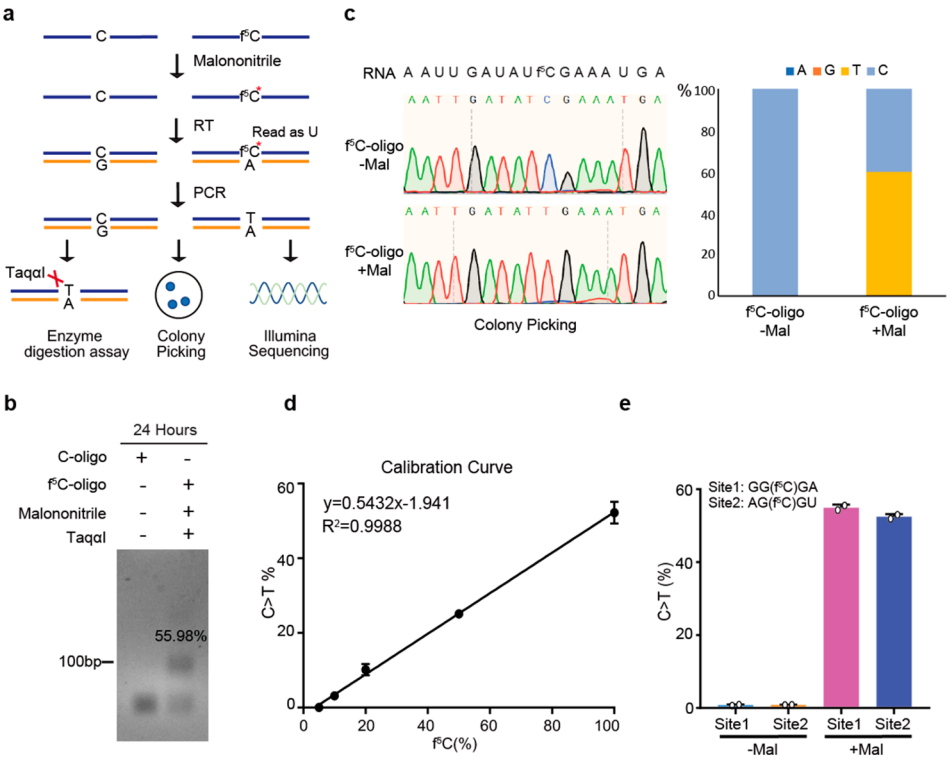

Figure 2. Quantitative sequencing of RNA f^{5}C by Mal-Seq. (a) Schematic of Mal-Seq workflow. (b) Taqα1 enzymatic digestion assay to detect base mutations mediated by malononitrile labeling. (c) Malononitrile-mediated C-to-T conversion measured by colony picking and Sanger sequencing. Left: Representative Sanger sequencing traces. Right: Quantification analysis (n = 10). (d) Calibration curve relating f^{5}C levels in RNA oligo1 and malononitrile-mediated C-to-T conversion. C-to-T mutations were measured by Illumina sequencing. Data are the mean ± s.d. (n = 2). (e) C-to-T conversion at two distinct f^{5}C-modified sites in RNA oligo2. Mutations were measured by Illumina sequencing. Data are the mean ± s.d. (n = 2).

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(Figure 2b and Figure S2). To characterize the nature of the sequence change, we performed Sanger sequencing, which indicated that 60% of the transcripts contained a C-to-T mutation, whereas the remaining 40% contained a C (Figure 2c). No other mutations were detected at the f^{5}C site or surrounding residues. To further confirm our result and generate a calibration curve relating f^{5}C stoichiometry and C-to-T conversion, we used high-throughput sequencing. Our data show a linear correlation between f^{5}C levels ranging from 5 to 100% stoichiometry and a malononitrile-induced C-to-T mutation (Figure 2d and Table S2) with a conversion factor near 0.5 (i.e., ∼50% C-to-T conversion after malononitrile treatment corresponds to 100% f^{5}C). Given the depth of coverage afforded by high-throughput sequencing analysis, we could also detect mutations to G/A or deletions at the f^{5}C site after malononitrile treatment, but the frequency of such events was low (0.1%). In addition, we tested RNA transcripts containing two or three f^{5}C modification sites within different sequence contexts and observed similar levels of C-to-T conversion at each site upon malononitrile treatment (Figure 2e, Figure S2b, and Table S3). Taken together, our results demonstrate that malononitrile-induced C-to-T mutations can be used to quantitatively sequence f^{5}C modifications on RNA at nucleotide resolution and within diverse sequence contexts. We named this approach Mal-Seq.

To demonstrate the utility of Mal-Seq, we applied our approach to characterize endogenous f^{5}C modification levels in the anticodon loop of mt-tRNA(Met). Studies have indicated the presence of f^{5}C at the C34 "wobble base" of mt-tRNA(Met) in a number of organisms, where it is proposed to facilitate the decoding of unconventional AUA and AUU Met codons among mitochondrial genes.[18] However, the lack of a unified, quantitative approach for characterizing f^{5}C modification levels has led to disparate findings regarding the prevalence and stoichiometry of f^{5}C levels in biological systems. We started by applying Mal-Seq to quantify f^{5}C on the wobble base of mt-tRNA(Met) from cultured human cells, where this modification has been best studied. Multiple groups have shown that f^{5}C biogenesis at this position requires the sequential action of m^{5}C methyltransferase NSUN3 followed by Fe(II), α-KG-dependent dioxygenase ALKBH1;[12,13,19,20] however, the quantification of modification levels has varied. Suzuki and coworkers used LC/MS analysis to show that C34 is fully modified to f^{5}C,[20] whereas two independent reports relying upon primer extension and bisulfite-based sequencing methods found a mixture of f^{5}C and m^{5}C at this position.[13,19] Therefore, we extracted total RNA from wild-type (WT) HEK293T cells and performed Mal-Seq using targeted RT-PCR of the anticodon region of mt-tRNA(Met). Our analysis shows 57.98 ± 0.16% malononitrile-induced C-to-T conversion, indicating that mt-tRNA(Met) is fully modified with f^{5}C at the wobble base (Figure 3b,c and Table S4), consistent with Suzuki's findings. In addition, we performed parallel analyses on RNA extracted from ALKBH1 or NSUN3 KO cells generated by CRISPR/Cas9 technology and found <0.3% C-to-T mutation (Figure 3c, Figure S3, and Table S4), confirming that both enzymes are required for f^{5}C installation on mt-tRNA(Met).

We next characterized the presence of f^{5}C on the wobble position of mt-tRNA(Met) in other eukaryotes. This modification has been found in organisms including squid,[21] flies,[22] chickens,[23] and cows,[14] but the extent of f^{5}C modification in these species is largely unknown. We obtained

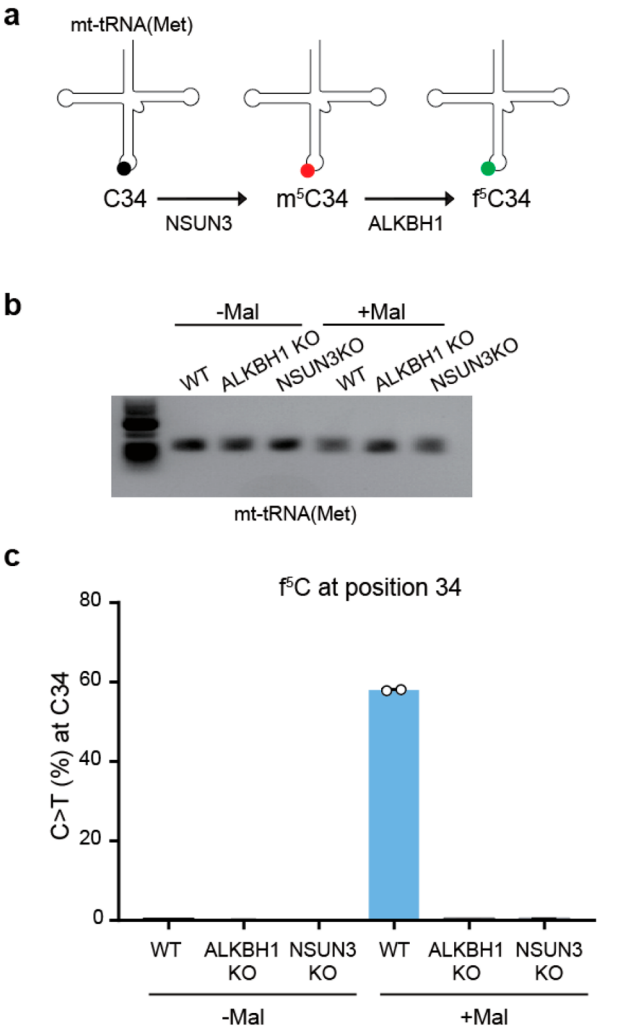

Figure 3. Mal-Seq reveals that f^{5}C34 in mt-tRNA(Met) is fully modified in HEK293T cells. (a) Schematic showing the formation of f^{5}C by NSUN3 and ALKBH1 on mt-tRNA(Met). (b) RT-PCR of mt-tRNA(Met) amplified from WT, ALKBH1 KO, and NSUN3 KO cell lines. (c) C-to-T mutation at C34 on mt-tRNA(Met) detected by Mal-Seq using RNA from WT, ALKBH1 KO, and NSUN3 KO cells. Data are the mean ± s.d. (n = 2).

total RNA from budding yeast, flies, *C. elegans*, and mice and characterized the f^{5}C levels by Mal-Seq using species-specific primers for each mt-tRNA(Met) (Figure S4). In yeast and flies, we found no evidence of f^{5}C on mt-tRNA(Met), indicating that this modification is absent or below our limit of detection (Figure 4a and Table S5). Budding yeast lack a clear ALKBH1 homologue, which is consistent with low f^{5}C modification. Whereas f^{5}C on mt-tRNA(Met) has been reported to occur in flies, the modification levels were partial, and quantitation was never performed.[22] In contrast, *C. elegans* showed 26.65 ± 1.65% Mal-Seq C-to-T conversion corresponding to ∼50% of the mt-tRNA(Met) modified with f^{5}C at the wobble position. This is in line with the recent characterization of a mitochondrial ALKBH1 homologue in this organism.[24] We also measured f^{5}C levels in different mouse tissues including the heart, brain, and liver. In these tissues, we observed 37.8−42.9% (liver: 37.8 ± 1.7%, heart: 41.5 ± 3.2%, brain: 42.9 ± 1.3%) C-to-T conversion corresponding to ∼70−80% f^{5}C

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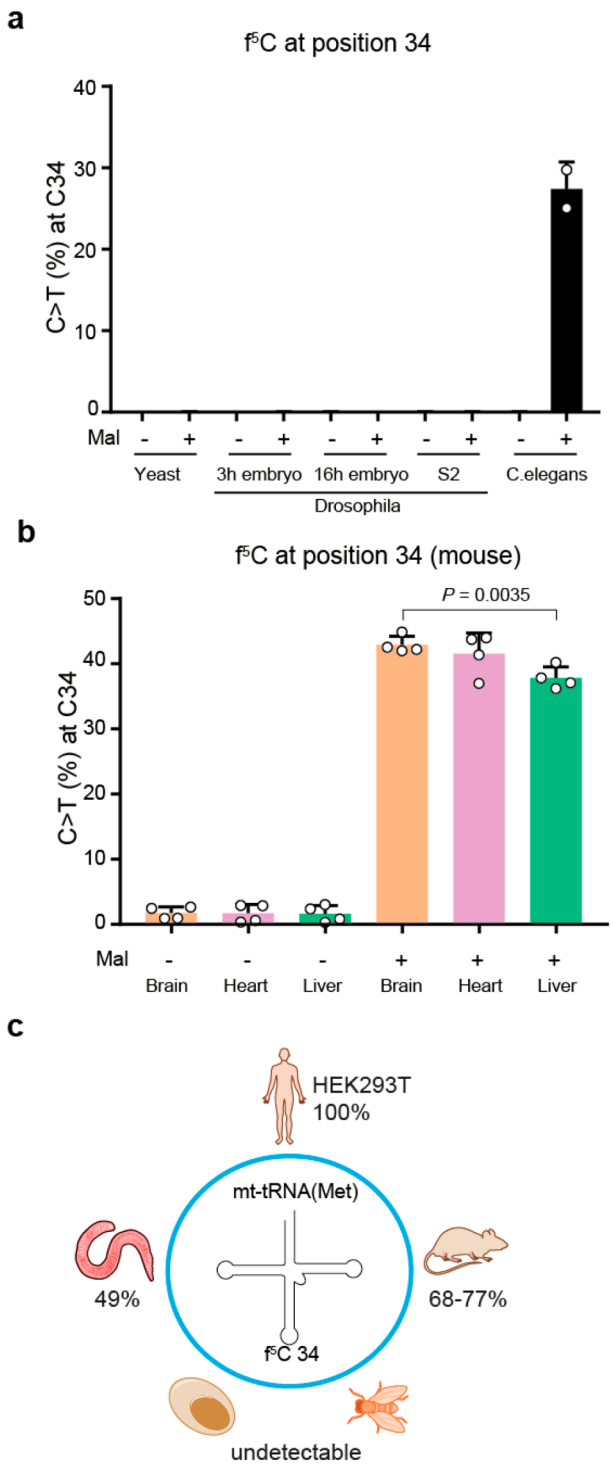

Figure 4. Detection of f^{5}C34 on mt-tRNA(Met) among different organisms. (a) Mal-Seq analysis of C34 on mt-tRNA(Met) using RNA extracted from *C. elegans*, budding yeast, and *D. melanogaster*. (b) Mal-Seq analysis of C34 on mt-tRNA(Met) in different mouse tissues. (c) Schematic showing f^{5}C levels on mt-tRNA(Met) in different organisms detected by Mal-Seq. Data are the mean ± s.d. (n = 2 for yeast, *C. elegans*, fly S2 cells; n = 4 for mouse tissues; n = 2 technical replicates for fly embryos). P values were determined using a two-sided unpaired Student's t test.

modification, with a slight decrease in the liver (Figure 4b and Table S5).

In this work we developed a chemical sequencing approach, Mal-Seq, for detecting and quantifying f^{5}C on RNA based on malononitrile-mediated C-to-T conversion during RT-PCR. Using model f^{5}C containing RNAs, we show that Mal-Seq can detect f^{5}C sites in diverse sequence contexts and that C-to-T mutation frequency correlates linearly with f^{5}C levels between 5 and 100% stoichiometry. An important limitation of our approach is that malononitrile-mediated C-to-T conversion at f^{5}C sites is partial (~50%); therefore, the identification and quantification of low-stoichiometry f^{5}C modifications (<10%) may pose a challenge.

We applied Mal-Seq to analyze f^{5}C modification at the wobble base of mt-tRNA(Met) in different organisms and different tissues types. Our results show that mt-tRNA(Met) is fully modified with f^{5}C in human HEK293T cells and demonstrate ~70−80% modification levels in the mouse tissues that we assayed, which is consistent with the important role of this modification in mitochondrial translation in mammals.[12,13,19] Interestingly, the modification levels are largely invariant in the different murine tissues that we sampled. The oxidation of m^{5}C to f^{5}C on mt-tRNA(Met) requires ALKBH1, which uses O_2 and α-KG as cofactors. In principle, the ALKBH1 activity (and, as a consequence, the mitochondrial translation efficiency) could be responsive to fluctuations in the levels of these central metabolites; whereas this hypothesis remains to be tested explicitly, our data suggest that the installation of f^{5}C on mt-tRNA(Met) is largely independent of the physiological fluctuations in metabolite levels across different tissues under the conditions tested. In addition, we found that high-level f^{5}C modification (>50%) is characteristic of mammals and is absent in lower eukaryotes (Figure 4c). Because recognition of mitochondrial Met AUA/AUU codons is important in many eukaryotes, other mechanisms must exist to support this role in systems lacking f^{5}C. Alternatively, a lower-level f^{5}C modification may be sufficient to satisfy the requirements of mitochondrial translation in these organisms.

Finally, the development of nucleotide resolution sequencing strategies for detecting an f^{5}C modification opens opportunities for mapping this modified base transcriptome-wide in different organisms. While our work was in review, two complementary strategies for the chemical sequencing of f^{5}C were reported by Meier[25] and Zhou,[26] relying on the hydride reduction of f^{5}C to dihydrouridine derivatives. In mammals, f^{5}C has been detected in total RNA by LC-MS analysis,[27] but it is unknown whether this modification occurs outside of the anticodon of mt-tRNA (Met). Interestingly, ALKBH1 has been shown to reside outside of the mitochondria in the nucleus,[28] raising the possibility of f^{5}C sites on non-mitochondrial RNAs. Chemical sequencing strategies, together with identification of the relevant writer enzymes, should enable a comprehensive investigation of the f^{5}C epitranscriptome and shed light on its role in biology. Such studies are under way and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00707.

Chemical and biological methods, high-throughput sequencing data, nucleoside mass spectrometry data, and uncropped gel and Western blot images (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

RT-PCR, reverse-transcription polymerase chain reaction; f^{5}C, 5-formylcytosine; m^{5}C, 5-methylcytidine; hm^{5}C, 5-hydroxymethylcytidine; α-kG, α-ketoglutarate

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