

Transcriptome-wide identification of 5-methylcytosine by deaminase and reader protein-assisted sequencing

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eLife Assessment

This potentially **useful** study introduces an orthogonal approach for detecting RNA modification, without chemical modification of RNA, which often results in RNA degradation and therefore loss of information. Compared to previous versions, the most recent one is improved and sufficiently aligned with the standards of the field to merit consideration by the research community, making the evidence **solid** according to said standards. Nevertheless, uncertainty regarding false positive and false negative rates remains, as it does for some of the alternative approaches. With more rigorous validation, the approach might be of particular interest for sites in RNA molecules where modifications are rare.

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Abstract

5-Methylcytosine (m^5C) is one of the post-transcriptional modifications in mRNA and is involved in the pathogenesis of various diseases. However, the capacity of existing assays for accurately and comprehensively transcriptome-wide m^5C mapping still needs improvement. Here, we develop a detection method named DRAM (deaminase and reader protein assisted RNA methylation analysis), in which deaminases (APOBEC1 and TadA-8e) are fused with m^5C reader proteins (ALYREF and YBX1) to identify the m^5C sites through deamination events neighboring the methylation sites. This antibody-free and bisulfite-free approach provides transcriptome-wide editing regions which are highly overlapped with the publicly available BS-seq datasets and allows for a more stable and comprehensive identification of the m^5C loci. In addition, DRAM system even supports ultra-low input RNA (10ng). We anticipate that the DRAM system could pave the way for uncovering further biological functions of m^5C modifications.

Introduction

Epigenetics refers to stable inheritance without changing the basic sequence of DNA, involving various forms such as DNA methylation, histone modification and RNA modification. In recent years, RNA sequencing technology has boosted research on RNA epigenetics. More than 170 RNA modifications have been identified, mainly including m⁶A, m⁵C, m¹A, m⁷G and others ^{1,2}. Notably, RNA m⁵C methylation represents a crucial post-transcriptional modification observed across different RNA types, such as tRNA, mRNA, rRNA, vault RNA, microRNA, long non-coding RNA and enhancer RNA ³⁻⁸. Numerous studies have revealed multiple molecular functions of m⁵C in numerous key stages of RNA metabolism, such as mRNA stability, translation, and nuclear export ^{5,9-13}. The dynamic alterations of m⁵C play integral roles in many physiological and pathological processes, such as early embryonic development ¹⁴, neurodevelopmental disorder ^{15,16} and multifarious tumorigenesis and migration ¹⁷⁻²⁰. Moreover, this modification significantly contributes to the regulation of gene expression ^{5,9-13,17}. Therefore, the detection of m⁵C sites appears to be essential for understanding their underlying effects on cellular function and disease states.

With the recent advances in sequencing techniques, several high-throughput assays have been developed for qualitative or quantitative analysis of m⁵C. To date, bisulfite-sequencing (BS-seq) has been proven to be the gold standard method for RNA m⁵C methylation analysis ^{5,21,22}. This approach chemically deaminates unmethylated cytosine to uracil, while keeping methylated cytosine unchanged. The m⁵C methylation sites can be identified by subsequent library construction and sequencing. However, bisulfite treatment of BS-seq is extremely detrimental to RNA, thus resulting in unstable detection of m⁵C in low abundance RNA or highly structured RNA, which directly affects the confidence of results ^{23,24}. Another major type of global m⁵C analysis depends on antibody-assisted immunoprecipitation of m⁵C methylated RNAs, such as m⁵C-RIP-seq ²⁵⁻²⁷, AZA-IP-seq ²⁸ or miCLIP-seq ⁷. These methods are unable to recognize methylation on mRNAs with low abundance and secondary structure. Moreover, these methods are highly dependent on antibody specificity, which usually leads to unspecific binding of RNA and a low amount of m⁵C-modified regions. Moreover, TAWO-seq, originally developed for the identification of hm⁵C, is also capable of m⁵C analysis, but it highly depends on the oxidation efficiency of perovskite, which usually causes false positives and unstable conversion ^{29,30}. Furthermore, the emerging third-generation sequencing, such as Nanopore-seq, can directly map m⁵C by tracking the characteristic changes of bases, but it still faces challenges of a high error rate ³¹⁻³³. These together largely hamper its wide application on transcriptome profiling of m⁵C (Supplementary Table 1). Hence, there is an urgent need for a simple, efficient, sensitive, and antibody-independent method for global m⁵C detection.

The RNA-binding protein ALYREF is the initially recognized nuclear m⁵C reader that binds directly to m⁵C sites in mRNA and plays key roles in promoting mRNA nuclear export or tumor progression ⁵. Another well-known m⁵C reader, YBX1, can also recognize m⁵C-modified mRNA through its cold-shock domain and participates in a variety of RNA-dependent events such as mRNA packaging, mRNA stabilization and translational regulation ^{9,18}. RNA affinity chromatography and mass spectrometry analyses using biotin-labelled oligonucleotides with or without m⁵C were performed in previous reports, which indicated that ALYREF and YBX1 had a more prominent binding ability to m⁵C-modified oligonucleotides ^{5,18}. YBX1 can preferentially recognize mRNAs with m⁵C modifications via key amino acids W65-N70 (WFNVRN) ¹⁸, while K171 is essential for the specific binding of ALYREF to m⁵C sites ⁵. Previous studies have shown that mutations in key amino acids responsible for recognising m⁵C binding in ALYREF and YBX1 lead to a significant reduction in their binding levels to m⁵C-containing oligonucleotides ^{5,18}. Nucleic acid deaminases, primarily categorized as cytosine deaminases and adenine deaminases, are zinc-dependent enzymes which facilitate the deamination of cytosine or adenine within DNA

or RNA substrates³⁴, APOBEC1, an evolutionarily conserved family member of APOBEC proteins, can specifically catalyze the deamination of cytosine in single-stranded RNA (ssRNA) or DNA (ssDNA) to uracil^{35–37}. TadA8e is an adenine deaminase optimized through re-engineering of TadA and it induces conversion of adenine to inosine (eventually read as guanine by transcriptases) in ssRNA or ssDNA^{38,39}. APOBEC1 and TadA8e, with their prominent deamination efficiency, have been employed for the development of precise and efficient base editors such as CBE and ABE8e, which find widespread application in studies related to genome editing^{37,38}.

Here we aim to establish a deaminase and m⁵C reader-assisted RNA methylation sequencing approach (DRAM-seq), which identifies the m⁵C sites through reader-mediated recognitions and deaminase-mediated point mutations neighboring the m⁵C methylation sites. This bisulfite-free and antibody-free method is anticipated to provide more comprehensive and cost-effective transcriptome-wide detection of m⁵C methylation, which may better assist on exploring its further regulatory mechanisms.

Results

Development of DRAM system for m⁵C detection

Our sequencing platform is inspired by the concept of the m⁶A DART-seq assay, in which C near the m⁶A site is converted into U without affecting sequences near non-m⁶A sites⁴⁰. Therefore, we hypothesized that, by utilizing the targeted binding of m⁵C readers, deaminase can be recruited to achieve deamination of cytosine or adenine in the vicinity of the m⁵C sites on single-stranded RNA, thereby facilitating the detection of the m⁵C site. This approach was named DRAM (deaminase and m⁵C reader-assisted RNA methylation sequencing). As RNA-binding proteins, ALYREF and YBX1 also could bind to RNAs without m⁵C modification^{5,18}. To exclude the false-positive detection of DRAM due to the non-m⁵C specific binding of ALYREF and YBX1, knockout of W65-N70 (WFNVRN) amino acids in YBX1 and K171A mutation in ALYREF were introduced separately, resulting in the DRAM^{mut} system (Fig. S1A-S1D). Subsequently, we verified the affinity ability of YBX1 and ALYREF for m⁵C-modified RNAs by RNA pull-down experiments. Consistent with previous reports^{5,18}, those two m⁵C readers preferentially bound RNAs containing m⁵C modifications. Furthermore, mutating key amino acids involved in their interaction with m⁵C significantly reduced their binding ability, indicating that ALYREF and YBX1 exhibit specificity for m⁵C-methylated mRNAs. (Fig. S1E-S1H). To confirm the recognition of m⁵C site by DRAM system, DRAM, DRAM^{mut} and Deaminase system were transfected into the human HEK293T cells, respectively. Finally, we considered the presence of m⁵C modification in the vicinity only if the deamination changes produced under DRAM induction were significantly different from those produced under DRAM^{mut} or Deaminase induction (Fig. 1A).

Previous studies have indicated that there is no uniform intrinsic signature motif sequence that can characterize all m⁵C sites^{5,26,41,42}. To comprehensively detect the m⁵C loci, the readers of m⁵C (ALYREF and YBX1) were separately fused to the C-terminus of the deaminases (APOBEC1 and TadA-8e), namely DRAM-ABE and DRAM-CBE system (Fig. 1B).

DRAM detection system is assayed in an m⁵C-dependent form

To confirm the recognition of m⁵C site by DRAM system, DRAM, DRAM^{mut} and Deaminase were transfected into the human HEK293T cells, respectively. To evaluate candidate DRAM constructs within a cellular environment, we performed fluorescence microscopy to analyze the expression of DRAM. The results showed that DRAM-ABE and DRAM-CBE were properly expressed in HEK293T cells (Fig. S2A-S2B). In addition, flow cytometry displayed ~60% of cells were GFP-positive (Fig. S2C). Two previously reported m⁵C sites in RPSA and AP5Z1 were selected for the

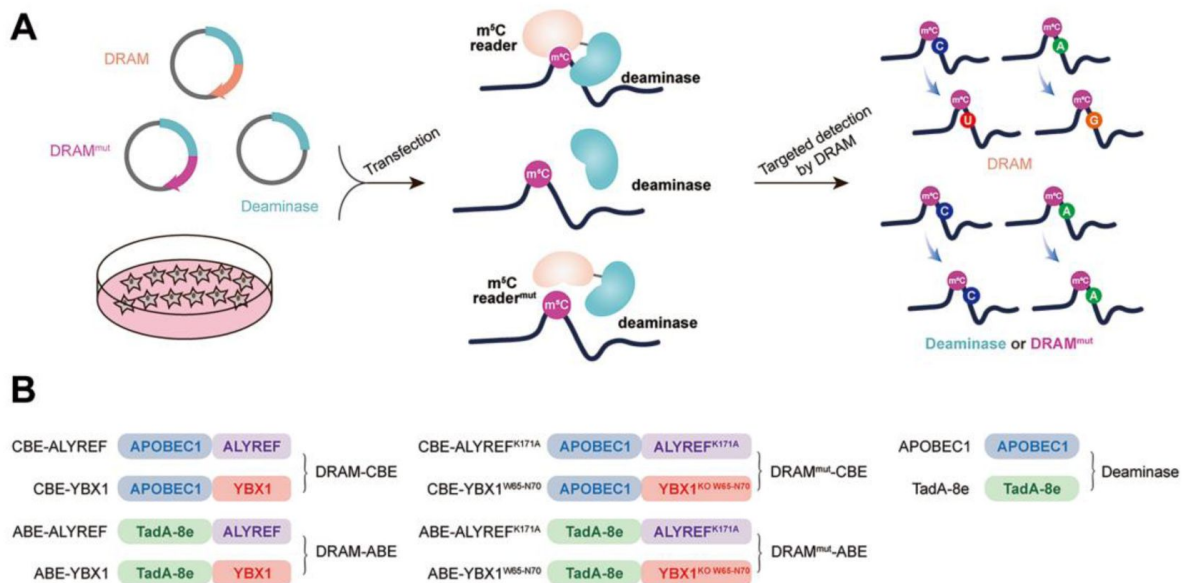


Fig.1

Development of DRAM system for m⁵C detection.

(A) Schematic diagram of the DRAM assay. DRAM, DRAM^{mut} and Deaminase system were transfected into HEK293T cells separately. After DRAM transfection, the deaminase was directed by m⁵C reader to the vicinity of the m⁵C site and induce C-to-U/A-to-G mutations, whereas transfection of the DRAM^{mut} or Deaminase system failed to effectively induce similar mutations due to the absence of the m⁵C-recognition-binding domain.

(B) The overall design of DRAM, DRAM^{mut} and Deaminase system.

analysis^{5,21}, and their methylation status was verified by bisulfite sequencing PCR. The deep sequencing results showed that the m⁵C fraction of RPSA and SZRD1 was 75.5% and 27.25%, respectively (**Fig. 2A** and **B**). Sanger sequencing following RT-PCR was then performed to determine the editing of neighbouring m⁵C sites by DRAM system in these two mRNA. Notably, adenine close to the m⁵C site in RPSA mRNA was mutated into guanine, resulting in an A-to-G editing rate of 14.7% by DRAM-ABE, whereas this was rarely observed with TadA-8e or DRAM^{mut}-ABE (**Fig. 2C**). DRAM-CBE induced C to U editing in the vicinity of the m⁵C site in AP5Z1 mRNA, with 13.6% C-to-U editing, while this effect was significantly reduced with APOBEC1 or DRAM^{mut}-CBE (**Fig. 2D**). Subsequently, in order to investigate whether the DRAM system can detect other types of RNA, such as tRNA, 28S rRNA, or others, we performed PCR amplification of the flanking sequences of the m⁵C sites 3782 and 4447 on 28S rRNA and several m⁵C sites on tRNA, such as the m⁵C48 and m⁵C49 sites of tRNA^{Val}, the m⁵C48 and m⁵C49 sites of tRNA^{Asp}, and the m⁵C48 site of tRNA^{Lys}. But Sanger sequencing showed that there was no valid A-to-G/C-to-U mutation detected, which is most likely due to the fact that ALYTEF and YBX1 are mainly responsible for the mRNA m⁵C binding proteins, and thus the DRAM system is more suitable for the mRNA m⁵C detection (Fig. S3). Taken together, the fusion of m⁵C reader and deaminase can effectively and selectively deaminate cytosine/adenine in the vicinity of the mRNA m⁵C sites.

NSUN2⁴³ and NSUN6⁴⁴, two family members of NOL1/NSUN protein, were both identified as m⁵C methyltransferase of mRNA⁴⁵. To verify that the detection of DRAM occurs in the presence of m⁵C, we performed knockdown experiments of NSUN2 and NSUN6 in HEK293T cells by base deletion, resulting in frameshift mutations that led to reduced expression of NSUN2 and NSUN6. These cells were then transfected with DRAM. The knockout efficiency has been confirmed by western blotting (**Fig. 2E**, **2F** and Fig. S4A,4B). It has been previously demonstrated that m⁵C methylation of AP5Z1 and RPSA is catalyzed by NSUN2 and NSUN6, respectively^{21,46}. In line with this, sanger sequencing following RT-PCT showed a significant reduction in C-to-U or A-to-G mutations near the m⁵C sites in methyltransferase-deficient cells compared with WT cells (**Fig. 2G** and **H**). Overall, these findings suggest that the DRAM detection system is assayed in an m⁵C-dependent form.

DRAM enables transcriptome-wide analysis of m⁵C methylation

Subsequently, we performed RNA-seq analysis after DRAM transfection by detecting C-to-U/A-to-G editing events to accomplish transcriptome-wide detection of m⁵C (**Fig. 3A**). To serve as positive controls, two previously published BS-seq datasets were also integrated^{5,21}. Mutations were detected near the m⁵C site in RPSA as A-to-G by DRAM-ABE (**Fig. 3B**), and DRAM-CBE detected the presence of C-to-U mutations near the AP5Z1 m⁵C site (**Fig. 3C**). However, the DRAM^{mut} and Deaminase systems induced few effective mutations close to these sites. Examination of multiple reported high-confidence RNA m⁵C sites showed that DRAM-seq editing events were also enriched in the vicinity of the BS-seq sites (**Fig. 3B**, **3C** and Fig. S5).

DRAM-seq analysis further confirmed that mutations in AP5Z1 and RPSA mRNA were reduced in methyltransferase knockout cells compared to wild-type cells (**Fig. 3D**, **3E**). Moreover, the knockout cells exhibited overall rare DRAM-seq editing events close to m⁵C sites in other mRNAs (Fig. S6). These indicated that DRAM-seq analysis was detected in an m⁵C-dependent manner. Unfortunately, motif analysis failed to identify any sequence preferences or consensus motifs associated with DRAM-edited sites mediated by loci associated with NSUN2 or NSUN6. (Fig. S4D).

A comparison of three biological replicates from each experimental group revealed a strong reproducibility of A-to-G/C-to-U mutations in HEK293T cells expressing DRAM-ABE and DRAM-CBE (Fig. S7). Moreover, the DRAM-edited mRNAs revealed a high degree of overlap across the three biological replicates (Fig. S4C). And a recent study by Wang et al. showed that ALYREF deletion

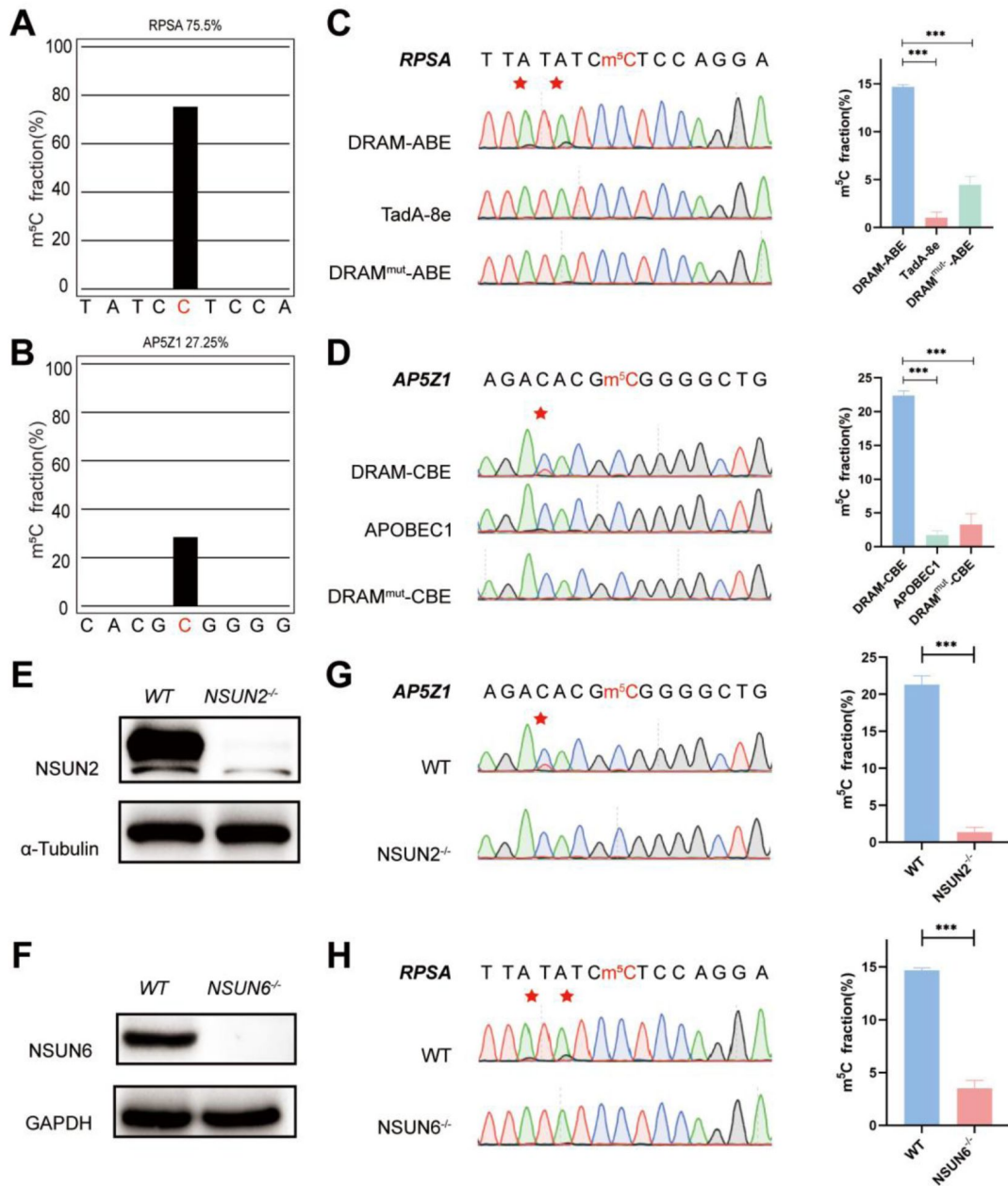


Fig.2

DRAM detection system was assayed in an m⁵C -dependent form.

(A, B) Two m⁵C sites from RPSA (A) and AP5Z1 (B) mRNA detected by deep sequencing of bisulfite sequencing PCR in HEK293T cells. The m⁵C sites are highlighted by red color. The m⁵C fraction of RPSA and AP5Z1 were 75.5% and 27.25% (The number of reads is greater than 1000). (C, D) Sanger sequencing following RT-PCR verified two m⁵C sites from RPSA (C) and AP5Z1 (D) mRNAs in DRAM-transfected HEK293T cells, respectively. HEK293T cells only expressing DRAM^{mut} or Deaminase were served as negative controls. The left panel illustrates the location of DRAM induced mutation sites, which is highlighted in red asterisk. The right panel shows the corresponding quantification of sanger sequencing.

(E, F) The knockout efficiency of NSUN2 (E) and NSUN6 (F) in HEK293T cell lines verified by Western blotting. The protein level of α-Tubulin and GAPDH were served as loading controls, separately.

(G, H) DRAM induced mutations close to m⁵C sites in AP5Z1 (G) and RPSA (H) mRNAs after NSUN2 and NSUN6 knockout in HEK293T cells. The left panel illustrates the location of DRAM induced mutation sites, which is highlighted in red asterisk. The right panel shows the corresponding quantification of sanger sequencing.

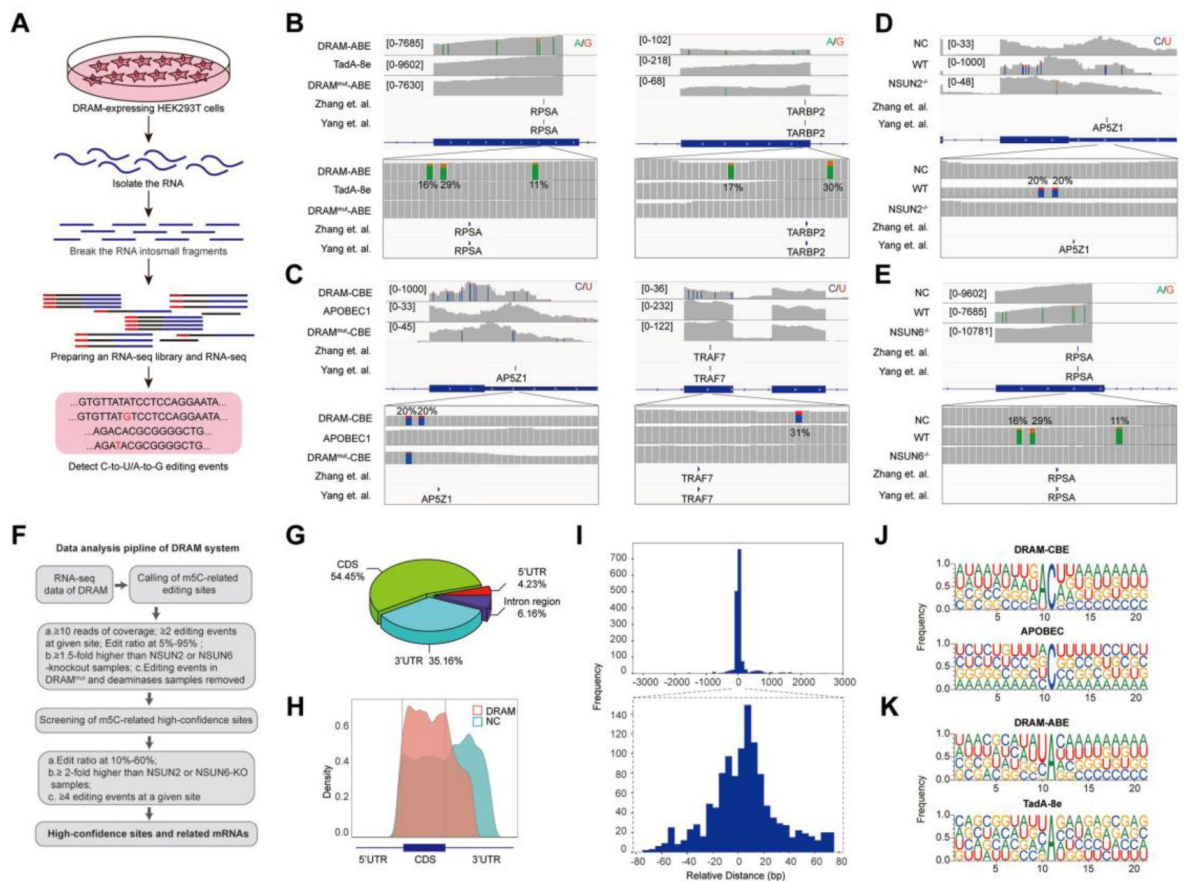


Fig.3

DRAM enables transcriptome-wide analysis of m⁵C methylation.

(A) Schematic of the DRAM-seq method.

(B, C) Integrative genomics viewer (IGV) browser traces of DRAM-seq data expressing the indicated constructs in RPSA (**B**, left panel), TARBP2 (**B**, right panel), AP5Z1 (**C**, left panel), and TRAF7 (**C**, right panel) mRNAs. C-to-U or A-to-G mutations found in at least 10% of reads are indicated by coloring. The previously published RNA BS-seq datasets from two individual studies were displayed as panel “Yang et al.” and “Zhang et al.”. (n(DRAM)=3 independent samples, n(Deaminase)=2 independent samples, and n(DRAM^{mut})=1 independent sample.)

(D, E) Integrative genomics viewer (IGV) browser traces of DRAM-seq data in wildtype and methyltransferases knockout cells in AP5Z1 (**D**) and RPSA (**E**) mRNAs. C-to-U or A-to-G mutations were found in at least 10% of reads are indicated by coloring. The previously published RNA BS-seq datasets from two individual studies were displayed as panel “Yang et al.” and “Zhang et al.”. n=3 independent samples.

(F) Screening process for DRAM-seq assays and principles for screening high-confidence genes.

(G) The pie chart shows the distribution of editing sites in different transcript region in cells expressing DRAM (n=3 independent samples).

(H) The density map showing the distribution of editing events across the mRNA transcripts detected by DRAM-seq.

(I) The frequency plot showing the distribution of the distances of edit events in DRAM-seq relative to the m⁵C sites from the published BS-seq datasets. The position of each m⁵C site of BS-seq is determined as 0, and the relative distance of each site to the nearest edit event in DRAM-seq is calculated and plotted. The plots are presented separately based on the cutoff of upstream and downstream 3000bp (above) and 80bp (below) windows.

(J, K) Motif analysis discovered within the ±20nt region around the C-to-U or A-to-G editing site in cells expressing DRAM-CBE (**J**), APOBEC1 (**J**), DRAM-ABE (**K**) and TadA-8e (**K**).

affects the expression of 94 mRNAs⁴⁷, and only 55.32% of these ALYREF-regulated mRNAs can be detected by the DRAM system (Fig. S4E). These findings suggest that DRAM selectively targets specific RNAs for editing, exhibiting a high degree of consistency across samples.

To obtain information on a set of high-confidence DRAM-seq data, we filtered the list of sites transfected with deaminase alone and screened the sequencing results with methyltransferase depleted, pooled editing events occurring in at least 10% of reads across multiple samples to obtain a set of high-confidence editing sites (Fig. 3F and Supplementary Table 2), and integrated genes with editing sites occurring in DRAM-ABE and DRAM-CBE (Fig. 3F and Supplementary Table 3). Previous studies have indicated that m⁵C sites are predominantly distributed in the coding sequences (CDS) and notably enriched near the initiation codon^{5,25,26,48–50}. To further delineate the characteristics of the DRAM-seq data, we compared the distribution of DRAM-seq editing sites within the gene structure, specifically examining their occurrences in the 5' untranslated region (5'UTR), 3' untranslated region (3'UTR), CDS and Intergenic/Intron region. Our analysis revealed that DRAM-seq editing events in cells expressing DRAM-ABE and DRAM-CBE were primarily located in the CDS and 3'UTR, indicating a non-random distribution of m⁵C (Fig. 3G, Fig. S8A and 8B). Moreover, plotting the distribution of DRAM-seq editing sites in mRNA segments (5'UTR, CDS, and 3'UTR) highlighted a significant enrichment in the CDS (Fig. 3H). In contrast, cells expressing the deaminase exhibited a distinct distribution pattern of editing sites, characterized by a prevalence throughout the 3'UTR (Fig. 3H). This finding reaffirms that the specific editing pattern observed in DRAM-seq across the transcriptome depends on its capacity to bind m⁵C.

Comparative analysis of the DRAM-seq editing sites with the previously published BS-seq m⁵C sites indicated that the likelihood of editing was notably higher in closer proximity to the m⁵C sites (Fig. 3I). Furthermore, the editing window of DRAM exhibited enrichment approximately 20bp before and after the m⁵C site (Fig. 3I). Investigation into the sequences surrounding the editing window revealed that AC motifs were the most significantly enriched in DRAM-CBE, whereas (U/C) A motifs were most notably enriched in DRAM-ABE. In contrast, the APOBEC1 and Tada-8e samples displayed no significantly enriched motifs, with mutations being more randomly orientated (Fig. 3J, 3K).

DRAM-seq provides stable and comprehensive identification of m⁵C loci

Subsequently, we then evaluated the ability of DRAM-seq to detect m⁵C across the entire transcriptome and compared its performance to that of the previously reported BS-seq. Although both previous studies employed bisulfite treatment, the resulting data obtained significant discrepancies due to variations in their treatment and analysis methodologies. We first compiled the overall distribution of mutant regions identified by DRAM-seq, presenting both the mutant sites detected by the DRAM system and those reported in previous studies^{5,18} across each chromosome (Fig. 4A). Our results indicated that DRAM-seq identified the presence of m⁵C modifications covering 79.6% of the genes detected by Yang et al.⁵ and 91.9% of the genes detected by Zhang et al.²¹ (Fig. 4B and D). Remarkably, certain pivotal regulators with diverse biological functions, such as ATG16L1 (coordinates autophagy pathway)⁵¹ and ARHGEF25 (plays an important role in actin cytoskeleton reorganisation)⁵², were identified by Zhang et al. and DRAM-seq, but not by Yang et al. (Fig. 4C). Conversely, FANCD2 (Maintains chromosome stability)⁵³ and RPL15 (components of the large ribosomal subunit)^{54,55}, were discovered by Yang et al. and DRAM-seq, but not by Zhang et al. (Fig. 4E). Hence, DRAM-seq appears to offer a more stable and comprehensive identification of the m⁵C loci.

To provide functional insights into m⁵C RNA-modified genes in HEK293T cells, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. These results highlighted the involvement of these genes in the regulation of diverse key biological processes,

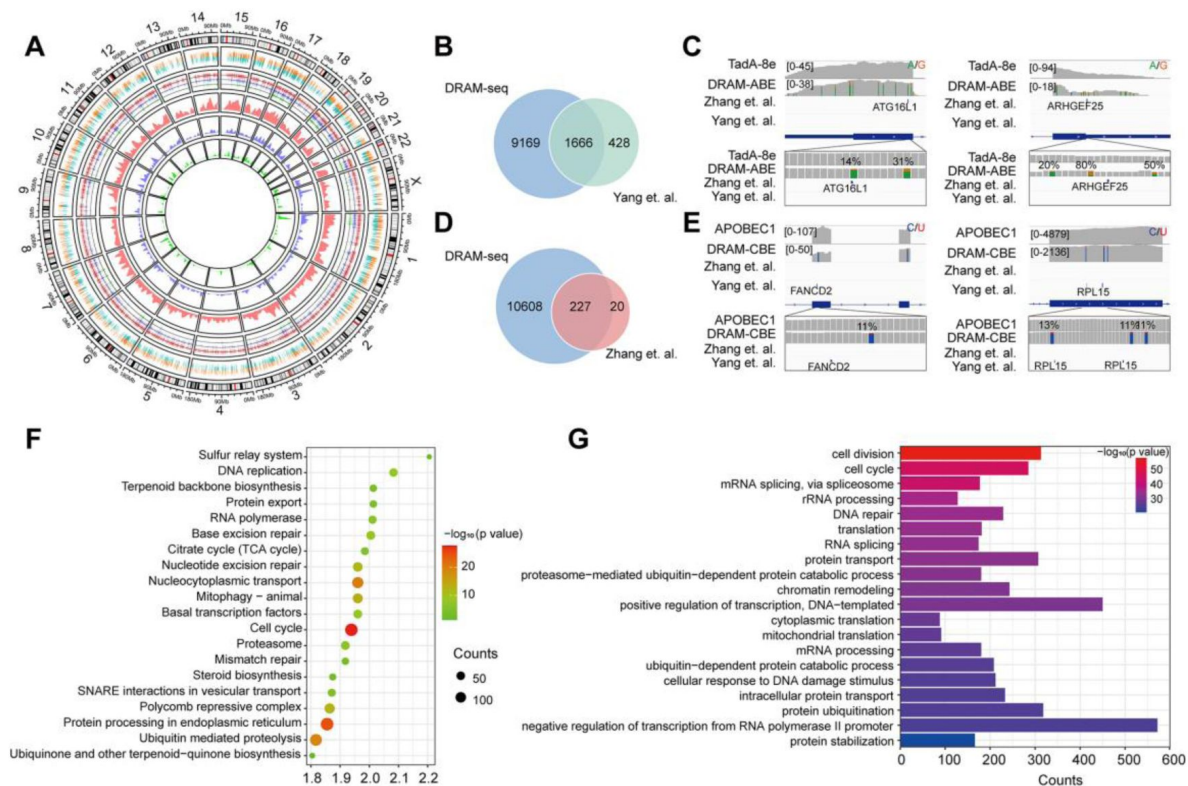


Fig.4

Stable and comprehensive cellular identification of m⁵C loci by DRAM-seq.

(A) Comparison of the overall distribution of genes with m⁵C modifications detected by DRAM-seq, Yang et al. and Zhang et al. on chromosomes. The mutation sites detected by DRAM-seq on each gene are categorized into dual-colored short lines, with positive strand mutations shown in orange and negative strand mutations in dark green. The line graph and kernel density plot in the inner ring represent the locations and distributions of overlapping genes detected by DRAM-seq (red), Yang et al. (blue) and Zhang et al. (light green).

(B) Venn diagram showing the overlap between DRAM-seq and Yang et al.'s edited genes.

(D) Venn diagram showing the overlap between DRAM-seq and Zhang et al.'s edited genes.

(C, E) Integrative genomics viewer (IGV) browser traces of DRAM-seq data expressing the indicated constructs in the ATG16L1 (B), ARHGEF25 (B), FANCD2 (D), and RPL15 (D) mRNAs. C-to-U/A-to-G mutations found in at least 10% of reads are indicated by coloring, and the m⁵C site found by BS-seq is also labelled.

(F) Genes with DRAM-seq editing events were analyzed for KEGG bioprocess enrichment.

(G) GO biological processes enrichment analysis of genes with DRAM-seq editing events. Statistical analyses were performed using the DAVID tool.

such as cell division, cell cycle, mRNA splicing, protein processing in the endoplasmic reticulum, nucleocytoplasmic transport, translation, DNA repair and others (**Fig.4F** [↗](#), **4G** [↗](#), Fig. S8C and S8D).

DRAM enables low-input m⁵C profiling

A significant challenge in m⁵C detection lies in the specificity of antibodies and the substantial amount of input RNA required for sequencing. RNA is susceptible to degradation during denaturation, sodium bisulfite treatment and desulfurization steps in the BS-seq assay⁵⁶ [↗](#). Immunoprecipitation-based m⁵C assays and LC-MS/MS also impose high demand for sample input⁷ [↗](#),²⁵ [↗](#),⁵⁷ [↗](#). Several experiments have highlighted the requirement of 100-500 ng of RNA for m⁵C-RIP-seq, while BS-seq necessitates an even more demanding 750-1000 ng of RNA²¹ [↗](#),²⁵ [↗](#),⁵⁸ [↗](#). To assess the detection limits of DRAM-Sanger, we attempted to amplify two representative m⁵C-containing sites in the RPSA and AP5Z1 transcripts from diluted RNA samples.

Remarkably, we successfully generated PCR products of these two mRNAs from cDNAs corresponding to 250 ng, 50 ng, and 10 ng of total RNA. Quantitative analysis by Sanger sequencing demonstrated nearly identical Sanger traces across these dilutions (**Fig.5A** [↗](#) and **B** [↗](#)). This finding underscores that the specificity of DRAM editing depended on its ability to bind m⁵C, and DRAM is proficient in low-input m⁵C analyses. Furthermore, cell viability was determined by CCK8 assay on HEK293T cells transfected with DRAM (**Fig.5C** [↗](#)). Importantly, there was no significant difference in the relative proliferative capacity of the cells compared to untransfected cells (NC), indicating that DRAM expression did not adversely affect cell viability (**Fig.5D** [↗](#)).

Transfection of the DRAM system in cells results in the transient overexpression of fusion proteins. To investigate how varying expression levels of these proteins influence A-to-G and C-to-U editing within the same m⁵C region, we conducted a gradient transfection using plasmid concentrations of 1500 ng, 1000 ng and 500 ng. This approach allowed us to progressively reduce the expression levels of the fusion proteins (**Fig. 5E** [↗](#) and **5F** [↗](#)). Sanger sequencing revealed that the editing efficiency of A-to-G and C-to-U within the m⁵C region significantly decreased as fusion protein expression diminished (**Fig. 5G** [↗](#) and **5H** [↗](#)). These findings suggest that the transfection efficiency of the DRAM system is concentration-dependent and that the ratio of editing efficiency to transfection efficiency may assist in the quantitative analysis of m⁵C using the DRAM system.

Discussion

In recent years, m⁵C methylation modifications have received increasing attention, with multiple reports detailing the distribution of RNA m⁵C methylation modifications across various species and tissues, elucidating their characteristics. Despite the relatively low abundance of m⁵C, its highly dynamic changes hold significant implications for the regulation of physiological and pathological processes⁵ [↗](#),²¹ [↗](#),⁴⁴ [↗](#). However, due to the limitations of sequencing methods and the variability of data processing, there remains ample room for progress in the study of m⁵C detection methods.

In this study, we developed a site-specific, depth-sequencing-free m⁵C detection method using DRAM-Sanger. This workflow relies on conventional molecular biology assays such as RT-PCR and Sanger sequencing, eliminating the need for specialized techniques and thereby simplifying the process of m⁵C detection.

DRAM-seq introduces a novel strategy for transcriptome-wide m⁵C detection, overcoming inherent limitations in existing methods. Notably, DRAM-seq covered around 80% of the high-confidence m⁵C-modified genes detected by BS-seq and identified more potential m⁵C sites. This can be attributed to the avoidance of bisulfite treatment by DRAM-seq, preventing RNA damage and

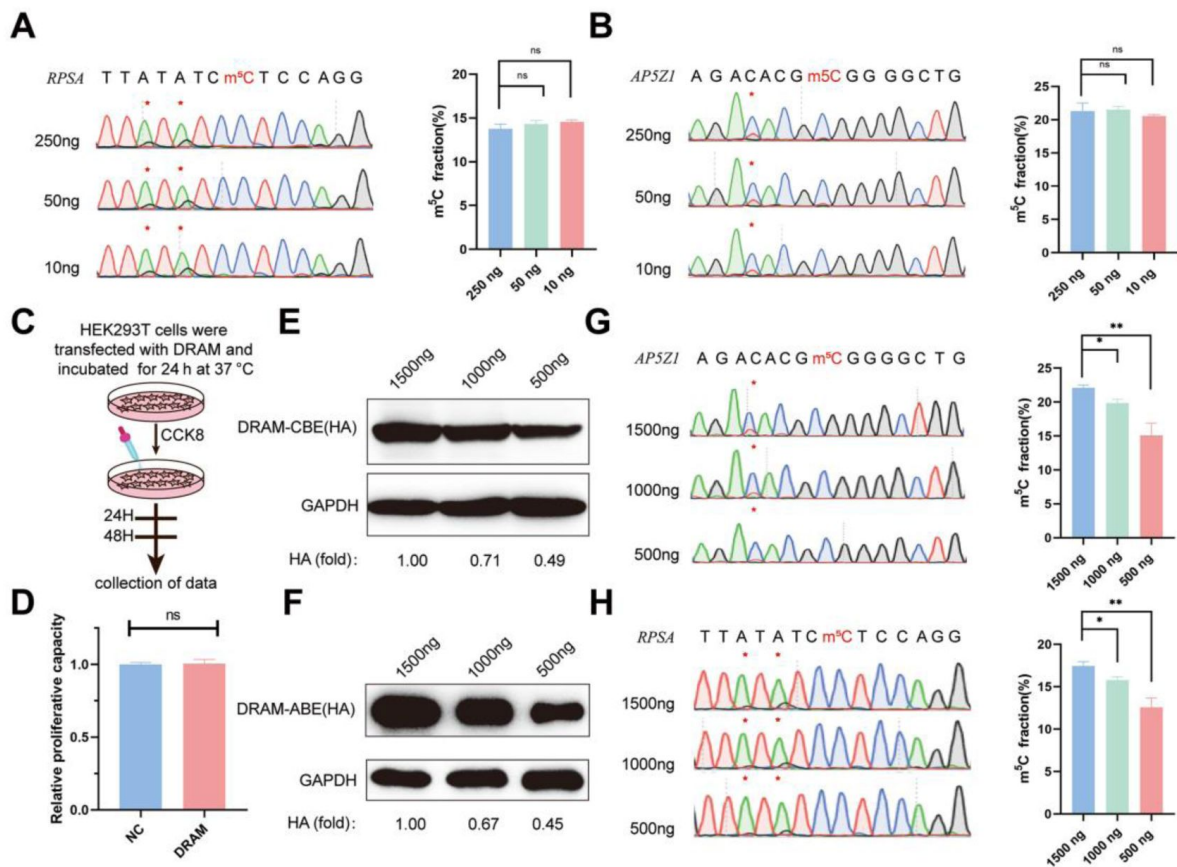


Fig.5

Low-input m⁵C detection and transfection efficiency of DRAM system.

(A, B) DRAM analysis of RPSA (A) and AP5Z1 (B) mRNAs with 250 ng, 50 ng, and 10 ng of input RNA. Representative Sanger sequencing plots are shown on the left panel, with mutation sites marked with asterisks. The mutation rates are quantified on the right panel.

(C) Flowchart illustrating Cell viability analysis by CCK8 reagent after DRAM transfection in HEK293T cells.

(D) Quantitative comparison of the relative proliferative capacity of DRAM-expressing and untransfected cells.

(E, F) The expression levels of DRAM-CBE (E) and DRAM-ABE (F) systems different plasmid transfection concentrations were verified by Western blotting.

(G, H) Editing of RPSA (G) and AP5Z1 (H) mRNA at varying concentrations of DRAM protein expression. The left panels indicate Sanger sequencing results following RT-PCR, while the corresponding quantifications of DRAM-induced mutations are shown in the right panels.

ensuring a more comprehensive representation of RNA samples. This feature also likely contributes to the observed stability of DRAM-seq in comparison to BS-seq. Additionally, DRAM-seq is not limited by antibody specificity and is resistant to chemical-induced damage.

A prominent challenge in existing m⁵C profiling methods is their reliance on substantial amounts of input RNA samples. In contrast, DRAM operates through the deamination activity of deamination activity of deaminase, preserving RNA integrity and preventing degradation. The notable advantage of DRAM lies in its capacity for low-input m⁵C detection. Our analysis demonstrates that DRAM requires as low as 10ng of total RNA for m⁵C detection. While DRAM is currently well-suited for detecting m⁵C on a transcriptome-wide scale, the potential for future applications involving third-generation sequencing could extend its utility to individual mRNAs, particularly m⁵C heterogeneity on mRNA splicing variants. In addition, the DRAM system depends on the specific recognition of m⁵C modifications on ssRNA by the reader protein, theoretically avoiding the false-positive effects of 5-hydroxymethylation modifications in other assays, such as BS-seq²¹[–23](#). This potential feature could enhance the accuracy of the DRAM assay, albeit it still requires careful validation.

In our study, m⁵C detection was performed following the transient transfection of the DRAM detection system into mammalian cells, which might result in a lower mutation rate at the corresponding site. Therefore, employing lentiviral-mediated transfection into cell lines of interest could potentially enhance the efficiency of m⁵C detection. Our results confirm that YBX1 and ALYREF exhibit specificity as m⁵C readers, binding preferentially to RNAs with m⁵C modifications, thereby validating the reliability of the DRAM detection system. However, mutations in the key amino acids responsible for m⁵C binding reduced their affinity while retaining some binding capacity. DRAM-seq analysis identified a substantial number of m⁵C sites. However, we cannot exclude the potential existence of false positive sites resulting from non-specific binding of the m⁵C reader. Further elucidation of the key amino acids directing ALYREF and YBX1's binding to m⁵C methylation sites should enable more accurate and sensitive m⁵C detection by DRAM-seq. Due to the lack of a fixed base composition for characterizing all m⁵C modification sites, DRAM has an apparent limitation in achieving single-base resolution for detecting m⁵C. This technical constraint may explain the absence of identifiable sequence specificity in our analysis of m⁵C sites catalyzed by NSUN2 and NSUN6, despite previous reports associating these methyltransferases with “G”-rich sequences and the “CUCCA” motif⁵⁹[–62](#). However, our present study proved that the measuring resolution of DRAM is around 40nt, which facilitates higher precision than that of m⁵C-RIP-seq (~100nt). In the future, with more in-depth analyses of m⁵C reader structures and the identification of new potential m⁵C readers, we expect to achieve more precise m⁵C localization and more comprehensive m⁵C modification detection. Moreover, the substitution of deaminases, such as A3A and A3G (the family members of APOBEC), could also potentially enhance the efficiency of the DRAM detection⁶⁰[–62](#).

Although the m⁵C assay can be performed using the DRAM system alone, comparing it with the DRAM^{mut} and deaminase controls could enhance the accuracy of m⁵C detection in specific regions. Given that the expression of DRAM fusion proteins significantly influences m⁵C detection, it is advisable to transfect the same batch of cells during the assay to ensure consistent transfection efficiency across experimental groups and thus can better standardize the detection.

One future direction of endeavour is the purification of DRAM fusion proteins to facilitate *in vitro* detection of RNA m⁵C methylation, which could extend the scope of DRAM-seq to diverse sample types. Another potential application for DRAM-seq could be the expression of drug-inducible DRAM systems *in vivo* using various animal models for m⁵C analysis. These will together provide novel insights into m⁵C modifications for biological and clinical research.

Conclusions

In summary, we developed a novel deaminase and reader protein-assisted RNA m⁵C methylation approach that detects the m⁵C region by deaminating As or Cs in close proximity to the m⁵C sites, which does not rely on antibodies or bisulfite, thus leading to unprecedentedly comprehensive transcriptome-wide RNA m⁵C methylation profiling. We anticipated that this system could pave the way for uncovering further biological functions of m⁵C modifications and facilitate the development of therapeutic interventions for associated diseases.

Materials and methods

Plasmid construction

ALYREF and YBX1 expression plasmids were purchased from MIAOLING BIOLOGY (<http://www.miaolingbio.com/>), and the ALYREF and YBX1 fractions were then amplified using specific primer. The ALYREF and YBX1 portions were amplified using pCMV-APOBEC1-YTH (Addgene plasmid no. 131636; <https://www.addgene.org/131636/>) and ABE8e (Addgene plasmid no. 138489; <https://www.addgene.org/138489/>) to amplify the deaminase portion and the essential plasmid construct proxies, and finally the fragments were recombined by the ClonExpress Ultra One Step Cloning Kit to complete the plasmid vector construction. Both DRAM^{mut}-ABE and DRAM^{mut}-CBE related vectors were obtained by introducing the corresponding key amino acid mutations using Fast Site-Directed Mutagenesis Kit (TIANGEN Biotech). The primer sequences used are listed in Supplementary Table 4.

Cell culture and plasmid transfection

HEK293T cell line (ATCC) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (CLARK BIOSCIENCE) and 1% penicillin (100 U/ml)-streptomycin (100 µg/ml). The cells were seeded in 12-well plates and transfected using Hieff TransTM Liposomal Transfection Reagent (Yeasten).

NSUN2-depleted cell lines were generated by cloning NSUN2-targeting single guide RNA sequences into the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid (Addgene plasmid no. 62988; <http://n2t.net/addgene:62988>). Plasmids were then transfected into HEK293T cells and Puromycin (Meilunbio) was added at a final concentration of 3 µg/ml to enrich the positively transfected cells 24 h after transfection. After 72 h, the cells were collected and used for genotyping by Sanger sequencing. NSUN6-depleted cell lines were generated in the same way. The primers used for genotyping and single guide RNA sequences are listed in Supplemental Table 4.

Cell viability measurements

HEK293T cells were transfected with DRAM plasmid and cultured at 37°C for 24 h. Subsequently, 1000 cells were seeded in 96-well plates. After waiting for the cells to attach to the wall, the cell activity was detected by Cell Counting Kit-8 (Meilunbio). Cell Counting Kit-8 contains WST-8, which in the presence of the electronically coupled reagent 1-Methoxy PMS can be reduced by mitochondrial dehydrogenase to the orange-colored metazan product Formazan, the absorbance of which is measured at 450 nm to analyze cellular activity.

Western blotting

For protein blotting, samples were lysed in RIPA Lysis Buffer (Meilunbio) with Phenylmethanesulfonyl fluoride (PMSF) and the BCA protein assay kit (Beyotime Biotechnology) was used to Protein concentration was measured. Total protein extracts were separated by SDS-PAGE on a 10% gel and then transferred to 0.22 nm polyvinylidene fluoride membranes (Boster).

Subsequently, the proteins were probed with specific antibodies after the blot was blocked with 5% non-fat milk (Boster). Images were quantified using ImageJ software and all data are expressed as mean $\pm$ SEM.

The following antibodies and concentrations were used: NSUN2 Polyclonal antibody (Proteintech; Cat No.20854-1-AP; 1:7500), NSUN6 Polyclonal antibody (Proteintech; Cat No. 17240-1-AP; 1:2000), RabbitAnti-GAPDH antibody (Bioss; bs-41373R; 1:2000), Alpha Tubulin Polyclonal antibody (Proteintech; Cat No. 11224-1-AP; 1:2000), HRP-labeled Goat Anti-Rabbit IgG(H+L) (Beyotime Biotechnology; A0208; 1:2000).

cDNA synthesis and Sanger sequencing

Total cellular RNA was extracted with TRIzol reagent (TIANGEN Biotech) and cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio) according to the manufacturer's recommendations. PCR was then performed using 2 $\times$ Taq PCR MasterMix II (TIANGEN Biotech) and primers flanking m⁵C target sites, and the purified PCR products were directly sequenced by Sanger sequencing. The Sanger sequencing results were analyzed using EditR 1.0.10 to calculate the mutation frequency⁶³. The primers used in this study are shown in Supplemental Table 4.

Real-time quantitative PCR

cDNA was synthesized using FastKing RT kit (with gDNase) (TIANGEN Biotech) according to the manufacturer's recommendations. RT-qPCR assay was performed using SuperReal PreMix Plus (SYBR Green) (TIANGEN Biotech). GAPDH was used as an endogenous control, and the expression levels were normalized to the control and calculated by the $2^{-\Delta\Delta C_t}$ formula. All samples were analyzed in triplicate and each mRNA quantification represents the average of at least three measurements. All data are expressed as mean $\pm$ SEM. The primers used in this study are shown in Supplemental Table 4.

Protein structure modelling

Protein structure simulations were performed using the SWISS-MODEL online website (<https://swissmodel.expasy.org/interactive>)⁶⁴. The SWISS-MODEL database is able to provide up-to-date annotated 3D protein models, which are generated from automated homology modelling of related model organisms and experimental structural information for all sequences in UniProtKB, with reliable structural information, and subsequently protein structure observations were performed using PyMOL⁶⁵.

Bisulfite sequencing PCR

We referenced bisulfite sequencing PCR, an assay established by Matthias Schaefer et al. We chemically deaminated cytosine in RNA using the EZ RNA methylation kit (50) (ZYMO RESEARCH) and then quantified m⁵C methylation levels based on PCR amplification of cDNA combined with deep sequencing²³.

RNA Conversion Reagent was premixed with prepared RNA samples, and the RNA was denatured at 70°C for 5 minutes, followed by a reaction period of 45 minutes at 54°C. Finally, the purified RNA samples were recovered after desulfurization by RNA Desulphonation Buffer. cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Bio) according to the manufacturer's recommendations. PCR was then performed using 2 $\times$ EpiArt HS Taq Master Mix (Dye Plus) (Vazyme) and m⁵C target site-specific Bisulfite Primer (primer sequences were designed at <https://zymoresearch.eu/pages/bisulfite-primer-seeker>), the products were purified by TIANGel Midi Purification Kit (TIANGEN Biotech), and the connectors for second-generation sequencing

were attached at both ends of the products for sequencing. Finally, deep sequencing was performed by HiTOM analysis to detect the methylation level (The number of reads >1000 in deep sequencing) ⁶⁶[66](#). The primers used in this study are shown in Supplemental Table 4.

Library construction and next-generation sequencing

1μg of total cellular RNA was used for sequencing library generation by NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA, Catalog #: E7530L) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEB Next First Strand Synthesis Reaction Buffer(5X). First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs was converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEB Next Adaptor with hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially 370~420 bp in length, the library fragments were purified with AMPure XP system (Beverly, USA). Then 3 μL USER Enzyme (NEB, USA) was used with size selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent 5400 system(Agilent, USA)and quantified by QPCR (library concentration ≥ 1.5 nM). The qualified libraries were pooled and sequenced on Illumina platforms with PE150 strategy in Novogene Bioinformatics Technology Co., Ltd (Beijing, China), according to effective library concentration and data amount required.

DRAM-seq analysis and calling of edited sites

The raw fastq sequencing data were cleaned by trimming the adapter sequences using Fastp (v0.23.1) and were aligned to the human genome (hg19) using STAR (v2.7.7) in paired-end mode. The aligned BAM files were sorted and PCR duplicates were removed using Samtools (v1.12). The cite calling pf DRAM-seq was performed using Bullseye, a previously customized pipeline to look for C-to-U or A-to-G edited sites throughout the transcriptome⁴⁰[40](#). Briefly, the sorted and deduplicated BAM files were initially parsed by parseBAM.pl script.

Then, Find_edit_site.pl script was employed to find C-to-U or A-to-G editing events by DRAM-seq with at least 10 reads of coverage, an edit ratio of 5%-95%, an edit ratio at least 1.5-fold higher than NSUN2 or NSUN6-knockout samples, and at least 2 editing events at a given site. Sites that were only found in one replicate of each DRAM protein variant were removed. Editing events appeared in cells expressing merely APOBEC1 or Tada8e were also removed. For high confidence filtering, we further adjusted the Find_edit_site.pl parameters to the edit ratio of 10%-60%, an edit ratio of control samples at least 2-fold higher than NSUN2 or NSUN6-knockout samples, and at least 4 editing events at a given site.

Metagene and motif analyses

Metagene analysis was performed using hg19 annotations according to previously reported tool, MetaplotR⁶⁷[67](#). For motif analysis, the 20bp flanking sequence of each DRAM-seq editing site was extracted by Bedtools (v2.30.0)⁶⁸[68](#). The motif logos were then plotted by WebLogo (v3.7.12)⁶⁹[69](#).

Replicates analysis

Independent biological replicates of DRAM-ABE or DRAM-CBE in DRAM-seq analysis were separately compared by computing the Pearson correlation coefficient between the number of C-to-U mutations per mRNA between any two replicate experiments.

GO and KEGG analysis

GO and KEGG analysis of DRAM-seq edited mRNAs was performed using the DAVID bioinformatic database ⁷⁰[70](#). GO terms with a P value of less than 0.05 were considered statistically significant.

RNA pulldown assay

The biotin-labeled RNA oligonucleotides with (Oligo-m⁵C) or without m⁵C (Oligo-C) were prepared in advance: 5'-biotin-GAGGUAUGAAXUGUAAGTT-3' (X = C or m⁵C, used in the ALYREF and ALYREF^{mut} group) and 5'-biotin-GAAAGGAGAUXGCCAUUAUCC-3' (X = C or m⁵C, used in the YBX1 and YBX1^{mut} group). Protein lysates were then isolated from HEK293T cells transfected with DRAM-YBX1, DRAM-YBX1^{mut}, DRAM-ALYREF or DRAM-ALYREF^{mut} for 24 h using lysis buffer. RNA pull-down assays were performed with the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo) following the manufacturer's instructions, and the results were finally analyzed by Western blotting.

Statistical analysis

All data are expressed as mean ± S.E.M of three independent determinations. Data were analyzed through a two-tailed t-test. A probability of $P < 0.05$ was considered statistically significant; *, $P < 0.05$, **, $P < 0.01$, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ and ****, $P < 0.0001$ denote the significance thresholds; ns denotes not significant.

Data and Materials Availability

The data supporting the findings of this study are available within the article and its Supplementary Information. Other data and reagents are available from the corresponding authors upon reasonable request.

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Additional files

Supporting information [🔗](#)

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Reviewer #2 (Public review):

The fledgling field of epitranscriptomics has encountered various technical roadblocks with implications as to the validity of early epitranscriptomics mapping data. As a prime example, the low specificity of (supposedly) modification-specific antibodies for the enrichment of modified RNAs, has been ignored for quite some time and is only now recognized for its dismal reproducibility (between different labs), which necessitates the development of alternative methods for modification detection. Furthermore, early attempts to map individual epitranscriptomes using sequencing-based techniques are largely characterized by the deliberate avoidance of orthogonal approaches aimed at confirming the existence of RNA modifications that have been originally identified.

Improved methodology, the inclusion of various controls, and better mapping algorithms as well as the application of robust statistics for the identification of false-positive RNA modification calls have allowed revisiting original (seminal) publications whose early mapping data allowed making hyperbolic claims about the number, localization and importance of RNA modifications, especially in mRNA. Besides the existence of m6A in mRNA, the detectable incidence of RNA modifications in mRNAs has drastically dropped.

As for m5C, the subject of the manuscript submitted by Zhou et al., its identification in mRNA goes back to Squires et al., 2012 reporting on >10.000 sites in mRNA of a human cancer cell line, followed by intermittent findings reporting on pretty much every number between 0 to > 100.000 m5C sites in different human cell-derived mRNA transcriptomes. The reason for such discrepancy is most likely of a technical nature. Importantly, all studies reporting on actual transcript numbers that were m5C-modified relied on RNA bisulfite sequencing, an NGS-based method, that can discriminate between methylated and non-methylated Cs after chemical deamination of C but not m5C. RNA bisulfite sequencing has a notoriously high background due to deamination artifacts, which occur largely due to incomplete denaturation of double-stranded regions (denaturing-resistant) of RNA molecules. Furthermore, m5C sites in mRNAs have now been mapped to regions that have not only sequence identity but also structural features of tRNAs. Various studies revealed that the highly conserved m5C RNA methyltransferases NSUN2 and NSUN6 do not only accept tRNAs but also other RNAs (including mRNAs) as methylation substrates, which in combination account for most of the RNA bisulfite-mapped m5C sites in human mRNA transcriptomes. Is m5C in mRNA only a result of the Star activity of tRNA or rRNA modification enzymes, or is their low stoichiometry biologically relevant?

In light of the short-comings of existing tools to robustly determine m⁵C in transcriptomes, other methods, like DRAM-seq, aiming to map m⁵C independently of ex situ RNA treatment with chemicals, are needed to arrive at a more solid "ground state", from which it will be possible to state and test various hypotheses as to the biological function of m⁵C, especially in lowly abundant RNAs such as mRNA.

Importantly, the identification of >10,000 sites containing m⁵C increases through DRAM-Seq, increases the number of potential m⁵C marks in human cancer cells from a couple of 100 (after rigorous post-hoc analysis of RNA bisulfite sequencing data) by orders of magnitude. This begs the question, whether or not the application of these editing tools results in editing artefacts overstating the number of actual m⁵C sites in the human cancer transcriptome.

[Editors' note: earlier reviews have been provided here: <https://doi.org/10.7554/eLife.98166.3.sa1>; <https://doi.org/10.7554/eLife.98166.2.sa1>; <https://doi.org/10.7554/eLife.98166.1.sa1>]

<https://doi.org/10.7554/eLife.98166.4.sa1>

Author response:

The following is the authors' response to the original reviews.

Responses to Reviewer's Comments:

To Reviewer #2:

(1) The use of two m⁵C reader proteins is likely a reason for the high number of edits introduced by the DRAM-Seq method. Both ALYREF and YBX1 are ubiquitous proteins with multiple roles in RNA metabolism including splicing and mRNA export. It is reasonable to assume that both ALYREF and YBX1 bind to many mRNAs that do not contain m⁵C.

To substantiate the author's claim that ALYREF or YBX1 binds m⁵C-modified RNAs to an extent that would allow distinguishing its binding to non-modified RNAs from binding to m⁵C-modified RNAs, it would be recommended to provide data on the affinity of these, supposedly proven, m⁵C readers to non-modified versus m⁵C-modified RNAs. To do so, this reviewer suggests performing experiments as described in Slama et al., 2020 (doi: 10.1016/j.ymeth.2018.10.020). However, using dot blots like in so many published studies to show modification of a specific antibody or protein binding, is insufficient as an argument because no antibody, nor protein, encounters nanograms to micrograms of a specific RNA identity in a cell. This issue remains a major caveat in all studies using so-called RNA modification reader proteins as bait for detecting RNA modifications in epitranscriptomics research. It becomes a pertinent problem if used as a platform for base editing similar to the work presented in this manuscript.

The authors have tried to address the point made by this reviewer. However, rather than performing an experiment with recombinant ALYREF-fusions and m⁵C-modified to unmodified RNA oligos for testing the enrichment factor of ALYREF in vitro, the authors resorted to citing two manuscripts. One manuscript is cited by everybody when it comes to ALYREF as m⁵C reader, however none of the experiments have been repeated by another laboratory. The other manuscript is reporting on YBX1 binding to m⁵C-containing RNA and mentions PARCLIP experiments with ALYREF, the details of which are nowhere to be found in doi: 10.1038/s41556-019-0361-y.

Furthermore, the authors have added RNA pull-down assays that should substitute for the requested experiments. Interestingly, Figure S1E shows that ALYREF binds equally well to unmodified and m⁵C-modified RNA oligos, which contradicts

doi:10.1038/cr.2017.55, and supports the conclusion that wild-type ALYREF is not specific m⁵C binder. The necessity of including always an overexpression of ALYREF-mut in parallel DRAM experiments, makes the developed method better controlled but not easy to handle (expression differences of the plasmid-driven proteins etc.)

Thank you for pointing this out. First, we would like to correct our previous response: the binding ability of ALYREF to m⁵C-modified RNA was initially reported in doi: 10.1038/cr.2017.55, (and not in doi: 10.1038/s41556-019-0361-y), where it was observed through PAR-CLIP analysis that the K171 mutation weakens its binding affinity to m⁵C - modified RNA.

Our previous experimental approach was not optimal: the protein concentration in the INPUT group was too high, leading to overexposure in the experimental group. Additionally, we did not conduct a quantitative analysis of the results at that time. In response to your suggestion, we performed RNA pull-down experiments with YBX1 and ALYREF, rather than with the pan-DRAM protein, to better validate and reproduce the previously reported findings. Our quantitative analysis revealed that both ALYREF and YBX1 exhibit a stronger affinity for m⁵C -modified RNAs. Furthermore, mutating the key amino acids involved in m⁵C recognition significantly reduced the binding affinity of both readers. These results align with previous studies (doi: 10.1038/cr.2017.55 and doi: 10.1038/s41556-019-0361-y), confirming that ALYREF and YBX1 are specific readers of m⁵C -modified RNAs. However, our detection system has certain limitations. Despite mutating the critical amino acids, both readers retained a weak binding affinity for m⁵C, suggesting that while the mutation helps reduce false positives, it is still challenging to precisely map the distribution of m⁵C modifications. To address this, we plan to further investigate the protein structure and function to obtain a more accurate m⁵C sequencing of the transcriptome in future studies. Accordingly, we have updated our results and conclusions in lines 294-299 and discuss these limitations in lines 109114.

In addition, while the m⁵C assay can be performed using only the DRAM system alone, comparing it with the DRAM^{mut} control enhances the accuracy of m⁵C region detection. To minimize the variations in transfection efficiency across experimental groups, it is recommended to use the same batch of transfections. This approach not only ensures more consistent results but also improve the standardization of the DRAM assay, as discussed in the section added on line 308-312.

(2) Using sodium arsenite treatment of cells as a means to change the m⁵C status of transcripts through the downregulation of the two major m⁵C writer proteins NSUN2 and NSUN6 is problematic and the conclusions from these experiments are not warranted. Sodium arsenite is a chemical that poisons every protein containing thiol groups. Not only do NSUN proteins contain cysteines but also the base editor fusion proteins. Arsenite will inactivate these proteins, hence the editing frequency will drop, as observed in the experiments shown in Figure 5, which the authors explain with fewer m⁵C sites to be detected by the fusion proteins.

The authors have not addressed the point made by this reviewer. Instead the authors state that they have not addressed that possibility. They claim that they have revised the results section, but this reviewer can only see the point raised in the conclusions. An experiment would have been to purify base editors via the HA tag and then perform some kind of binding/editing assay in vitro before and after arsenite treatment of cells.

We appreciate the reviewer's insightful comment. We fully agree with the concern raised. In the original manuscript, our intention was to use sodium arsenite treatment to downregulate NSUN mediated m⁵C levels and subsequently decrease DRAM editing efficiency, with the aim of monitoring m⁵C dynamics through the DRAM system. However, as the reviewer pointed

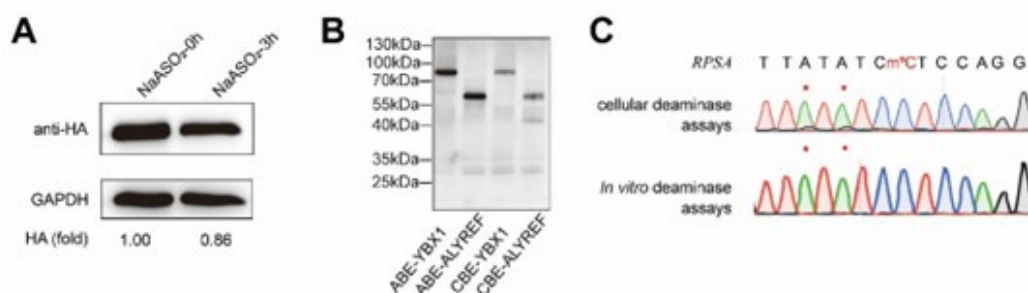
out, sodium arsenite may inactivate both NSUN proteins and the base editor fusion proteins, and any such inactivation would likely result in a reduced DRAM editing.

This confounds the interpretation of our experimental data.

As demonstrated in Author response image 1A, western blot analysis confirmed that sodium arsenite indeed decreased the expression of fusion proteins. In addition, we attempted in vitro fusion protein purification using multiple fusion tags (HIS, GST, HA, MBP) for DRAM fusion protein expression, but unfortunately, we were unable to obtain purified proteins. However, using the Promega TNT T7 Rapid Coupled In Vitro Transcription/Translation Kit, we successfully purified the DRAM protein (Author response image 1B). Despite this success, subsequent in vitro deamination experiments did not yield the expected mutation results (Author response image 1C), indicating that further optimization is required. This issue is further discussed in line 314-315.

Taken together, the above evidence supports that the experiment of sodium arsenite treatment was confusing and we determined to remove the corresponding results from the main text of the revised manuscript.

Author response image 1.



(3) The authors should move high-confidence editing site data contained in Supplementary Tables 2 and 3 into one of the main Figures to substantiate what is discussed in Figure 4A. However, the data needs to be visualized in another way than excel format. Furthermore, Supplementary Table 2 does not contain a description of the columns, while Supplementary Table 3 contains a single row with letters and numbers.

The authors have not addressed the point made by this reviewer. Figure 3F shows the screening process for DRAM-seq assays and principles for screening highconfidence genes rather than the data contained in Supplementary Tables 2 and 3 of the former version of this manuscript.

Thank you for your valuable suggestion. We have visualized the data from Supplementary Tables 2 and 3 in Figure 4A as a circlize diagram (described in lines 213-216), illustrating the distribution of mutation sites detected by the DRAM system across each chromosome. Additionally, to improve the presentation and clarity of the data, we have revised Supplementary Tables 2 and 3 by adding column descriptions, merging the DRAM-ABE and DRAM-CBE sites, and including overlapping m⁵C genes from previous datasets.

Responses to Reviewer's Comments:

To Reviewer #3:

The authors have again tried to address the former concern by this reviewer who questioned the specificity of both m⁵C reader proteins towards modified RNA rather than unmodified RNA. The authors chose to do RNA pull down experiments which serve as a proxy for proving the specificity of ALYREF and YBX1 for m⁵C modified RNAs. Even though this reviewer asked for determining the enrichment factor of the reader-base editor fusion proteins (as wildtype or mutant for the identified m⁵C specificity motif) when presented with m⁵C-modified RNAs, the authors chose to use both reader proteins alone (without the fusion to an editor) as wildtype and as respective m⁵C-binding mutant in RNA in vitro pull-down experiments along with unmodified and m⁵C-modified RNA oligomers as binding substrates. The quantification of these pull-down experiments (n=2) have now been added, and are revealing that (according to SFigure 1 E and G) YBX1 enriches an RNA containing a single m⁵C by a factor of 1.3 over its unmodified counterpart, while ALYREF enriches by a factor of 4x. This is an acceptable approach for educated readers to question the specificity of the reader proteins, even though the quantification should be performed differently (see below).

Given that there is no specific sequence motif embedding those cytosines identified in the vicinity of the DRAM-edits (Figure 3J and K), even though it has been accepted by now that most of the m⁵C sites in mRNA are mediated by NSUN2 and NSUN6 proteins, which target tRNA like substrate structures with a particular sequence enrichment, one can conclude that DRAM-Seq is uncovering a huge number of false positives. This must be so not only because of the RNA bisulfite seq data that have been extensively studied by others, but also by the following calculations: Given that the m⁵C/C ratio in human mRNA is 0.02-0.09% (measured by mass spec) and assuming that 1/4 of the nucleotides in an average mRNA are cytosines, an mRNA of 1.000 nucleotides would contain 250 Cs. 0.02-0.09% m⁵C/C would then translate into 0.05-0.225 methylated cytosines per 250 Cs in a 1000 nt mRNA. YBX1 would bind every C in such an mRNA since there is no m⁵C to be expected, which it could bind with 1.3 higher affinity. Even if the mRNAs would be 10.000 nt long, YBX1 would bind to half a methylated cytosine or 2.25 methylated cytosines with 1.3x higher affinity than to all the remaining cytosines (2499.5 to 2497.75 of 2.500 cytosines in 10.000 nt, respectively). These numbers indicate a 4999x to 1110x excess of cytosine over m⁵C in any substrate RNA, which the "reader" can bind as shown in the RNA pull-downs on unmodified RNAs. This reviewer spares the reader of this review the calculations for ALYREF specificity, which is slightly higher than YBX1. Hence, it is up to the capable reader of these calculations to follow the claim that this minor affinity difference allows the unambiguous detection of the few m⁵C sites in mRNA be it in the endogenous scenario of a cell or as fusion-protein with a base editor attached?

We sincerely appreciate the reviewer's rigorous analysis. We would like to clarify that in our RNA pulldown assays, we indeed utilized the full DRAM system (reader protein fused to the base editor) to reflect the specificity of m⁵C recognition. As previously suggested by the reviewer, to independently validate the m⁵C-binding specificity of ALYREF and YBX1, we performed separate pulldown experiments with wild-type and mutant reader proteins (without the base editor fusion) using both unmodified and m⁵C-modified RNA substrates. This approach aligns with established methodologies in the field (doi:10.1038/cr.2017.55 and doi: 10.1038/s41556-019-0361-y). We have revised the Methods section (line 230) to explicitly describe this experimental design.

Although the m⁵C/C ratios in LC/MS-assayed mRNA are relatively low (ranging from 0.02% to 0.09%), as noted by the reviewer, both our data and previous studies have demonstrated that ALYREF and YBX1 preferentially bind to m⁵C-modified RNAs over unmodified RNAs, exhibiting 4-fold and 1.3-fold enrichment, respectively (Supplementary Figure 1E–1G).

Importantly, this specificity is further enhanced in the DRAM system through two key mechanisms: first, the fusion of reader proteins to the deaminase restricts editing to regions near m^5C sites, thereby minimizing off-target effects; second, background editing observed in reader-mutant or deaminase controls (e.g., DRAM^{mut}-CBE in Figure 2D) is systematically corrected for during data analysis.

We agree that the theoretical challenge posed by the vast excess of unmodified cytosines. However, our approach includes stringent controls to alleviate this issue. Specifically, sites identified in NSUN2/NSUN6 knockout cells or reader-mutant controls are excluded (Figure 3F), which significantly reduces the number of false-positive detections. Additionally, we have observed deamination changes near high-confidence m^5C methylation sites detected by RNA bisulfite sequencing, both in first-generation and high-throughput sequencing data. This observation further substantiates the validity of DRAM-Seq in accurately identifying m^5C sites.

We fully acknowledge that residual false positives may persist due to the inherent limitations of reader protein specificity, as discussed in line 299-301 of our manuscript. To address this, we plan to optimize reader domains with enhanced m^5C binding (e.g., through structure-guided engineering), which is also previously implemented in the discussion of the manuscript.

The reviewer supports the attempt to visualize the data. However, the usefulness of this Figure addition as a readable presentation of the data included in the supplement is up to debate.

Thank you for your kind suggestion. We understand the reviewer's concern regarding data visualization. However, due to the large volume of DRAM-seq data, it is challenging to present each mutation site and its characteristics clearly in a single figure. Therefore, we chose to categorize the data by chromosome, which not only allows for a more organized presentation of the DRAM-seq data but also facilitates comparison with other database entries. Additionally, we have updated Supplementary Tables 2 and 3 to provide comprehensive information on the mutation sites. We hope that both the reviewer and editors will understand this approach. We will, of course, continue to carefully consider the reviewer's suggestions and explore better ways to present these results in the future.

(3) A set of private Recommendations for the Authors that outline how you think the science and its presentation could be strengthened

NEW COMMENTS to TEXT:

Abstract:

"5-Methylcytosine (m^5C) is one of the major post-transcriptional modifications in mRNA and is highly involved in the pathogenesis of various diseases."

In light of the increasing use of AI-based writing, and the proof that neither DeepSeek nor ChatGPT write truthfully statements if they collect metadata from scientific abstracts, this sentence is utterly misleading.

m^5C is not one of the major post-transcriptional modifications in mRNA as it is only present with a m^5C/C ratio of 0.02- 0.09% as measured by mass-spec. Also, if m^5C is involved in the pathogenesis of various diseases, it is not through mRNA but tRNA. No single published work has shown that a single m^5C on an mRNA has anything to do with disease. Every conclusion that is perpetuated by copying the false statements given in the many reviews on the subject is based on knock-out phenotypes of the involved writer proteins. This reviewer wishes that the authors would abstain from the common practice

that is currently flooding any scientific field through relentless repetitions in the increasing volume of literature which perpetuate alternative facts.

We sincerely appreciate the reviewer's insightful comments. While we acknowledge that m⁵C is not the most abundant post-transcriptional modification in mRNA, we believe that research into m⁵C modification holds considerable value. Numerous studies have highlighted its role in regulating gene expression and its potential contribution to disease progression. For example, recent publications have demonstrated that m⁵C modifications in mRNA can influence cancer progression, lipid metabolism, and other pathological processes (e.g., PMID: 37845385; 39013911; 39924557; 38042059; 37870216).

We fully agree with the reviewer on the importance of maintaining scientific rigor in academic writing. While m⁵C is not the most abundant RNA modification, we cannot simply draw a conclusion that the level of modification should be the sole criterion for assessing its biological significance. However, to avoid potential confusion, we have removed the word "major".

COMMENTS ON FIGURE PRESENTATION:

Figure 2D:

The main text states: "DRAM-CBE induced C to U editing in the vicinity of the m⁵C site in AP5Z1 mRNA, with 13.6% C-to-U editing, while this effect was significantly reduced with APOBEC1 or DRAM^{mut}-CBE (Fig.2D)." The Figure does not fit this statement. The seq trace shows a U signal of about 1/3 of that of C (about 30%), while the quantification shows 20+ percent

Thank you for your kind suggestion. Upon visual evaluation, the sequencing trace in the figure appears to suggest a mutation rate closer to 30% rather than 22%. However, relying solely on the visual interpretation of sequencing peaks is not a rigorous approach. The trace on the left represents the visualization of Sanger sequencing results using SnapGene, while the quantification on the right is derived from EditR 1.0.10 software analysis of three independent biological replicates. The C-to-U mutation rates calculated were 22.91667%, 23.23232%, and 21.05263%, respectively. To further validate this, we have included the original EditR analysis of the Sanger sequencing results for the DRAM-CBE group used in the left panel of Figure 2D (see Author response image 2). This analysis confirms an m⁵C fraction (%) of $22/(22+74) = 22.91667$, and the sequencing trace aligns well with the mutation rate we reported in Figure 2D. In conclusion, the data and conclusions presented in Figure 2D are consistent and supported by the quantitative analysis.

Author response image 2.

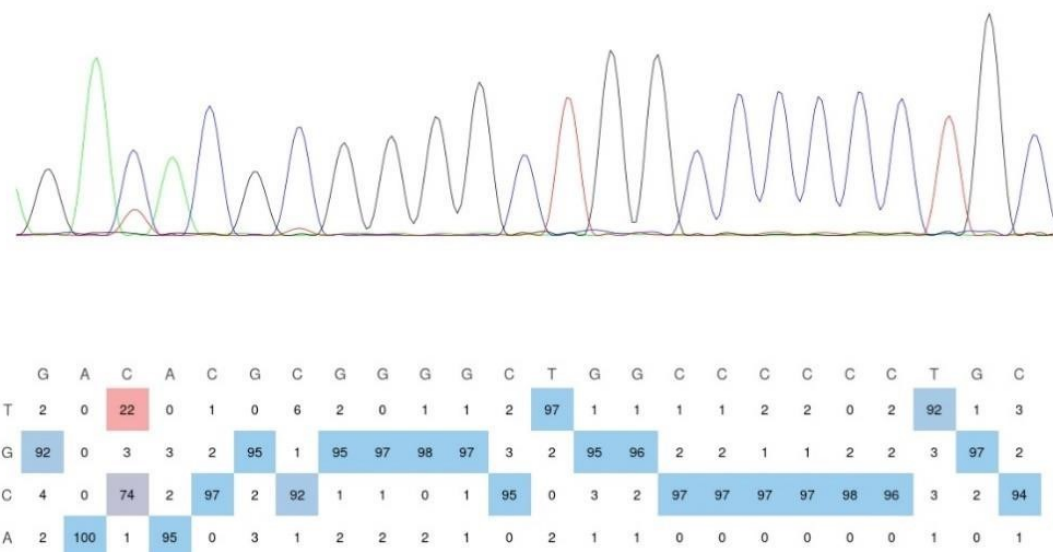


Figure 4B: shows now different numbers in Venn-diagrams than in the same depiction, formerly Figure 4A

We sincerely thank the reviewer for pointing out this issue, and we apologize for not clearly indicating the changes in the previous version of the manuscript. In response to the initial round of reviewer comments, we implemented a more stringent data filtering process (as described in Figure 3F and method section) : "For high-confidence filtering, we further adjusted the parameters of Find_edit_site.pl to include an edit ratio of 10%–60%, a requirement that the edit ratio in control samples be at least 2-fold higher than in NSUN2 or NSUN6knockout samples, and at least 4 editing events at a given site." As a result, we made minor adjustments to the Venn diagram data in Figure 4A, reducing the total number of DRAM-edited mRNAs from 11,977 to 10,835. These changes were consistently applied throughout the manuscript, and the modifications have been highlighted for clarity. Importantly, these adjustments do not affect any of the conclusions presented in the manuscript.

Figure 4B and D: while the overlap of the DRAM-Seq data with RNA bisulfite data might be 80% or 92%, it is obvious that the remaining data DRAM seq suggests a detection of additional sites of around 97% or 81.83%. It would be advised to mention this large number of additional sites as potential false positives, unless these data were normalized to the sites that can be allocated to NSUN2 and NSUN6 activity (NSUN mutant data sets could be subtracted).

Thank you for pointing this out. The Venn diagrams presented in Figure 4B and D already reflect the exclusion of potential false-positive sites identified in methyltransferasedeficient datasets, as described in our experimental filtering process, and they represent the remaining sites after this stringent filtering. However, we acknowledge that YBX1 and ALYREF, while preferentially binding to m⁵C-modified RNA, also exhibit some affinity for unmodified RNA. Although we employed rigorous controls, including DRAM^{mut} and deaminase groups, to minimize false positives, the possibility of residual false positives cannot be entirely ruled out. Addressing this limitation would require even more stringent filtering methods, as discussed in lines 299–301 of the manuscript. We are committed to

further optimizing the DRAM system to enhance the accuracy of transcriptome-wide m^5C analysis in future studies.

SFigure 1: It is clear that the wild type version of both reader proteins are robustly binding to RNA that does not contain m^5C . As for the calculations of x-fold affinity loss of RNA binding using both ALYREF -mut or YBX1 -mut, this reviewer asks the authors to determine how much less the mutated versions of the proteins bind to a m^5C -modified RNAs. Hence, a comparison of YBX1 versus YBX1 -mut (ALYREF versus ALYREF -mut) on the same substrate RNA with the same m^5C -modified position would allow determining the contribution of the so-called modification binding pocket in the respective proteins to their RNA binding. The way the authors chose to show the data presently is misleading because what is compared is the binding of either the wild type or the mutant protein to different RNAs.

We appreciate the reviewer's valuable feedback and apologize for any confusion caused by the presentation of our data. We would like to clarify the rationale behind our approach. The decision to present the wild-type and mutant reader proteins in separate panels, rather than together, was made in response to comments from Reviewer 2. Below, we provide a detailed explanation of our experimental design and its justification.

First, we confirmed that YBX1 and ALYREF exhibit stronger binding affinity to m^5C -modified RNA compared to unmodified RNA, establishing their role as m^5C reader proteins. Next, to validate the functional significance of the DRAM^{mut} group, we demonstrated that mutating key amino acids in the m^5C -binding pocket significantly reduces the binding affinity of YBX1^{mut} and ALYREF^{mut} to m^5C -modified RNA. This confirms that the DRAM^{mut} group effectively minimizes false-positive results by disrupting specific m^5C interactions.

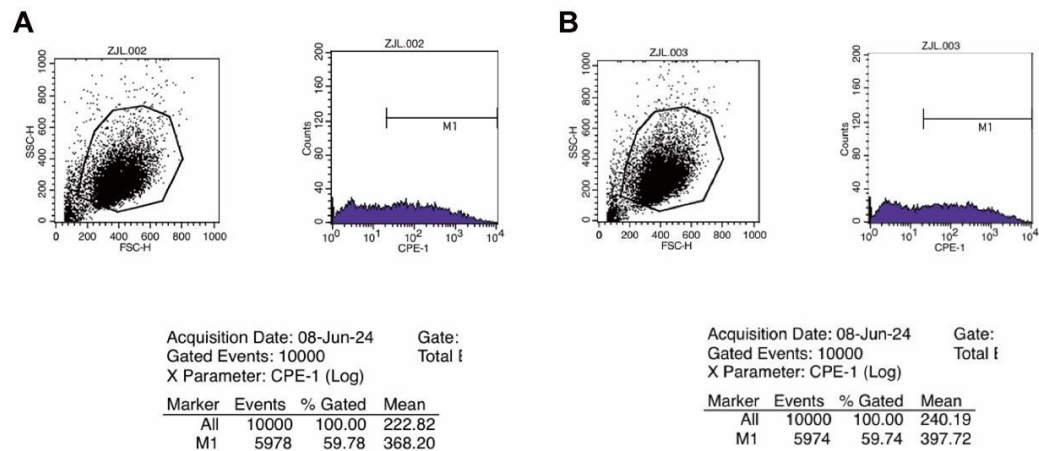
Crucially, in our pull-down experiments, both the wild-type and mutant proteins (YBX1/YBX1^{mut} and ALYREF/ALYREF^{mut}) were incubated with the same RNA sequences. To avoid any ambiguity, we have included the specific RNA sequence information in the Methods section (lines 463–468). This ensures a assessment of the reduced binding affinity of the mutant versions relative to the wild-type proteins, even though they are presented in separate panels.

We hope this explanation clarifies our approach and demonstrates the robustness of our findings. We sincerely appreciate the reviewer's understanding and hope this addresses their concerns.

SFigure 2C: first two panels are duplicates of the same image.

Thank you for pointing this out. We sincerely apologize for incorrectly duplicating the images. We have now updated Supplementary Figure 2C with the correct panels and have provided the original flow cytometry data for the first two images. It is important to note that, as demonstrated by the original data analysis, the EGFP-positive quantification values (59.78% and 59.74%) remain accurate. Therefore, this correction does not affect the conclusions of our study. Thank you again for bringing this to our attention.

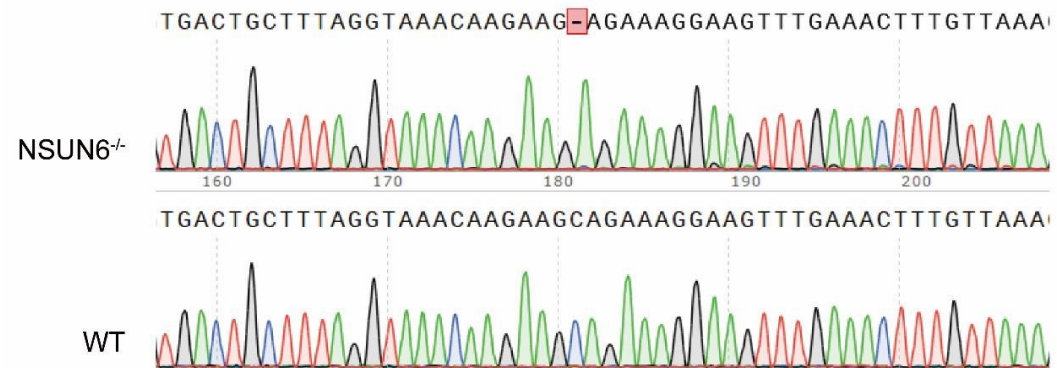
Author response image 3.



SFigure 4B: how would the PCR product for NSUN6 be indicative of a mutation? The used primers seem to amplify the wildtype sequence.

Thank you for your kind suggestion. In our NSUN6^{-/-} cell line, the NSUN6 gene is only missing a single base pair (1bp) compared to the wildtype, which results in frame shift mutation and reduction in NSUN6 protein expression. We fully agree with the reviewer that the current PCR gel electrophoresis does not provide a clear distinction of this 1bp mutation. To better illustrate our experimental design, we have included a schematic representation of the knockout sequence in SFigure 4B. Additionally, we have provided the original sequencing data, and the corresponding details have been added to lines 151-153 of the manuscript for further clarification.

Author response image 4.



SFigure 4C: the Figure legend is insufficient to understand the subfigure.

Thank you for your valuable suggestion. To improve clarity, we have revised the figure legend for SFigure 4C, as well as the corresponding text in lines 178-179. We have additionally updated the title of SFigure 4 for better clarity. The updated SFigure 4C now demonstrates

that the DRAM-edited mRNAs exhibit a high degree of overlap across the three biological replicates.

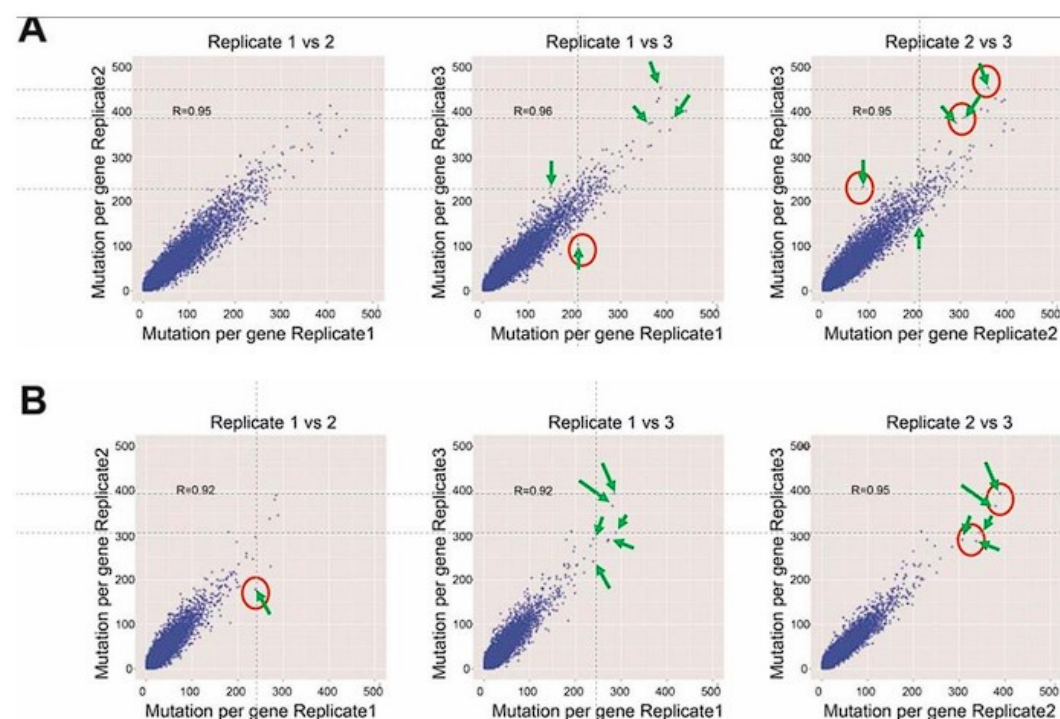
SFigure 4D: the Figure legend is insufficient to understand the subfigure.

Thank you for your kind suggestion. We have revised the figure legend to provide a clearer explanation of the subfigure. Specifically, this figure illustrates the motif analysis derived from sequences spanning 10 nucleotides upstream and downstream of DRAMedited sites mediated by loci associated with NSUN2 or NSUN6. To enhance clarity, we have also rephrased the relevant results section (lines 169-175) and the corresponding discussion (lines 304-307).

SFigure 7: There is something off with all 6 panels. This reviewer can find data points in each panel that do not show up on the other two panels even though this is a pairwise comparison of three data sets (file was sent to the Editor) Available at https://elife-rp.msubmit.net/elife-rp_files/2025/01/22/00130809/02/130809_2_attach_27_15153.pdf

Response: We thank the reviewer for pointing this out. We would like to clarify the methodology behind this analysis. In this study, we conducted pairwise comparisons of the number of DRAM-edited sites per gene across three biological replicates of DRAM-ABE or DRAM-CBE, visualized as scatterplots. Each data point in the plots corresponds to a gene, and while the same gene is represented in all three panels, its position may vary vertically or horizontally across the panels. This variation arises because the number of mutation sites typically differs between replicates, making it unlikely for a data point to occupy the exact same position in all panels. A similar analytical approach has been used in previous studies on m6A (PMID: 31548708). To address the reviewer's concern, we have annotated the corresponding positions of the questioned data points with arrows in Author response image 5.

Author response image 5.



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