

Decoding m⁶Am by simultaneous transcription-start mapping and methylation quantification

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eLife Assessment

This study presents a new quantitative method, CROWN-seq, to map the cap-adjacent RNA modification N⁶,2'-O-dimethyladenosine (m⁶Am) with single nucleotide resolution. Using thoughtful controls and well-validated reagents, the authors provide **compelling** evidence that the method is reliable and reproducible. Additionally, the study provides **important** evidence that m⁶Am may increase transcription in modified mRNAs. However, the data only demonstrates a correlation between m⁶Am and transcriptional regulation rather than causality. Overall, this study is poised to advance m⁶Am research, being of broad interest to the RNA biology and gene regulation fields.

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Abstract

N⁶,2'-O-dimethyladenosine (m⁶Am) is a modified nucleotide located at the first transcribed position in mRNA and snRNA that is essential for diverse physiological processes. m⁶Am mapping methods assume each gene uses a single start nucleotide. However, gene transcription usually involves multiple start sites, generating numerous 5' isoforms. Thus, gene levels annotations cannot capture the diversity of m⁶Am modification in the transcriptome. Here we describe CROWN-seq, which simultaneously identifies transcription-start nucleotides and quantifies m⁶Am stoichiometry for each 5' isoform that initiates with adenosine. Using CROWN-seq, we map the m⁶Am landscape in nine human cell lines. Our findings reveal that m⁶Am is nearly always a high stoichiometry modification, with only a small subset of cellular mRNAs showing lower m⁶Am stoichiometry. We find that m⁶Am is associated with increased transcript expression and provide evidence that m⁶Am may be linked to transcription initiation associated with specific promoter sequences and initiation mechanisms. These data suggest a potential new function for m⁶Am in influencing transcription.

Introduction

m⁶Am (*N*⁶,2'-*O*-dimethyladenosine) is the most common modified nucleotide in mRNA. m⁶Am is found specifically at the first transcribed position of mRNAs, termed the transcription-start nucleotide (TSN), which reflects the transcription-start site (TSS) in DNA. During transcription, the TSN typically acquires a 2'-*O*-methyl modification^{1,2}, which is deposited by CMTR1 (Cap-specific mRNA nucleoside-2'-*O*-methyltransferase 1)³. In the case of mRNAs that use adenosine as the TSN, the initial 2'-*O*-methyladenosine (Am) can be further methylated on the *N*⁶ position of the adenine nucleobase to form m⁶Am by PCIF1 (Phosphorylated CTD Interacting Factor 1)⁴.

Studies using PCIF1 depletion (i.e., m⁶Am depletion) have revealed that m⁶Am has important roles in cell physiology. In normal cells, PCIF1 depletion does not appear to affect cell growth or viability⁴. However, in oxidative stress conditions, PCIF1 deficiency leads to impaired cell growth⁴. In cancer cells, PCIF1 depletion markedly enhances cell death during anti-PD1 therapy⁵. During viral infection, PCIF1 depletion results in increased HIV replication⁶, impaired SARS-Cov-2 infection⁷, and increased VSV immunogenicity⁸. These studies indicate that m⁶Am has important roles in diverse cellular contexts.

A major goal has been to identify and characterize the m⁶Am- and Am-containing mRNAs. Initial chromatographic studies in the 1970s demonstrated that cellular mRNAs can exist in m⁶Am and Am forms, with the Am form being more predominant⁹. To map m⁶Am modified genes, several antibody-based methods were developed, including miCLIP¹⁰, m⁶Am-seq¹¹, m6ACE-seq¹², and m6Am-exo-seq¹³. These methods can identify m⁶Am sites (miCLIP and m6ACE-seq), m⁶Am peaks (m⁶Am-seq), or m⁶Am containing genes (m⁶Am-exo-seq). Am genes were identified when m⁶Am was not detected but the reported TSN in publicly available datasets was A. The remaining genes were annotated as Gm, Cm, or Um based on public TSS annotations.

However, despite these transcriptome-wide m⁶Am maps, the effect of m⁶Am on mRNA is unclear. By examining m⁶Am genes, along with the change in mRNA stability and translation, small and inconsistent effects have been observed from different labs^{4,13–15}.

We considered the possibility that the difficulty in establishing m⁶Am function may be due to flaws in the way that genes are designated as m⁶Am genes. Previous m⁶Am mapping studies treated m⁶Am like other modified nucleotides, which are internal. In studies of internal nucleotides, such as m⁶A, isoform diversity is generally not considered since these different isoforms rarely impact the detection of the nucleotide. In contrast, m⁶Am is highly affected by isoform diversity since it is located at the 5' end. Most genes generate multiple transcript 5' isoforms that each use a different TSSs¹⁶. However, previous m⁶Am mapping studies assumed that each gene has a single TSN, whose identity was based on existing gene annotations. Therefore, existing m⁶Am maps that assign a specific start nucleotide to each gene cannot be accurate, since most genes produce a range of transcripts, with possibly multiple start nucleotides. For this reason, m⁶Am mapping and functional studies of m⁶Am need to be performed in a way that considers the 5' isoform diversity of most genes.

Another concern is that the existing m⁶Am mapping studies designated each gene as either m⁶Am or Am. However, it is possible that m⁶Am levels can be variable, with only a fraction of transcripts containing m⁶Am and the remainder containing Am. Stoichiometric maps of m⁶Am can potentially reveal the degree to which a transcript would be influenced by m⁶Am-dependent pathways.

Quantitative m⁶Am mapping is especially important for small nuclear RNAs (snRNAs), which also contain m⁶Am at their TSN. Initial biochemical characterization of snRNAs revealed that the first nucleotide was Am¹⁷, but subsequent studies showed that nearly half of all snRNAs are initially methylated to m⁶Am, and then demethylated by FTO to Am^{10,12}. Thus, m⁶Am is a transient intermediate in snRNA biogenesis. Notably, m⁶Am levels can be highly regulated in snRNAs¹⁰, however, current m⁶Am mapping methods are unable to quantify changes in m⁶Am stoichiometry.

To understand the transcriptome-wide distribution of m⁶Am, we developed CROWN-seq (**C**onversion **R**esistance detection **O**n **W**hole-transcriptomic transcription-start **N**⁶,2'-*O*-dimethyladenosine by **s**equencing), an antibody-free quantitative m⁶Am mapping method. Using CROWN-seq, we define the overall repertoire of 5' isoforms for each gene, and the specific isoforms that use m⁶Am as the TSN across nine different cell lines. We find that annotations of genes based on a single start nucleotide do not capture the diversity of 5' transcript isoforms for most genes. Instead, m⁶Am is more accurately assessed for each 5' isoform separately. Nearly all A-initiated transcript isoforms have very high m⁶Am stoichiometry, and that transcripts containing Am as the TSN are relatively rare. Transcript isoforms that contain m⁶Am are more highly expressed, and loss of m⁶Am due to depletion of PCIF1 leads to reduced expression of transcript isoforms containing m⁶Am. However, we find that this effect is not due to decreased mRNA stability. Instead, the depletion of PCIF1 affects transcripts based on upstream core promoter elements. Our data suggest that transcription mechanisms that utilize specific core promoter sequences achieve high expression, which might be linked to a transcription-promoting effect of m⁶Am. Overall, our quantitative transcriptome-wide transcription-start nucleotide m⁶Am maps reveal a markedly distinct m⁶Am profile than previously measured, show that m⁶Am is the predominant modified nucleotide relative to Am in mRNA, and suggest roles of m⁶Am in transcription.

Results

ReCappable-seq reveals high transcript isoform diversity at the 5' end

In previous m⁶Am mapping studies, genes were annotated to be m⁶Am, Am, Gm, Cm, or Um^{4,10,13,14}, based on the assumption that each gene has one major transcription-start nucleotide. To determine how often genes are characterized by a single major TSS we used ReCappable-seq¹⁸, a method for quantitative measurement of transcription-start sites. ReCappable-seq is similar to traditional TSS-seq methods which involve ligation of an oligonucleotide to the 5' end of mRNAs¹⁹, thus precisely marking the TSN. However, ReCappable-seq adds an enrichment step for capped mRNA fragments to significantly reduce background signals from internal sites that are derived from RNA cleavage. Thus, ReCappable-seq provides a highly sensitive and precise mapping of TSNs at single-nucleotide resolution (Note S1, “The comparison of transcription-start mapping methods”).

By analyzing ReCappable-seq data in HEK293T cells, we found that protein-coding genes tend to have multiple TSNs. Among the 9,199 genes analyzed, we identified 87,624 TSNs (see Methods). On average, a gene uses 9.5±9 (mean and s.d., hereafter) TSNs (**Figure 1A**). Only ~9% of genes contain a single TSN (**Figure 1A**). Thus, most genes cannot be characterized by a single start nucleotide.

As an example, *SRSF1*, which was previously classified as a m⁶Am gene, has ~37 different 5' isoforms in HEK293T cells, of which ~41.5% do not use an A-TSN (**Figure 1B**). As another example, *ADAR* was previously classified as a Gm gene, but ~71.0% of transcripts use A-TSNs

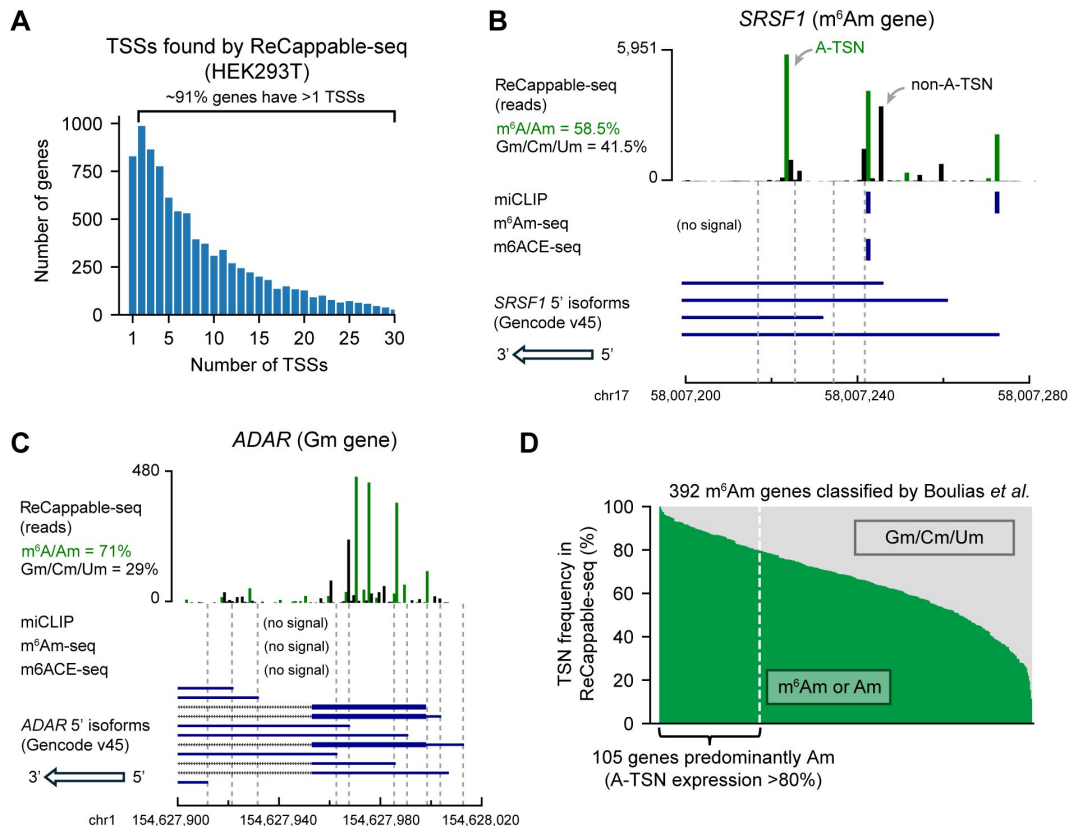


Figure 1.

Many m^6Am genes are mistakenly annotated

(A) Genes tend to have multiple TSSs. Shown is a histogram displaying the number of TSSs per protein-coding gene in HEK293T cells. TSSs (87,624 TSSs from 9,199 genes) were mapped using ReCappable-seq. These TSSs have expression levels ≥ 1 TPM (transcription-start nucleotide per million).

(B) and **(C)** Examples of genes that were mistakenly classified by previous studies¹⁴. *SRSF1* **(B)** was previously designated as m^6Am because of the miCLIP signals overlapping with A-TSSs. However, based on ReCappable-seq, ~41.5% of the reads are mapped to non-A-TSSs in *SRSF1*. Notably, one of the most expressed m^6Am A-TSS (chr17:58,007,228) was mistakenly considered as internal m^6A because this position was not previously annotated as a TSS⁴⁴. *ADAR* **(C)** was previously classified as Gm. There is no m^6Am signal based on miCLIP¹⁴, m^6Am -seq¹¹, or m6ACE-seq¹² mapped to *ADAR*. However, ~71% of the transcripts from *ADAR* are A-initiated.

(D) Previously classified m^6Am genes express considerable levels of non-A-initiated transcripts. Each column represents a gene previously classified as m^6Am gene by miCLIP¹⁴. For each gene, the percentage of transcript isoforms starting with m^6Am/Am (in green) or Gm/Cm/Um (in gray) are shown. The percentage was calculated by weighting each transcript isoform by its expression level. The TSN frequencies were obtained using ReCappable-seq in HEK293T cells.

(Figure 1C). These observations are not artifacts of ReCappable-seq because similar results were also found using other TSN mapping methods (Figure S1A, S1B).

Conceivably m⁶Am genes produce multiple 5' isoforms, but the isoforms predominantly use A-TSNs. If this were the case, then the gene could indeed be considered an m⁶Am gene if all the A-TSNs were methylated to m⁶Am. We considered a gene to be predominantly composed of A-TSNs if >80% of transcripts start with A. Using this criterion, we found that only ~24% of m⁶Am genes determined by miCLIP¹⁴ are primarily composed of A-TSNs (Figure 1D). Similar observations were also found in other m⁶Am mapping methods^{4,13} (Figure S1C, D).

Our ReCappable-seq analysis also suggested that previous m⁶Am mapping methods may not have detected the diversity of m⁶Am in the transcriptome. ReCappable-seq identified many more A-TSNs than the total number of previously mapped m⁶Am sites. For example, in both *SRSF1* and *ADAR*, many A-TSNs are seen using ReCappable-seq, however, m⁶Am signals were only found at a few of these A-TSNs by either miCLIP¹⁴, m⁶Am-seq¹¹, or m6ACE-seq¹² (Figure 1B, C). This might suggest that only a few A-TSNs are m⁶Am modified. However, it is also possible that the antibody-based mapping methods do not have the resolution or sensitivity to distinguish between m⁶Am at different 5' isoforms. Notably, previous m⁶Am mapping studies exhibited very low overlap with each other. miCLIP¹⁴, m⁶Am-seq¹¹ and m6ACE-seq¹² together identified 7,480 m⁶Am sites in HEK293T cells (Figure S1E and Table S1). Among these sites, only 1.1% (84) are found in all three methods and 9.7% (728) are found in at least two studies (Figure S1E and Table S1). Taken together, these data demonstrate a variety of concerns about existing m⁶Am mapping studies.

CROWN-seq integrates TSN mapping and m⁶Am quantification

To understand the distribution of m⁶Am in the transcriptome, we sought to develop a method to identify the entire repertoire of TSNs among all the 5' transcript isoforms for each gene. In this way, we can identify the specific 5' isoforms for each gene that contain m⁶Am. Additionally, we wanted a quantitative method rather than the qualitative assessment provided by previous antibody-based methods. Recently, chemical methods using sodium nitrite were developed for m⁶A analysis^{20–22}. This method identifies m⁶A by chemically deaminating (“converting”) unmethylated A's into inosines (I's), while leaving m⁶A's intact. During sequencing, the A-to-I conversions are readily detected because I's are reverse transcribed into G's. This approach leads to precise and robust m⁶A quantification²⁰. Because of the chemical similarity between m⁶Am and Am, we explored the potential use of sodium nitrite conversion to map and quantify m⁶Am.

We first asked if Am is susceptible to deamination by sodium nitrite. To test this, we performed sodium nitrite conversion on a m⁷G-ppp-Am-initiated transcript (see Methods). We applied the sodium nitrite conversion protocol used in GLORI, which includes glyoxal treatment to prevent modification of guanosine residues²⁰. After sodium nitrite treatment, the RNA was reverse transcribed and sequenced. The conversion rate of Am was quantified by counting A or G reads at the first nucleotide position. In this assay, Am was completely converted (Figure S2A), indicating that sodium nitrite efficiently converts Am and thus can be used for m⁶Am quantification.

We considered the possibility that GLORI data²⁰ could be mined to measure m⁶Am stoichiometry at previously mapped m⁶Am sites¹⁴. We noticed that many A's at these TSNs were highly converted in GLORI (Figure S2B), suggesting prevalent Am. This is inconsistent with mass spectrometry analysis of mRNA cap structures from us¹ and others^{2,4}, which has suggested that m⁶Am is very prevalent while Am is relatively rare in mRNA. A potential cause of the high level of Am at TSNs predicted by GLORI could be the extensive RNA fragmentation that occurs with sodium nitrite treatment. RNA fragments that have 5' ends that align to the TSNs of overlapping transcript isoforms can confound the measurement of m⁶Am stoichiometry (Figure S2C, S2D). Thus, GLORI cannot distinguish between true TSNs and internal bases that are found

at the 5' end of RNA fragments. To overcome this limitation, we sought to develop a method that selectively analyzes A-TSNs and thereby removes the confounding effect of overlapping transcripts.

We developed CROWN-seq, which selectively analyzes TSNs throughout the transcriptome (**Figure 2A**). In this method, Am residues in mRNA are converted to Im using sodium nitrite. Next, we specifically isolate the 5' ends of mRNA by replacing the m⁷G cap with a desthiobiotin affinity tag using a decapping-and-recapping strategy¹⁸. By enriching the m⁷G-proximal sequence, we can simultaneously sequence the TSN of all transcripts, including both m⁶Am and non-m⁶Am TSNs. This is conceptually different from existing m⁶Am mapping methods which only examine the m⁶Am transcripts. For A-TSNs, m⁶Am stoichiometry can be quantified by counting the number of A reads (reflecting m⁶Am) or G reads (reflecting Am). In this way, we not only obtain TSN locations but also m⁶Am stoichiometry in the same RNA molecule.

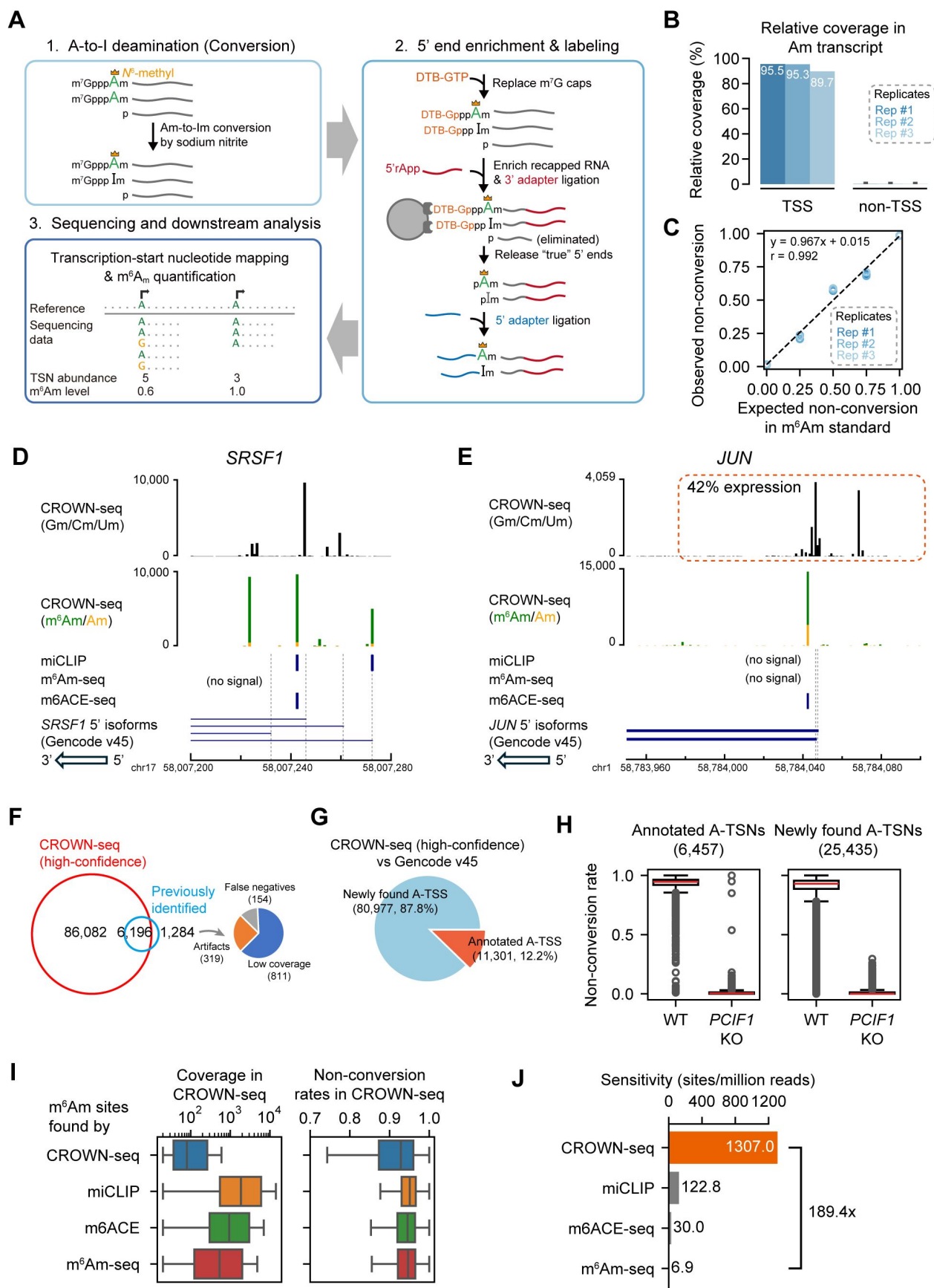


Figure 2.

CROWN-seq correctly maps and quantifies m⁶Am.

(A) Schematic of CROWN-seq. RNAs are firstly treated with sodium nitrite, which causes Am at the transcription-start position to be converted to Im. To isolate the TSN, m⁷G caps are replaced with 3'-desthiobiotin (DTB) caps. These DTB caps are enriched on streptavidin beads, while uncapped background RNA fragments are uncapped and washed away. After washing, an enriched pool of transcript 5' ends is released from the beads by cleaving the triphosphate bridge, leaving 5' monophosphate ends that are ligated to an adapter. After adapter ligation, cDNA was synthesized and amplified for Illumina sequencing. During sequencing, the converted sequences were aligned to a reference genome. The TSNs can be determined as the first base immediately after the 5' adapter sequence. To quantify m⁶Am stoichiometry, we count the number of A (m⁶Am) and G (Am) bases at the TSN position.

(B) CROWN-seq enriches reads that contain the TSN. The relative coverage of reads mapped to the TSS and non-TSS regions across the m⁷G-ppp-Am-initiated RNA standard was calculated. The average relative coverage of reads that map to the TSS and to non-TSS positions are shown for three replicates. The 95% CI of the relative coverages is shown using error bars.

(C) CROWN-seq exhibits high quantitative accuracy for measuring m⁶Am stoichiometry. RNA standards (Table S2 [\[link\]](#)) were prepared with 0%, 25%, 50%, 75%, and 100% m⁶Am stoichiometry. To make m⁶Am standards in different m⁶Am levels, we generated both Am transcripts and m⁶Am transcripts by *in vitro* transcription with cap analogs m⁷G-ppp-Am and m⁷G-ppp-m⁶Am. Five transcripts were made in the Am and m⁶Am form and mixed to achieve the indicated m⁶Am stoichiometry. These transcripts have identical 5' ends and different barcodes (Table S2 [\[link\]](#)). Linear least-squares regression was performed to calculate the correlation between expected non-conversion rates and the observed average non-conversion rates for each standard. All TSNs shown in this plot have high sequencing coverage, ranging from 656 to 21,545 reads.

(D) CROWN-seq results for *SRSF1*. CROWN-seq shows that 54.0% of *SRSF1* transcripts initiate with A. Among the A-initiated transcripts, 93.4% were resistant to conversion (A's, shown in green), and therefore m⁶Am. As a result, *SRSF1* has 50.4% m⁶Am transcripts, 3.6% Am transcripts, and 46.0% non-A-initiated transcripts. Notably, a previous miCLIP study identified an internal m⁶A site:⁴⁴[\[link\]](#) which we found was m⁶Am at the TSN based on CROWN-seq.

(E) CROWN-seq results for *JUN*. CROWN-seq shows that ~58% of *JUN* transcripts initiate with A. Unlike *SRSF1* which A-TSNs are highly methylated, *JUN* A-TSNs are only ~75% methylated. As a result, *JUN* has 43.5% m⁶Am transcripts, 14.5% Am transcripts, and 42% non-A-initiated transcripts.

(F) CROWN-seq identifies most m⁶Am sites identified in previous studies. 7,480 m⁶Am sites in HEK293T cells found either by miCLIP¹⁴[\[link\]](#), m⁶Am-seq¹¹[\[link\]](#), or m⁶ACE-seq¹²[\[link\]](#) were analyzed. The high-confidence sites in CROWN-seq were defined as A-TSN with ≥20 unique mapped reads. The results shown are from HEK293T cells, which is the same cell line used in all previous studies. Among the 1,284 sites uniquely found in other studies, 811 sites are also mapped by CROWN-seq but at lower coverage (1-19 reads); 319 sites are mapped very far (>100 nt) away from any TSS annotation and thus can be considered as false positives; the remaining 154 sites are mapped very closely to known TSSs and may be false negative results in CROWN-seq.

(G) Many A-TSNs identified in CROWN-seq in HEK293T cells are not annotated. In this analysis, A-TSSs in **(F)** were intersected with the TSS annotation in Gencode v45. Only 12.2% of A-TSSs found by CROWN-seq are previously annotated.

(H) CROWN-seq exhibits high accuracy in TSN discovery. In this analysis, we compared the non-conversion of A-TSNs between wild-type and *PCIF1* knockout cells. For the 6,457 A-TSNs annotated by Gencode v45, most of them have high non-conversion rates in wild-type cells and very low non-conversion rates in *PCIF1* knockout cells, indicating correct TSN mapping. Similar to the annotated TSNs, 25,435 newly found A-TSNs were also found to have differential

m⁶Am between wild-type and *PCIF1* knockout. Thus, these newly found A-TSNs were also mostly true positives. In this analysis, only A-TSNs mapped by at least 20 reads in both wild-type and *PCIF1* knockout HEK293T cells were used. The details of these sites can be found in [Table S3](#).

(I) The previously identified m⁶Am sites are biasedly in higher expression and higher m⁶Am stoichiometry. Shown are the sequencing coverage (left) and non-conversion rates (right) of different sets of m⁶Am sites in HEK293T CROWN-seq data. In total, 98,147 sites found by CROWN-seq, 2,129 sites found by miCLIP¹⁴, 3,693 sites found by m⁶ACE-seq¹², and 1,610 sites found by m⁶Am-seq¹¹ are shown.

(J) CROWN-seq has much higher sensitivity in m⁶Am discovery than all existing m⁶Am mapping methods. In this analysis, sensitivity is defined as m⁶Am/A-TSN found per million mapped reads. For CROWN-seq, sensitivity was defined as the slope of linear regression result between sequencing depth and A-TSN number among different samples in this study (see [Figure S2G](#)). For other methods, sensitivity was defined as the number of reported m⁶Am sites over the number of reads in all libraries required for m⁶Am identification.

To increase the accuracy of m⁶Am quantification, we made several optimizations to the ReCappable-seq protocol to markedly increase the read depth of TSNs. These include steps for on-bead adapter ligation and the introduction of unique molecular identifiers (UMIs) in the library preparation (see Methods).

Benchmarking CROWN-seq using m⁶Am-modified standards

To test TSN enrichment in CROWN-seq, we used a m⁷G-ppp-m⁶Am standard spiked into cellular mRNA. Among three replicates, we observed that ~93% of the reads mapped to the TSN ([Figure 2B](#)), confirming the enrichment of TSN. To further assess the enrichment of TSNs, we performed GLORI on the same sample. However, in GLORI only a few reads map to the TSN ([Figure S2C](#)). Thus, the decapping-and-recapping approach markedly enriches for TSNs.

We next wanted to determine the quantitative accuracy of CROWN-seq. To test this, we performed CROWN-seq on a mixture of RNA standards with predefined ratios of m⁶Am and Am (see Methods and [Table S2](#)). We found a highly linear correlation between the expected m⁶Am levels and the observed non-conversion rates measured by CROWN-seq across three replicates (Pearson's $r = 0.992$, [Figure 2C](#)). Taken together, CROWN-seq achieves both precise TSS mapping and m⁶Am quantification in m⁶Am standards.

CROWN-seq markedly expands the number of mapped m⁶Am sites

To assess the ability of CROWN-seq to map and quantify m⁶Am throughout the transcriptome, we performed CROWN-seq on poly(A)-selected RNA from HEK293T. In total, we identified 219,195 high-confidence TSNs, of which 92,278 were A-TSNs (Note S2, “Selection of m⁶Am identification cutoffs”). These TSNs were highly reproducible across biological and technical replicates ([Figure S2E](#)). Among the A-TSNs, 89,898 were from protein-coding genes, and 219 were from snRNA or snoRNA. Notably, among the mRNA A-TSNs, nearly all had high non-conversion rates ([Figure S2F](#)), indicating that nearly all A-TSNs contain high stoichiometry m⁶Am.

In contrast to previous m⁶Am mapping methods, CROWN-Seq reveals the diversity of TSNs among all the transcript isoforms for each gene. For example, in the case of *SRSF1*, m⁶Am is readily visible along with multiple other TSNs comprising Gm, Cm, or Um ([Figure 2D](#)).

CROWN-seq also shows that A-TSNs can have intermediate m⁶Am stoichiometry. For example, *JUN* expresses a 5' transcript isoform with an A-TSN, of which ~75% of transcript copies are m⁶Am modified ([Figure 2E](#)). Overall, CROWN-seq provides a comprehensive assessment of all TSNs in a gene and reveals the fraction of each A-TSN that is m⁶Am.

To confirm the accuracy of the mapped m⁶Am TSNs, we examined the 7,480 m⁶Am sites reported by miCLIP¹⁴, m⁶Am-seq¹¹, or m⁶ACE-seq¹². Among these sites, the vast majority (~82.8%, 6,196 of 7,480) were also found among the high-confidence A-TSNs in CROWN-seq (**Figure 2F**). For the remaining 1,284 sites, 811 are also found in CROWN-seq, but in lower sequencing depth; 319 were located far away (>100 nt) from any known TSSs and thus may be artifacts. Thus, CROWN-seq is highly reliable in detecting known m⁶Am sites. The low consistency between previous m⁶Am mapping studies likely reflects incomplete m⁶Am mapping in previous methods.

CROWN-seq clearly identified vastly more A-TSNs than all the other m⁶Am mapping methods combined (12.3-fold, 92,278 vs. 7,480). Notably, only ~12.2% of the newly found A-TSNs in CROWN-seq are annotated in Gencode v45 (**Figure 2G**, S2G), which primarily relies on CAGE data (see gene annotation guidelines by HAVANA²³). Notably, the newly identified TSNs with high coverage tend to overlap with or locate proximally to known TSSs, while the ones with low coverage tend to locate further to the known TSSs (**Figure S2G**). The newly identified A-TSNs could be artifacts or could be actual TSNs that were undetected by previous TSS-mapping studies. We suspect that these are true A-TSNs for two reasons: First, as part of the mapping criteria, a minimum of 20 independent reads across all replicates was required for TSN identification. Second, if these sites were RNA fragments, they would not contain m⁶Am. However, these A-TSNs show high stoichiometry of m⁶Am (i.e., non-conversion) in CROWN-seq (**Figure 2H**, S2F) which is lost in *PCIF1* knockout cells (**Figure 2H**), except for some outliers such as TSNs of *S100A6*, *IFI27*, and *ALDH1A1* (see **Table S3**). Thus, the marked increase in the number of m⁶Am sites revealed by CROWN-seq reflects the preferential enrichment for mRNA 5' ends, which leads to high sensitivity and read depth at TSNs transcriptome-wide.

In contrast to m⁶Am sites identified in CROWN-seq, m⁶Am that were identified in previous m⁶Am mapping methods tended to derive from high abundance transcripts or high abundance TSNs (**Figure 2I**). Because of the high read depth at TSNs, CROWN-seq enables the detection of m⁶Am at more m⁶Am sites than previous methods (**Figure 2J**, **Figure S2H**). Although we used a 20-read cutoff for mapping m⁶Am, m⁶Am sites identified with fewer reads are also likely to represent true TSNs. These m⁶Am TSNs typically show high non-conversion (e.g., 2 or 3 reads among a total of 3 reads) in HEK293T cells but zero non-conversions in *PCIF1* knockout cells (**Figure S2I**). The *PCIF1* dependence of these sites is consistent with a true m⁶Am TSN and further highlights the sensitivity of CROWN-seq for mapping m⁶Am at TSNs.

CROWN-seq reveals consistently high m⁶Am stoichiometry in mRNA across diverse human cell lines

Although our data showed that m⁶Am in mRNA generally exhibits very high stoichiometry (**Figure S2F**), we considered the possibility that these results were unique to HEK293T cells. Several studies have shown that *PCIF1* expression can vary considerably in different cell lines^{5,24}, which may indicate that m⁶Am stoichiometry is dependent on the cell line. We therefore wanted to determine the m⁶Am landscape across cell lines with varying levels of *PCIF1*.

We selected several cell lines for this analysis. First, we chose HEK293T, A549, HepG2, and K562 cells, which have also been characterized using multiple orthogonal datasets²⁵. Second, we selected colorectal cancer cells (i.e., HT-29 and HCT-116), since *PCIF1* depletion in these cells affects their migration and sensitivity to immunotherapy⁵. These colorectal cancer cells have high *PCIF1* expression based on western blotting and RT-qPCR, while the non-cancerous colon cell line CCD841 CoN has very low *PCIF1* expression⁵. Third, we selected cells with very low CTBP2 expression, a proposed coactivator of *PCIF1*²⁴. These cells, which include Jurkat E6.1 and Huh-7, as well as the previously mentioned K562 and HepG2 cells, would be expected to have low m⁶Am levels based on their low CTBP2 expression²⁴ (**Figure S3A**).

For each cell line, we performed CROWN-seq using two to four replicates. In total, we obtained 514 million aligned reads (Table S3 [↗](#)). In each cell line, 14,650 to 58,768 mRNA A-TSNs with at least 50 reads were analyzed (Table S3 [↗](#)). The 50-read threshold provides highly consistent quantification of m⁶Am stoichiometry between replicates (Figure S3B [↗](#) and Note S2).

Quantification of m⁶Am across all TSNs showed that mRNA m⁶Am stoichiometry is generally high. For most of the cells, the average m⁶Am stoichiometry is 0.895±0.03 (Figure 3A [↗](#)), indicating high overall mRNA m⁶Am levels. Some cell lines, for example, Jurkat E6.1, HT-29, and Huh-7 cells show very high and less variable m⁶Am levels (0.933±0.1, 0.924±0.1, and 0.916±0.1, respectively); while other cell lines such as CCD841 CoN, HCT-116, and K-562 have relatively low and more variable m⁶Am levels (0.825±0.2, 0.877±0.1, and 0.891±0.1, respectively). It should be noted that in all cell lines, the m⁶Am stoichiometry is still high compared with other mRNA modifications²⁰ [↗](#).

We considered the possibility that the high m⁶Am stoichiometry might be caused by RNA structure that blocks access to sodium nitrite leading to non-conversion. However, essentially complete conversion was seen in *PCIF1* knockout HEK293T cells, which makes it likely that m⁶Am is the cause of non-conversions. Also, we found that A-TSNs completely converted in 5' ends predicted to be highly structured, suggesting that RNA structure does not impair conversion in CROWN-seq (Figure S3C [↗](#)).

The differences in m⁶Am stoichiometry are related to *PCIF1* expression (Figure 3B [↗](#), S3D). For example, CCD841 CoN cells, which have very low *PCIF1* expression based on our measurements (Figure 3B [↗](#), S3D) and previous measurements⁵ [↗](#), exhibit the lowest median m⁶Am stoichiometry at ~0.878. However, even this stoichiometry is still relatively high. Thus, m⁶Am levels are affected by *PCIF1* expression, but m⁶Am can be considered as a high stoichiometry modification across all tested cell lines. On the other hand, the proposed *PCIF1* coactivator CTBP2, exhibited a weak correlation to mRNA m⁶Am (Figure 3C [↗](#)).

Several mRNAs show low m⁶Am stoichiometry

Although most A-TSNs in mRNA exhibit high m⁶Am stoichiometry, some exhibit stoichiometry below 0.8, and even below 0.5 (Figure 3A [↗](#) and Table S3 [↗](#)). To identify A-TSNs with low m⁶Am, we examined each A-TSN and calculated its average stoichiometry across all cell lines (Figure 3D [↗](#)). For each A-TSN we also assessed its variability by calculating the range of m⁶Am levels measured across cell lines (Figure 3D [↗](#)). This analysis demonstrates that a significant subset of A-TSNs have low stoichiometry, with some showing variability depending on the cell type. For example, *JUN* contains a lowly methylated A-TSN, as shown above in HEK293T cells (Figure 2E [↗](#)), and also exhibits low stoichiometry in many other cell lines (Figure 3E [↗](#)). In addition, *ENO1*, *MYC*, and *ACTB* also show low m⁶Am stoichiometry in some of their A-TSNs (Figure 3E [↗](#)).

We next used Gene Ontology (GO) analysis to determine if the low m⁶Am A-TSNs are associated with specific cellular functions. The GO analysis of Cellular Component categories showed a marked enrichment of genes linked to cellular respiration, ribosomal subunits, spliceosome, and nucleosome (which are mostly histone genes) (Figure 3F [↗](#), Table S4 [↗](#)). Similar results were found in the Biological Processes GO analysis (Table S4 [↗](#)). In addition to these genes, we also noticed HOX genes contain lowly methylated A-TSNs (Figure S3E [↗](#)).

Among all different gene categories, histone genes exhibited the lowest overall m⁶Am stoichiometry (Figure 3G [↗](#)). Notably, histone genes have unique mechanisms of gene expression. Many histone genes are located in gene clusters (i.e., clusters 6p22.1-2 and 1q21.1-2) and transcribed in histone locus bodies²⁶ [↗](#). These clustered histone genes tend to contain upstream TATA-box and downstream T-rich sequences (Figure S3F [↗](#)). In contrast, non-clustered histone

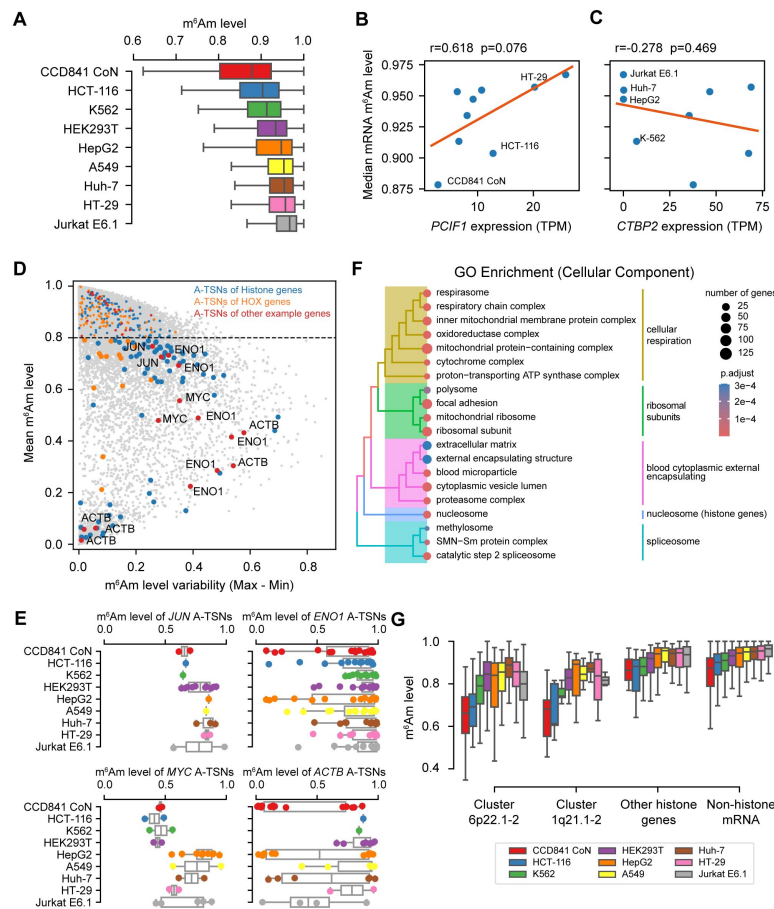


Figure 3.

CROWN-seq reveals m^6Am landscape in mRNA.

(A) Boxplot showing the overall mRNA m^6Am levels (i.e., m^6Am stoichiometry) among different cell lines. The exact m^6Am levels of the mRNA m^6Am sites in this analysis can be found in Table S3. Only m^6Am sites with ≥ 50 reads mapped in at least one cell line were analyzed.

(B) mRNA m^6Am stoichiometry is positively correlated with *PCIF1* expression. In this plot, *PCIF1* expression was estimated by the number of reads mapped to *PCIF1* TSSs. The read counts were normalized into transcription-start nucleotide per million (TPM) for gene expression comparison. Three cell lines (CCD841 CoN, HCT-116, and HT-29) whose *PCIF1* expression was estimated by western blots and RT-qPCR by Wang *et al.* are highlighted. Pearson's r and p -value in this analysis were obtained by linear regression.

(C) Overall mRNA m^6Am stoichiometry is not correlated with *CTBP2* expression. Similar to **(B)**, *CTBP2* expression was estimated by CROWN-seq. Four cell lines with very low *CTBP2* expression are highlighted.

(D) Some A-TSNs have relatively low and more variable m^6Am stoichiometry among cell lines. In this plot, the variability of the m^6Am stoichiometry of a site, which is defined as the maximum m^6Am subtracted by the minimum m^6Am stoichiometry among all cell lines is shown on the X-axis; the average m^6Am level of a site among all cell lines is shown on the Y-axis. Several example genes are indicated in different colors.

(E) Boxplots and dotplots showing the m^6Am levels of different A-TSNs in *JUN*, *ENO1*, *MYC*, and *ACTB*. These genes contain A-TSNs with relatively low m^6Am stoichiometry. In this plot, the exact m^6Am levels of individual A-TSNs are shown in dots, while the median and IQR of the m^6Am levels are shown in boxplots. Only m^6Am sites with ≥ 50 reads mapped were analyzed.

(F) Gene Ontology enrichment (Cellular Components) of genes containing lowly methylated m^6Am sites. The full Gene Ontology results can be found in Table S4.

(G) A-TSNs in histone genes tend to have relatively low m^6Am stoichiometry. In this plot, histone genes are categorized by their genomic localizations. Histone gene cluster 6p22.1-2 and 1q21.1-2 are the two major histone gene clusters. For histone gene cluster 6p22.1-2, 55 to 173 A-TSNs are shown in different cell lines; for histone gene cluster 1q21.1-2, 9 to 14 A-TSNs are shown; for other histone genes, 24 to 109 A-TSNs are shown.

genes tend to have high m^6Am stoichiometry (**Figure 3G**) and show different promoter sequence contexts (**Figure S3F**). This data suggests that transcription mechanisms might be important for determining m^6Am stoichiometry.

m^6Am stoichiometry is linked to the sequence of core promoter

The differential methylation in histone genes based on their transcription mechanisms raises the possibility that transcription initiation mechanisms might affect m^6Am stoichiometry. Because m^6Am is the first nucleotide in mRNA, its deposition may be highly influenced by early transcription events. Notably, PCIF1 binds to RNA polymerase II²⁷ and is enriched in promoter regions²⁸, which may be important for methylation of the 5' end of mRNAs. We therefore considered the possibility that different transcription mechanisms may be linked to different levels of m^6Am .

As a first test, we examined whether nucleotide preferences upstream (which would reflect sequences involved in transcription initiation) or downstream of the A-TSN are linked to m^6Am stoichiometry. We binned A-TSNs based on the m^6Am stoichiometry and examined nucleotide preferences at each position. Using this approach, we found that the nucleotides upstream of the A-TSN were markedly different for A-TSNs with low vs. high m^6Am stoichiometry (**Figure S4A**). For example, at positions -4 and -1, there was a clear positive correlation between the use of C and m^6Am stoichiometry (**Figure 4A**). The correlation of these nucleotide positions that lie in the promoter region to m^6Am stoichiometry suggests that transcriptional mechanisms might influence m^6Am stoichiometry.

We also observed strong nucleotide preferences at positions downstream of the A-TSN. These include nucleotide preferences at +2 (**Figure 4A**). These could reflect sequence preferences for PCIF1; however, this position is also part of transcription-initiation motifs (see below), and thus the contribution of transcription mechanisms and direct sequence preferences of PCIF1 are difficult to deconvolve.

To more directly determine specific transcription mechanisms linked to m^6Am , we examined how specific sequence motifs around A-TSNs correlate with m^6Am stoichiometry. We found markedly different sequence motifs surrounding highly methylated A-TSNs compared to lowly methylated A-TSNs (**Figure 4B**). A-TSNs with the highest m^6Am stoichiometry (top 5th-percentile, 0.991 average stoichiometry) are enriched in an $SSCA_{+1}GC$ (S=C/G) motif, which is similar but distinct from the well-known $BBCA_{+1}BW$ (B=C/G/T, W=A/T) transcription initiator motif²⁹, largely because of the C at the +3 position (**Figure 4B**). In contrast, the A-TSNs with the lowest m^6Am stoichiometry (bottom 5th-percentile, 0.435 average stoichiometry) were enriched in an unconventional $VA_{+1}RR$ (V=A/C/G, R=A/G) motif (**Figure 4B**).

We next classified each A-TSN based on whether they use the $SSCA_{+1}GC$ or $VA_{+1}RR$ motifs, or if they contain the conventional $BBCA_{+1}BW$ and BA_{+1} Inr-like motifs (**Figure S4B**). This plot shows that $BBCA_{+1}BW$ and BA_{+1} motifs exhibit intermediate m^6Am stoichiometry (**Figure 4C**). Overall, these data indicate that m^6Am stoichiometry is strongly related to the TSS motif in the core promoter, which implies that m^6Am formation is linked to the transcription initiation process. Because transcription initiation is also affected by other elements in the core promoter²⁹, we also asked whether these transcription-related elements, such as TATA-box and transcription factor-binding sites, are associated with higher or lower m^6Am stoichiometry. We first analyzed the relationship between m^6Am and elements including the TATA-box, BREu, BRED, and DCE²⁹. In this analysis, A-TSNs from promoters containing TATA-box exhibited lower m^6Am stoichiometry, especially those of histone genes (**Figure 4D**). On the other hand, other elements, such as BREu and BRED, which are motifs for recruitment of TFIIB²⁹, and DCE, which binds by TAF1²⁹, showed little correlation with m^6Am stoichiometry (**Figure S4C**). Thus, the presence of the TATA box exhibited the strongest effect and predicted lower m^6Am stoichiometry.

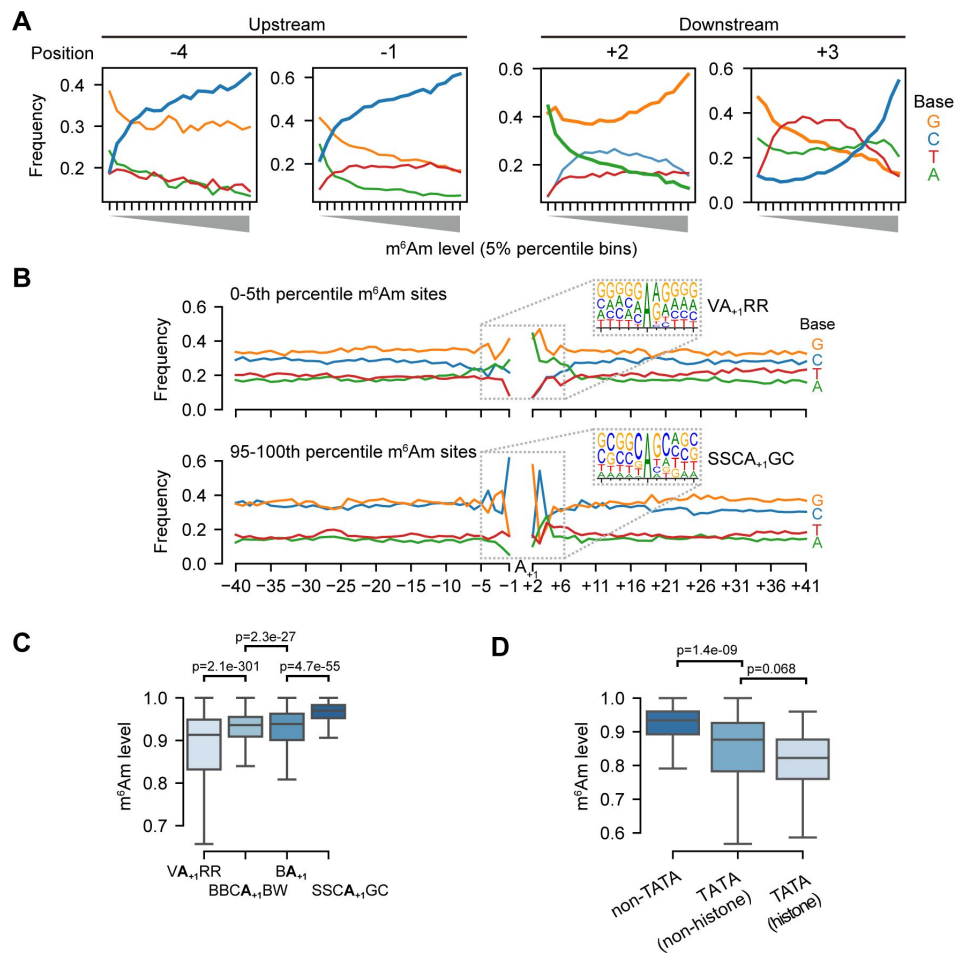


Figure 4.

m⁶Am stoichiometry is related to core promoter sequence.

(A) m⁶Am stoichiometry is related to base composition in both upstream and downstream of A-TSNs. In these plots, 58,723 A-TSNs are grouped into twenty 5-percentile bins (X-axis). For each bin, the frequency of A, T, C, and G bases at each position relative to the A-TSN are plotted on the Y-axis. Among different positions in the promoter region, C's in -4, -1, and +3, as well as G's in +2 are positively correlated with high m⁶Am; while A's in +2 are negatively correlated with high m⁶Am. Results for other promoter positions can be found in [Figure S4A](#).

(B) Motif analysis of A-TSNs with the lowest 5% m⁶Am stoichiometry (upper) and the A-TSNs with the highest 5% m⁶Am stoichiometry (lower). The core promoter region (-40 to +41) was screened for enriched motifs. The lowest 5% A-TSNs exhibited a VA₊₁RR TSS (V=A/C/G, R=A/G) motif, while the highest 5% A-TSNs exhibited a SSCA₊₁GC (S=C/G) motif. The sequence contexts for all A-TSNs are shown in [Figure S3E](#).

(C) A-TSNs expressed from different core promoters exhibit different m⁶Am stoichiometry. Core promoters containing the VA₊₁RR motif produce transcripts with relatively low m⁶Am stoichiometry. Transcripts using the SSCA₊₁GC motif exhibited relatively high m⁶Am stoichiometry. In comparison, the m⁶Am stoichiometry in conventional A-TSNs from either BBCA₊₁BW or BA₊₁ is also shown and exhibits intermediate m⁶Am stoichiometry. In this analysis, 14,788, 7,981, 34,578, and 1,376 A-TSNs were used for each of the four motifs. P-values, Student's t-test, two-sided.

(D) TATA-box containing core promoters exhibit relatively low m⁶Am stoichiometry. For this analysis, the TATA-box is defined as TATAWAWR²⁹. Because many TATA-boxes found in our A-TSN dataset are outside the classic -31 to -24 region, we extended the region for the TATA-box search to -36 to -19. Since histone genes preferentially contain TATA box, we separately plotted TATA-box-containing histone genes (N=155) and TATA-box-containing non-histone genes (N=28). 58,540 A-TSNs without TATA-box are also shown. P-values, Student's t-test, two-sided.

We next analyzed the relationship between m^6Am and transcription factor-binding sites (TFBS). To test this, we screened A-TSNs for the presence of 401 transcription-factor binding sites and examined the relationship between the binding sites and m^6Am stoichiometry (see Methods). Several TFBSs, such as those for NANOG and FOXJ3, exhibited a slight negative correlation to m^6Am (Figure S4D [↗](#)); while other TFBS, such as SP2 and KLF4, exhibited a slight positive correlation to m^6Am (Figure S4E [↗](#)). Overall, no specific TFBS exhibited a strong effect on m^6Am stoichiometry (Figure S4F [↗](#)).

Taken together, our data show a linkage between transcriptional mechanisms and m^6Am stoichiometry.

m^6Am does not substantially influence mRNA stability or translation

Previous studies sought to determine the effect of m^6Am on mRNA stability and translation based on gene-level annotations of the starting nucleotide^{4,14,15,30} [↗](#). However, the gene level annotations do not take into account the potential for many transcription-start nucleotides (Figure S5 [↗](#)). Rather than using a binary metric of m^6Am or non- m^6Am , we developed a metric that reports the fraction of all TSNs for each gene that contain m^6Am . This “ m^6Am gene index” is the ratio of m^6Am TSNs over all TSNs, as measured by CROWN-seq, for each gene. Using the m^6Am gene index, we reanalyzed the previously published translation efficiency^{4,14} [↗](#) and RNA stability¹⁴ [↗](#) data in HEK293T cells. We found that genes with low or high m^6Am gene index do not show differences in translation (Figure S6A, S6B [↗](#)) or RNA stability (Figure S6C [↗](#)) in *PCIF1* knockout cells compared to wild-type.

m^6Am is involved in efficient transcription of A-initiated transcripts

We next wanted to examine other potential functions of m^6Am . Although we found no clear effect of m^6Am on mRNA stability, we asked if m^6Am influences transcript expression levels. To test this, we quantified the abundance of each A-TSN isoform in HEK293T and A549 cells. For these experiments, we added a mixture of pre-capped ERCC spike-ins (see Methods) to the RNA samples before performing TSN expression quantification by ReCappable-seq. This ERCC spike-in mixture calibrates sequencing results and increases TSN expression quantification accuracy (see Methods).

In this analysis, we binned A-TSNs into percentiles based on their m^6Am stoichiometry. Here we could see that transcripts with the highest levels of m^6Am also exhibited the highest overall expression levels (Figure 5A [↗](#), S6D, and Table S5 [↗](#)). This suggests that m^6Am is associated with higher transcript expression.

To determine if m^6Am causes the increased expression of A-TSN transcripts, we measured the expression change for each A-TSN in wild-type and *PCIF1* knockout HEK293T and A549 cells (Table S5 [↗](#)). We found that A-TSNs with higher m^6Am stoichiometry exhibit significantly reduced expression in *PCIF1* knockout, while A-TSNs with the lowest m^6Am stoichiometry were almost unchanged (Figure 5B [↗](#), S6E). In contrast, G-TSNs were slightly increased in *PCIF1* knockout cells (Figure 5C [↗](#), S6F). These data suggest that m^6Am promotes the expression of A-TSN transcripts.

We were surprised that *PCIF1* depletion leads to a selective decrease in the expression of A-TSN transcripts in the highest percentile bin but had little to no effect in the other bins. Each bin has very high m^6Am stoichiometry (~0.77 in the lowest bin and ~0.98 in the highest bin in HEK293T) (Figure 5B [↗](#), S6E). Thus, if m^6Am is a stabilizing mark, we should see reduced expression in all bins. We therefore considered other possibilities that might explain why *PCIF1* depletion affects transcript levels in some bins but not others.

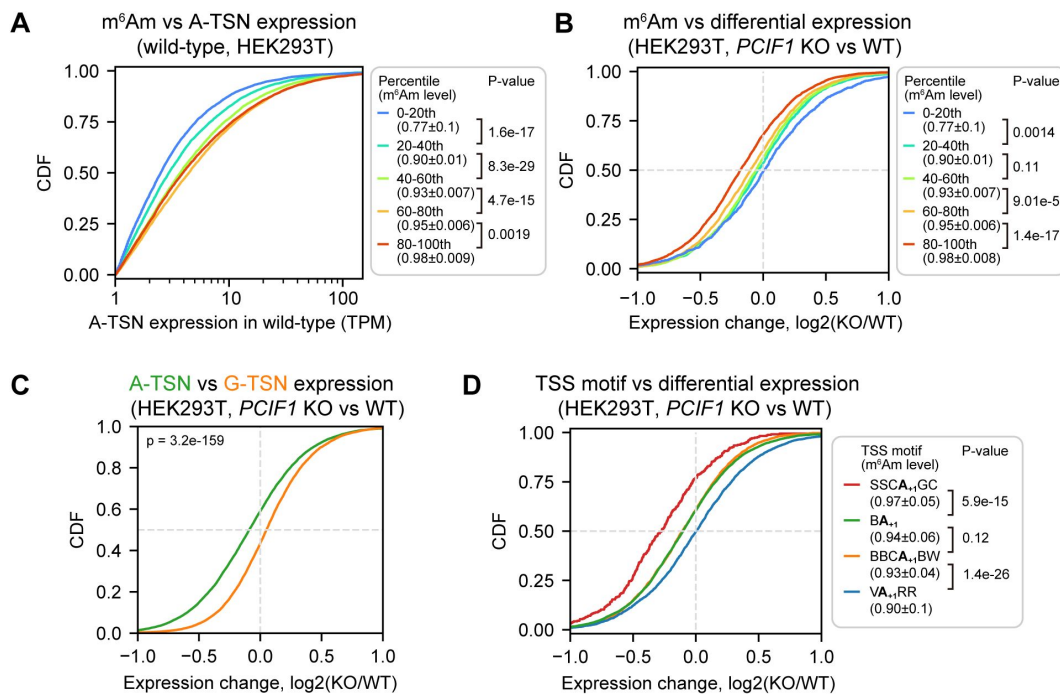


Figure 5.

PCIF1 knockout leads to m⁶Am and TSS motif-related A-TSN expression changes.

(A) m⁶Am stoichiometry is positively related to A-TSN expression in wild-type HEK293T cells. In this cumulative distribution plot, the expression of each A-TSN was quantified by ReCappable-seq, for all A-TSNs in each indicated m⁶Am stoichiometry bin. A-TSNs (n=58,723) were grouped into five bins based on m⁶Am stoichiometry quantified by CROWN-seq. In total, 5,125, 6,962, 7,991, 8,368, and 8,009 A-TSNs are shown in each bin (from low m⁶Am to high m⁶Am). These A-TSNs have an average TPM ≥ 1 in two ReCappable-seq replicates and coverage ≥ 50 in CROWN-seq. P-values, Student's t-test for TPM (log-transformed), two-sided.

(B) The expression level of high m⁶Am stoichiometry A-TSNs is reduced in *PCIF1* knockout. Shown is a cumulative distribution plot of A-TSN expression change in HEK293T cells upon *PCIF1* knockout. The differential expression of A-TSN was calculated by DESeq2⁴⁵. Similar to **(A)**, the A-TSNs were binned based on the m⁶Am stoichiometry. In total, 3,269, 2,272, 3,218, 3,813, and 3,369 A-TSNs are shown in each bin (from low m⁶Am to high m⁶Am). A-TSNs with a baseMean (i.e., the average of the normalized count among replicates) ≥ 100 were used in the differential expression test (two replicates were used for both wild-type and *PCIF1* knockout) and coverage ≥ 50 reads in CROWN-seq. P-values, Student's t-test, two-sided.

(C) Shown are cumulative distribution plots of expression changes of A-TSNs and G-TSNs after *PCIF1* depletion. 14,516 A-TSNs and 9,667 G-TSNs with expression levels quantified by ReCappable-seq are shown. These A-TSNs and G-TSNs have baseMean ≥ 100 during the differential expression test. P-values, Student's t-test, two-sided.

(D) Similar to **(B)**, A-TSNs that use different TSS motifs exhibit different changes in expression upon *PCIF1* knockout. In total, 352 A-TSNs using SSCA₊GC, 7,928 A-TSNs using BA₊, 2,958 A-TSNs using BBCA₊BW, and 2,760 A-TSNs using VA₊RR are shown. These A-TSNs have baseMean ≥ 100 during differential expression test (2 replicates for both wild-type and *PCIF1* knockout) and coverage ≥ 50 reads in CROWN-seq. P-values, Student's t-test, two-sided.

An important difference between A-TSN in different bins is that they tend to use different TSS motifs (see **Figure 4C**). We therefore asked if the effect of m⁶Am depletion is linked to the TSS motifs. For this analysis, we classified A-TSNs based on the presence of SSCA₊₁GC, VA₊₁RR, or other TSS motifs (i.e., BBCCA₊₁GC and BA₊₁). Here we found that the identity of the TSS motif was highly associated with the degree of transcript reduction in *PCIF1* knockout cells (**Figure 5D**, S6G). Notably, transcripts that use the SSCA₊₁GC motif showed the largest drop in expression. In contrast, A-TSNs that use the VA₊₁RR TSS motif showed almost no change in expression in *PCIF1* knockout cells (**Figure 5D**, S6G).

Taken together, these data suggest that the effect of m⁶Am is not related to mRNA stability but instead is related to transcription. Our data suggest that certain transcription initiation complexes, such as those that use the SSCA₊₁GC motif, rely on m⁶Am for efficient expression. However, other TSS motifs do not rely as strongly on m⁶Am to achieve efficient expression. These data suggest that m⁶Am may have important roles in the transcription processes.

CROWN-seq reveals diverse m⁶Am stoichiometry in snRNA and snoRNA

In addition to mRNAs, m⁶Am is also found on snRNAs and snoRNA^{10,12}. However, the stoichiometry and dynamics of m⁶Am in these RNAs are unknown. Using CROWN-seq we quantified m⁶Am stoichiometry in several snRNAs, including U1, U2, U4, U4ATAC, U5, U7, U11, and U12. These snRNAs are transcribed by RNA polymerase II³¹, are capped, and use A-TSNs¹⁰. Among these snRNAs, we identified 51 m⁶Am sites, of which 29 were unannotated 5' variants located close to the annotated TSNs (Table S6).

Compared with mRNA, m⁶Am in snRNA exhibited a very different distribution of stoichiometry (Figure S2F). First, snRNA m⁶Am sites exhibited generally low m⁶Am stoichiometry, typically below 0.3. Second, m⁶Am stoichiometry between different snRNA genes was much more variable than in mRNA (**Figure 6A**). Third, some snRNA genes show highly variable stoichiometry in different cell lines.

For example, among U1 snRNA genes, *U1.22* exhibited relatively high m⁶Am levels (~0.47-0.80) in all cell lines, while *RNU1-3* and *RNU1-4* show relatively low m⁶Am levels (~0.09-0.45, **Figure 6A** and Table S6). U5 snRNAs have the highest variability m⁶Am stoichiometry between cell types (**Figure 6A**). The U5 snRNA isoform *RNU5E-1* varies 31.6-fold in the m⁶Am level between HEK293T (0.0185) and Jurkat E6.1 cells (0.586). In contrast, m⁶Am in U2 and U7 snRNA are generally high (at 0.35-0.76 and 0.48-0.89, respectively) and not variable between cell lines (**Figure 6A**).

In addition to snRNA, we also examined 17 A-TSNs in C/D box snoRNA including U3, U8, and U13. These snoRNAs are involved in rRNA 2'-O-methylation during pre-rRNA processing³². m⁶Am stoichiometry in snoRNA is highly related to snoRNA species and snoRNA isoform. For example, among different U3 snoRNA isoforms, A-TSNs of *U3.18* have much higher m⁶Am stoichiometry than others (e.g., *SNORD3A*) (**Figure 6B**). These data indicate that snoRNA also has highly variable m⁶Am stoichiometry.

FTO specifically controls m⁶Am stoichiometry in snRNA and snoRNA

We next sought to understand why m⁶Am stoichiometry is low in snRNA and snoRNA. We considered the possibility that the overall m⁶Am stoichiometry in snRNA is correlated with *PCIF1* expression, as we saw with mRNA (**Figure 3B**). However, we found a poor correlation of overall m⁶Am stoichiometry in snRNA with *PCIF1* expression (Pearson's *r* = 0.204, *P*-value=0.597, **Figure 6C**).

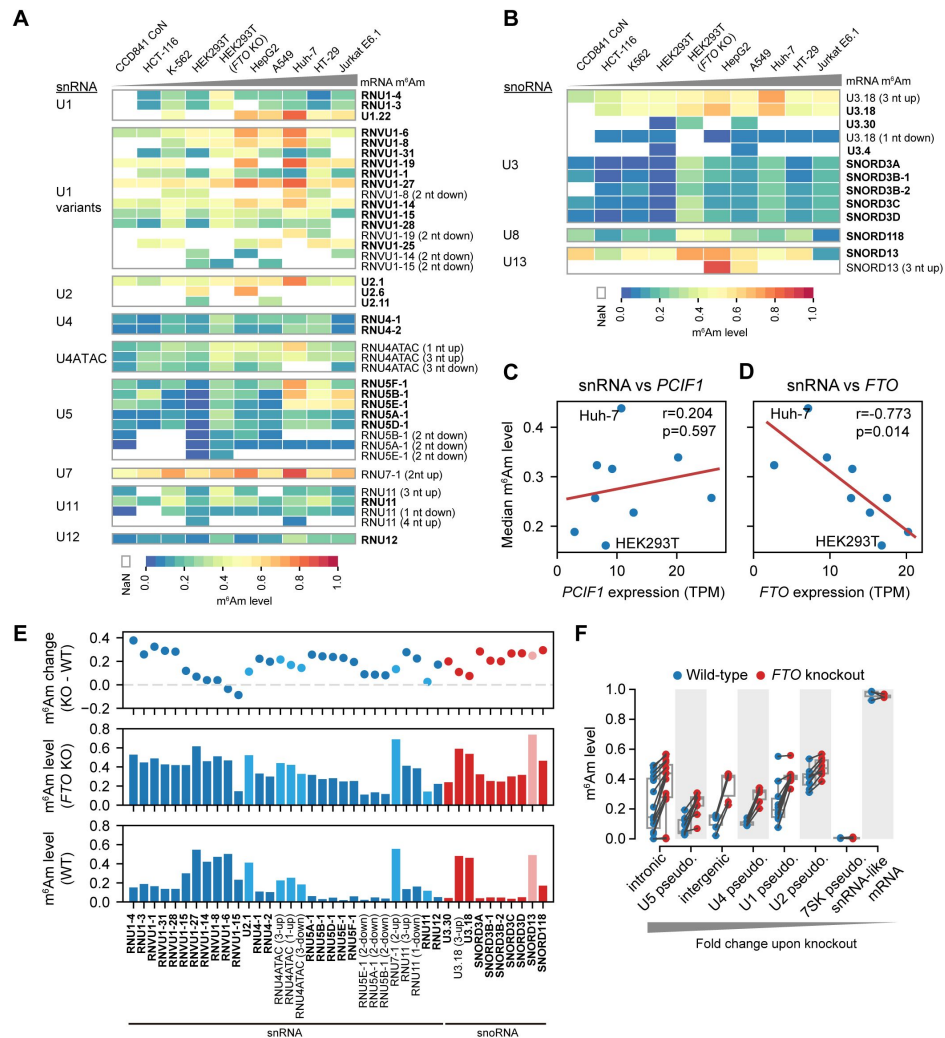


Figure 6.

CROWN-seq reveals m⁶Am landscape in snRNA and snoRNA.

(A) Heatmaps showing m⁶Am stoichiometry in different snRNA gene families and isoforms. Cell lines in the column are ranked by the overall mRNA m⁶Am stoichiometry. The name of each snRNA isoform is shown on the right. A-TSNs already annotated in Gencode v45 are highlighted in bold. For newly found A-TSNs, the relative distance between the new A-TSN and the nearest annotated A-TSN is shown in brackets. Note that Gencode v45 contains snRNA annotation from different databases. For example, *RNU1-4* and *U1.22* are both U1 snRNA, however, *RNU1-4* is from the HGNC database and *U1.22* is from the RFAM database.

(B) Similar to **(A)**, Heatmaps show the m⁶Am stoichiometry in different snoRNA isoforms.

(C) and **(D)**, snRNA methylation levels are not well correlated with *PCIF1* expression, but negatively correlated with *FTO* expression. The RNA expression levels of *PCIF1* and *FTO* were estimated by reading counts in CROWN-seq, which were converted into TPM to normalize the sequencing depth. Linear regressions were performed to obtain Pearson's r and p -value of the correlations.

(D) *FTO* depletion leads to increased m⁶Am level (i.e., m⁶Am stoichiometry) in many kinds of snRNA and snoRNA. In this plot, the difference in m⁶Am levels between wild-type and *FTO* knockout cells is shown in the first row. The exact m⁶Am levels in *FTO* knockout and wild-type cells are shown in the second and third rows. Different kinds of snRNA and snoRNA are shown in different colors.

(E) *FTO* depletion leads to increased m⁶Am stoichiometry in snRNA and snoRNA pseudogenes. In this plot, shown are the annotated pseudogenes of U1, U2, U4, U5, and 7SK, as well as the newly identified snRNA/snoRNA pseudogenes in intronic and intergenic regions. Several mRNAs exhibited 5' ends resembling snRNA pseudogenes. However, these snRNA-like mRNA 5' ends showed high and stable m⁶Am stoichiometry in both wild-type and *FTO* knockout cells.

We next considered FTO, a highly efficient demethylase for m⁶Am in snRNA^{10,12}. In contrast to PCIF1, FTO expression exhibited a strong negative correlation with snRNA methylation levels (Pearson's $r = -0.773$, $P\text{-value} = 0.014$, **Figure 6D**). Notably, HEK293T cells, which were tested in our previous study¹⁰, exhibited the highest FTO expression and the lowest snRNA m⁶Am stoichiometry (**Figure 6D**). Some other cell lines, such as Huh-7, have lower FTO expression and thus have relatively higher m⁶Am stoichiometry in snRNAs (**Figure 6D**).

We next wanted to determine how FTO affects m⁶Am stoichiometry in snRNAs. Using CROWN-seq on *FTO* knockout HEK293T cells, we observed prominent m⁶Am level increases in nearly all snRNA and snoRNA (**Figure 6E**). Most of the snRNA isoforms exhibited an overall increase in m⁶Am stoichiometry by ~ 0.2 upon *FTO* knockout. However, a notable subset of snRNAs were not affected by FTO depletion. For example, the *RNVU1-8* isoform has little change in m⁶Am stoichiometry. *RNVU1-8* has an unusually high m⁶Am stoichiometry at ~ 0.47 compared to other U1 snRNA isoforms in wild-type cells (**Figure 6E**).

Notably, FTO depletion does not increase m⁶Am levels in snRNA and snoRNA to the levels seen in mRNA (i.e. > 0.9 stoichiometry). This suggests that the low m⁶Am levels in snRNA and snoRNA are not solely due to FTO-mediated demethylation. Instead, these snRNAs are likely to be inefficiently methylated by PCIF1 and are then demethylated by FTO in order to achieve their overall low m⁶Am stoichiometry.

We also found FTO demethylates m⁶Am in snRNA pseudogenes. Overall, we mapped 69 A-TSNs in annotated snRNA/snoRNA pseudogenes. These pseudogenes exhibited increased methylation upon *FTO* knockout (**Figure 6F** and **Table S6**). We also identified 202 snRNA/snoRNA pseudogene-like transcripts (see **Table S6**). These transcripts exhibited very high similarity to the annotated snRNA/snoRNA pseudogenes, and therefore likely reflect previously unannotated pseudogenes (see Methods). Upon *FTO* knockout, A-TSNs in these unannotated pseudogenes also exhibited increased m⁶Am levels (**Figure 6F**).

FTO has minimal effects on m⁶Am and m⁶A at 5' ends of mRNA

We next asked whether FTO levels affect m⁶Am levels in mRNA. To address this question, we compared FTO RNA expression and median mRNA m⁶Am stoichiometry in all nine cell lines. This analysis shows a weak negative correlation between FTO expression and mRNA m⁶Am (Pearson's $r = -0.239$, $P\text{-value} = 0.535$, **Figure S7A**).

To further assess whether FTO affects m⁶Am levels in mRNA, we quantified m⁶Am level changes in mRNA in wild-type and *FTO* knockout HEK293T cells. Overall, we observed a very small increase in mRNA m⁶Am with only a few m⁶Am sites having notably increased methylation level upon *FTO* knockout (**Figure S7B**). Thus, only select m⁶Am sites in mRNA are efficiently demethylated by FTO.

Although CROWN-seq focuses on m⁶Am measurements, the reads in CROWN-seq can contain internal m⁶A sites if they are close to the TSN. m⁶A sites are readily detected because they do not undergo conversion with sodium nitrite. We therefore examined the stoichiometry of these 5'-proximal m⁶A sites in *FTO* knockout HEK293T cells. We identified internal m⁶A sites that were mapped with at least 50 reads in both wild-type and *FTO* knockout cells and had a non-conversion rate of ≥ 0.2 in either genotype. In total, we identified 235 high-confidence m⁶A sites which were found by both CROWN-seq and GLORI²⁰ (**Table S6**). These m⁶A sites exhibited the expected DRm⁶ACU motif (**Figure S7C**). However, these sites only showed small changes in non-conversion rates ($P\text{-value} = 0.00037$, paired t-test) (**Figure S7D** and **Figure S7E**). It should be noted that our conclusion about the effect of FTO on internal m⁶A is restricted to specific m⁶A sites around 5' ends since most internal m⁶A sites are not found in the 5' fragments examined in CROWN-seq.

Taken together, FTO has a strong preference for demethylating m⁶Am in snRNA, snoRNA, and their pseudogenes, compared to mRNA. FTO is a major determinant of the overall m⁶Am levels of these transcripts in different cell lines.

Discussion

Functional studies of m⁶Am require highly accurate transcriptome-wide maps. However, m⁶Am mapping studies have relied on the assumption that each gene can be considered to have a single start nucleotide. To overcome this, we developed CROWN-seq, which maps the TSNs for all 5' transcript isoforms, and measures the exact stoichiometry of m⁶Am at all A-TSNs. CROWN-seq reveals a markedly distinct distribution of m⁶Am than previously recognized, largely due to inaccuracies in previous maps, and the problem with assigning each gene to a single start nucleotide. In addition, the quantitative measurements of m⁶Am in CROWN-seq show that the earlier idea that many mRNAs contain transcription-start nucleotide Am is largely incorrect since nearly all A-TSNs exhibit high stoichiometry m⁶Am. Overall, this study establishes the first quantitative, transcript isoform-specific m⁶Am map in mammalian cells. The m⁶Am maps reveal that m⁶Am is associated with increased transcript abundance, with functions of m⁶Am more correlated with transcription initiation than stability.

By selectively capturing and examining only 5' ends, CROWN-seq achieves exceptional read depth at the TSN, enabling highly accurate identification and quantification of m⁶Am. Notably, CROWN-seq is an antibody-free method and thus avoids the problem of immunoprecipitation of both m⁶Am- and m⁶A-containing fragments. This dual-specificity of antibodies creates ambiguities in m⁶Am mapping. Additionally, antibody binding cannot provide quantitative measurements of m⁶Am. In contrast, CROWN-seq uses a sodium nitrite-based chemical method for m⁶Am identification, which we show fully converts Am to Im, but leaves m⁶Am intact. Thus, the fraction of A-TSNs that contain m⁶Am or Am can be readily determined by sequencing, where all Am nucleotides are read as G. The exceptional read depth of CROWN-seq enables quantification of m⁶Am at single-nucleotide resolution, resulting in vastly more m⁶Am sites than all previous m⁶Am mapping methods combined. Although CROWN-seq involves many chemical and enzymatic steps, m⁶Am quantification by CROWN-seq is very accurate and robust, which was examined by m⁶Am standards, consistency across different replicates, and *PCIF1* knockout data. Notably, chemical conversion-based methods tend to have artifacts in regions with stable RNA secondary structures^{33,34}, where the nucleotides cannot efficiently interact with the chemical reagent. However, we found that CROWN-seq is very reliable even for highly structured 5' ends (Figure S3C), which might be due to the high accessibility of the TSN, the high stringency of the conversion steps²⁰, or RNA denaturation due to glyoxal³⁵.

It is worth mentioning that there is no golden standard for transcription-start nucleotide (site) mapping accuracy estimation. For CROWN-seq, we first tested the mapping accuracy by *in vitro* transcribed RNA oligos, which shows that ~93% of the 5' ends can be mapped correctly. However, in practice, *in vitro* transcription might initiate at non-specific TSSs, resulting in 5' ends not overlapping with the desired TSSs^{36,37}. Thus, the mapping accuracy can be underestimated in this assay. Since mRNA A-TSNs in the cells are known to be highly methylated by *PCIF1*^{1,4}, we considered that the presence or absence of m⁶Am at mapped A-TSNs can be used to assess the accuracy of TSN identification. True A-TSNs should have m⁶Am. In CROWN-seq essentially all previously annotated A-TSNs and newly found A-TSNs exhibited high non-conversion rates. These A-TSNs were well converted upon *PCIF1* knockout. This indicates very high TSN mapping accuracy, even at the many previously unannotated TSNs described here. These previously unannotated TSNs were likely missed because traditional transcription-start mapping methods and pipelines lack the sensitivity to discover them. These unannotated TSNs might have specific

molecular functions. Future studies might focus on the biology of these unannotated TSNs, for example, whether these unannotated TSNs, compared to major TSNs, are associated with different mRNA processing events, such as alternative splicing.

We performed CROWN-seq in nine different cell types with the goal of understanding common principles that guide m⁶Am formation in mRNA. In all cell types, m⁶Am was a very high stoichiometry modification, with some exceptions. We found a correlation between PCIF1 expression and m⁶Am stoichiometry, but even cells with very low PCIF1 expression exhibited high m⁶Am stoichiometry. The CROWN-seq data is highly consistent with recent mass spectrometry analysis of mRNA caps by us¹ and others^{2,4}. These mass spectrometry studies purified the entire cap structure comprising the m⁷G, the triphosphate linker, and the first nucleotide. In these studies, m⁷G-ppp-m⁶Am was highly prevalent while m⁷G-ppp-Am abundance was typically 1/10 as m⁷G-ppp-m⁶Am in nearly all cell lines^{1,2}. This mass spectrometry data was the first suggestion that transcription-start nucleotide Am was not a prevalent modification in mRNA, as had been suggested by early chromatography studies⁹. We suspect that the high levels of Am seen in these early analyses of mRNA can be explained by contaminating snRNA or rRNA fragments, which are highly difficult to remove, even with multiple rounds of poly(A) purification³⁸. It remains possible that there are cell types or cellular contexts that remain to be discovered with low m⁶Am (i.e., high Am) levels. However, it is clear that high m⁶Am stoichiometry is a general feature of most or all cell types in this study.

The initial m⁶Am maps relied on published TSN annotations. In the first m⁶Am map, annotations were based on FANTOM5¹⁶, which primarily uses CAGE datasets to define the start nucleotide. However, these annotations selected a single start nucleotide even if multiple TSS signals from CAGE peaks were detected for a gene^{4,14}. It should be noted that some genes may primarily use m⁶Am for all 5' transcript isoforms. These genes would therefore have a high m⁶Am gene index. Genes with a high m⁶Am gene index are likely to be preferentially affected by PCIF1 depletion or pathways that affect m⁶Am.

Based on the small range of m⁶Am stoichiometry in A-initiated mRNAs, it is unlikely that the variability in stoichiometry has functional significance for most mRNAs. Instead, our data suggest that mRNAs initiate with either Gm, Cm, Um, or Am, where Am is highly m⁶Am modified. mRNAs that initiate with m⁶Am may have shared regulatory mechanisms that distinguish them from mRNAs that initiate with Gm, Cm, and Um. Additionally, genes that primarily initiate with m⁶Am, either because they have only one major transcription-start site, or because all their transcription-start nucleotides are A, would be highly influenced by m⁶Am-dependent regulatory mechanisms. Currently, cellular pathways that target m⁶Am-initiated mRNAs are not well known.

Our study revealed a link between m⁶Am and transcription. This effect was detectable because of the highly quantitative nature of m⁶Am measurement in CROWN-seq. Although all A-TSNs show high stoichiometry, there are differences in the overall m⁶Am stoichiometry between transcripts, e.g., ~0.85 stoichiometry vs. 0.95 stoichiometry which can readily be detected by CROWN-seq. We found that these differences are often related to the specific TSS motif. For example, the Inr-like SSCA₊₁GC TSS motif was associated with the highest m⁶Am stoichiometry, while transcripts using the VA₊₁RR TSS motif exhibited relatively lower m⁶Am stoichiometry. This finding highlights that the major role of m⁶Am might be linked to transcription regulation, which is supported by a recent study by An et al.³⁹.

We then examined the effects of PCIF1 depletion on m⁶Am transcript abundance. We found that transcripts with higher methylation in wild-type cells tend to have a larger reduction in RNA expression level upon *PCIF1* knockout. Further analysis showed that transcripts that use the SSCA₊₁GC TSS motif exhibited significantly reduced expression in *PCIF1* knockout cells. In contrast, transcripts that use the VA₊₁RR TSS motif were largely unaffected. Notably, transcripts normally have small differences in methylation (i.e., methylation level at 0.9 vs 0.98). Thus, m⁶Am

is unlikely to be a general stabilization mark in mRNA since it only affects transcripts based on promoter sequences. Instead, these different stoichiometries of m⁶Am are likely to be the consequence of different transcription mechanisms. Thus, it will be important to assess how these different transcription mechanisms use m⁶Am for gene expression.

PCIF1 is known to be associated with RNA polymerase II and is recruited to promoter regions during transcription²⁸. Thus, PCIF1 is ideally positioned to regulate transcription processes. It is interesting to speculate that m⁶Am may provide a mark that enhances subsequent elongation and thus maintains high overall expression for transcription initiation complexes that assemble on the SSCA₊₁GC TSS motif. Other transcription initiation complexes, such as those using the VA₊₁RR TSS motif, may not need this mechanism. However, our data cannot provide further details on whether the loss of m⁶Am is related to exact mechanisms such as transcription initiation selection, elongation, or premature termination. Notably, the recent study by An et al.³⁹ suggested that the loss of m⁶Am is related to enhanced premature termination and therefore leads to reduced RNA 5' end expression. An et al. proposed that m⁶Am can sequester PCF11, a m⁶Am reader, and thereby promote transcription by reducing premature transcription termination³⁹. However, it is still unclear whether the transcripts from the SSCA₊₁GC TSS motif are indeed more preferentially bound by PCF11. To better understand how PCIF1 regulates transcription, assays with transcription-start nucleotide resolution will be required.

Although m⁶Am and m⁶A are chemically similar, these two modifications appear to have very different biological functions. It is well known that m⁶A is a mark for RNA instability through the recruitment of YTHDF proteins⁴⁰. However, we find no correlation between m⁶Am and RNA instability. Additionally, our previous YTHDF1, YTHDF2, and YTHDF3 iCLIP studies did not show binding at mRNAs 5' ends⁴¹, which suggests that YTHDF proteins do not bind m⁶Am. Thus, specific m⁶Am-binding proteins might enable its unique functions in transcription.

Although most studies of m⁶Am and PCIF1 focus on mRNAs, we find that m⁶Am in snRNAs exhibit substantially higher variability and regulation than that in mRNA. Early biochemical studies of snRNA composition demonstrated that the first nucleotide was generally Am in all Pol II-derived snRNAs¹⁰. CROWN-seq generally supports this finding since most snRNAs have low m⁶Am stoichiometry. However, the previous study mainly focused on HEK293T cells¹⁰, which have very low m⁶Am in snRNA. In this study, nine different cell types were sequenced. These cell lines showed highly variable m⁶Am in snRNA. In some cases, several snRNAs can reach m⁶Am stoichiometry up to 0.70-0.89. These data raise the possibility that m⁶Am may affect snRNA functions, such as splicing and gene transcription^{12,42}, and *PCIF1* knockout phenotypes may be due to altered snRNA.

Notably, m⁶Am in snRNA is highly regulated by FTO, which is consistent with our earlier findings¹⁰. However, the previous study did not have transcript isoform level resolution in analyzing the effect of FTO demethylation. With CROWN-seq, we find that FTO has markedly different effects on different snRNAs, where some snRNAs appear highly demethylated by FTO while others are insensitive to FTO. Some snoRNA, and snRNA/snoRNA pseudogenes are also demethylated by FTO. Notably, FTO depletion affects numerous aspects of cell function⁴³. Our results thus raise the possibility that FTO-depletion phenotypes may result from increased m⁶Am levels in snRNAs, snoRNAs, or their pseudogenes.

Limitations of the study

One limitation of CROWN-seq is that it can be difficult to align sequencing reads to the genome. Unlike normal reads, which contain A, G, C, and U, most reads in CROWN-seq comprise only G, C, and U due to the conversion of A's. This makes it difficult to align reads to highly similar genes, such as snRNA isoforms and pseudogenes which have very similar 5' ends. For this reason, only a small fraction of reads from snRNA and pseudogenes were uniquely mapped to one genomic location and were used in this analysis. To better understand m⁶Am in these 5' ends with similar

sequences, future optimization is desired to increase the read lengths, which can help distinguish these sequences from each other. This requires technical innovations in reducing RNA fragmentation during sodium nitrite conversion, which comes from acid-catalyzed depurination and backbone cleavage²¹.

In this study, we quantified m⁶Am in nine different cell lines, which cover a wide range of PCIF1 expression levels. Although we found high m⁶Am stoichiometries in all cell lines, it is possible that some cells or tissues have more variable m⁶Am levels. In our previous study, mass spectrometry showed that the CCRF-SB cell line has relatively low m⁶Am stoichiometry at ~67.6%. However, these cells exhibit very slow growth as reported previously. As a result, we were unable to obtain enough amounts of mRNA needed for CROWN-seq. Future CROWN-seq studies may lead to the identification of cell types or contexts with dynamic m⁶Am landscapes.

The last limitation of this study is that the focus of this study was to quantify m⁶Am and to predict potential functions using *PCIF1* knockout cells. However, it is possible that *PCIF1* has non-catalytic functions that may contribute to the *PCIF1* knockout phenotype. Future experiments using catalytic-dead *PCIF1* can be useful to distinguish between the catalytic and non-catalytic functions of *PCIF1*.

Methods

Experimental model and subject details

Cell lines

HEK293T (wild-type, *PCIF1* knockout, and FTO knockout cells), A549 (wild-type and *PCIF1* knockout), HCT-116, Huh-7, and HT-29 cells were maintained in DMEM (Gibco #11995065). HepG2 and CCD841 CoN cells were maintained in EMEM (ATCC #30-2003). K562 and Jurkat E6.1 cells were maintained in RPMI1640 (Gibco #11875093). All media was supplemented with 10% FBS and 1X penicillin-streptomycin (Gibco #15140148). Cells were grown at 37 °C with 5% CO₂. We followed the instructions from the manufacturer to maintain the cells.

Methods details

RNA extraction and mRNA purification

Cellular total RNA in TRIzol LS (ThermoFisher #10296028) was extracted by Direct-zol RNA Miniprep kit (Zymo #R2070) or by Phenol Chloroform extraction. mRNA was purified by NEBNext Oligo d(T)25 Beads (NEB #E7499) or Dynabeads Oligo (dT)25 (Ambion #61002) based on mRNA purification from total RNA protocol of Dynabeads Oligo (dT)25 (Ambion #61002).

m⁶Am standard preparation

We used *in vitro* transcription to prepare m⁷G capped m⁶Am- or Am-initiated transcripts, which are based on HiScribe® T7 mRNA Kit with CleanCap® Reagent AG (NEB #E2080S). We first obtained DNA templates from IDT gBlock. In total, five DNA templates which are identical except for the 6-nt long barcode 42-nt downstream to the TSS were used (Table S2). The DNA templates contain 5'-TAATACGACTCACTATAAG-3' T7 promoter for *in vitro* transcription. We used CleanCap® Reagent AG (3' OMe) (TriLink #N-7413), which is included in NEB #E2080S, to generate m⁷G-ppp-Am modified transcripts. We used CleanCap® Reagent M6 (TriLink #N-7453) to generate the m⁷G-ppp-m⁶Am modified transcripts. The RNAs made by *in vitro* transcription were DNase I treated, purified, and then quantified by both Agilent TapeStation (RNA high sensitivity assay). We then

mixed the Am and m⁶Am modified oligos to generate m⁶Am standards with expected m⁶Am stoichiometry at 0%, 25%, 50%, 75%, and 100% m⁶Am stoichiometry. Notably, the guaranteed purity of the CleanCap® Reagent M6 is >95%. The CleanCap® Reagent M6 can contain m⁷G-ppp-AmG analog, which results in the reduced non-conversion rate in CROWN-seq.

Genomic assembly and annotations

The genomic sequence and annotations of Gencode v45 primary assembly were used in this study.

GLORI experiment

To validate whether sodium nitrite conversion can convert Am into Im, we spiked ~0.01 ng Am transcripts (ERCC-00057-1-TCGTCG) into ~250 ng poly(A) selected mRNA for GLORI assay. Ligation-based GLORI protocol was used in this study. Notably, the Am transcripts were decapped by mRNA Decapping Enzyme (NEB #M0608S) in advance. We first fragmentized the input RNA into ~200 nt long fragments (NEBNext Magnesium RNA Fragmentation Module (NEB #E6150S), 94°C, 2 minutes). The fragmentized RNAs were then A-to-I converted based on the GLORI protocol²⁰, we converted the glyoxal-protected RNA by 750 mM NaNO₂ at 16°C for 8 hours and 4°C overnight. The RNA was then deprotected in a deprotection buffer at 95°C for 10 minutes. The deprotected RNA was then T4 PNK (NEB #M0210S) treated and processed to ligation-based small RNA-seq library preparation⁴⁶. Notably, the 5' adapter in library preparation contains an 11 nt UMI sequence (see Table S7).

GLORI data processing

GLORI libraries were analyzed based on a modified mRNA bisulfite sequencing pipeline⁴⁷. The first 10 bases in GLORI libraries made with eCLIP protocol were first extracted by a customized script. GLORI reads were first quality trimmed by Cutadapt⁴⁸. For the GLORI library generated by eCLIP protocol, the parameters are `--max-n 0 --trimmed-only -a AGATCGGAAGAGCGTCGTG -e 0.1 -q 30 -m 40 --trim-n`; for GLORI library prepared by ligation based protocol generated in this study, the parameters are `-m 32 -j 4 -q 20 -e 0.25 -a AGATCGGAAGAGCACACGTG -A ATATNNNNNNNNNNAGATCGGAAGAGCGTCGTG`. After pre-processing, the reads were firstly A-to-G converted and aligned to A-to-G (positive strand) and T-to-C (negative strand) converted reference genome and transcriptome by Hisat2-2.1.0⁴⁹. Parameters in alignment: `-k 5 -fr -rna-strandness FR --no-temp-splicesite --no-mixed`. Only unique alignments were used. After alignment, the base information in sequences was restored so that m⁶Am signals can be reflected by the A-to-G mismatches. No further transcriptome alignment was performed on the unmapped reads. After alignment, a customized script based on Pysam⁵⁰ was used to pileup every single base to obtain the A, C, G, and U counts. Every single base was assigned to a transcript isoform if possible (order: mRNA > lncRNA > functional RNAs > pseudogenes). Non-conversion rate is defined as the number of A counts against the sum of A count and G count. Filters were applied to obtain high-quality non-converted A (m⁶A/m⁶Am) signals in a gene-specific manner: (1) only genes with at least 1000 counts were analyzed; (2) gene-specific non-conversion rates were computed for Binomial test on the frequency of non-conversion and sites with Binomial test P-value < 0.05 were used; (3) reads with more than 3 non-converted As were considered as noise and discarded; (4) sites with more than 5% signals were discarded due to the site may fall in a conversion-resistant region; (5) Only sites with no less than 20 reads covered and non-conversion rates over 0.1 were considered as m⁶A/m⁶Am sites. (6) Non-conversion rates of the same site from different replicates were averaged. Details of this pipeline can be found at https://github.com/jhfoxliu/GLORI_pipeline.

ReCappable-seq library preparation

A modified ReCappable-seq protocol¹⁸ was developed to reduce background, reduce material loss, and increase the utility of mapped reads. Several steps of library construction are now performed while the 5' desthiobiotinylated cap is bound to streptavidin beads. This reduces the

opportunity for carry-through of random fragmentation products to occur that would previously result in non-cap-derived 5' ends to be ligated. Next, 5' adapters with unique molecular indexes (UMIs) are used to permit robust PCR duplicate removal. Finally, ~160 spike-in mRNAs from SIRV-ERCC Spike-in mixture (Lexogen #051.03) with single defined 5' termini are used, which are used during analysis to build a dynamic thresholding pipeline that exclude false positive start sites. A complete step-by-step protocol as performed here will be available on the accompanying GitHub page (see Data and Code Availability).

5 µg total RNA was used as input for all experiments. RNA was denatured at 65 °C for 2 minutes before reaction mixes were added. First, 5'-phosphorylated RNAs were dephosphorylated using 25 U Quick CIP (NEB #M0525L) in a 50 µL reaction for 30 minutes at 37 °C. The reaction was cleaned using a Zymo RCC-5 column following the manufacturer's >200 nt protocol and eluted with 20 µL water. m⁷G capped RNAs were then decapped using 200 U yDcpS (NEB #M0463S) for 1 hour at 37 °C. This unique decapping enzyme liberates m⁷GMP, resulting in mRNAs with a 5'-diphosphate. The reaction was cleaned and eluted as before. Next, the 5'-diphosphorylated mRNAs were recapped with desthiobiotin-GTP (DTB-GTP) using vaccinia capping enzyme (5 µL VCE buffer, 0.5 µL inorganic pyrophosphatase (NEB #M0361S), 5 µL DTB-GTP (5 mM; NEB #N0761S), 50 U VCE (#M2080S)) for 45 minutes at 37 °C. The reaction was clean as before, however, a total of 4 washes were performed to ensure the complete removal of excess DTB-GTP. RNA was then fragmented by incubating at 95 °C for 2.5 minutes in a 25 µL reaction containing 100 mM Tris-HCl pH 8.0 and 2 mM MgCl₂. Fragmented RNA was placed on ice and brought to 30 µL with water. Streptavidin beads (NEB #S1421S) were washed in a high salt wash buffer (10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA) and resuspended in the high salt buffer at 4 mg/mL. 30 µL beads were added to 30 µL fragmented RNA and incubated for 45 minutes at room temperature with agitation. Beads were washed twice in a high salt buffer, twice in a lower salt buffer (10 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA), and twice in PNK wash buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.2% Tween). Beads were next resuspended in 40 µL PNK reaction mix (8 µL 5X pH 6.5 PNK buffer (350 mM Tris-HCl pH 6.5, 50 mM MgCl₂, 5 mM DTT), 1 µL T4 PNK (NEB #M0201S), 1 µL RNaseOUT (ThermoFisher #10777019) and incubated at 37 °C for 30 minutes with agitation to remove 3' phosphates resulting from the fragmentation. Beads were washed once in PNK wash, once in the high salt wash, then twice again in PNK wash. Next, a 3' adapter (see [Table S7](#)) was added to RNA by resuspending beads in 40 µL 3' ligation reaction mix (4 µL T4 RNA ligase buffer, 2 µL T4 RNA ligase 2 truncated KQ (NEB #M0373L), 1 µL RNaseOUT, 2 µL L7 adapter (20 µM stock, see [Table S7](#)), 16 µL of 50% PEG-8000) and incubated at 25 °C for 2 hours. The beads were washed once in PNK wash, once in high salt wash, twice in lower salt wash, then resuspended in 30 µL lower salt wash containing 1 mM biotin (ThermoFisher #B20656) to elute DTB-capped RNA fragments. The eluted RNA was cleaned by ethanol-AMPure XP (1.8 volumes AMPure XP, then 1.5 volumes 100% ethanol). To increase stringency, the streptavidin bead enrichment was repeated omitting enzymatic steps and instead washing three times with high salt and then three times with lower salt wash, and the eluate was cleaned again by ethanol-AMPure XP. The DTB-GTP cap was removed using 0.5 U/µl RppH (NEB #M0356S) in 1X ThermoPol buffer (NEB #M0356S) and incubating at 37 °C for 1 hour. The resulting 5'-monophosphate RNA fragments were purified by ethanol-AMPure XP. 30 pmol of a 5' adapter was ligated for 3 hours at 25 °C with 2 U/µL T4 RNA ligase 1 (NEB #M0437M). This RNA adapter contains an 11 nt UMI followed by a fixed sequence (AUAU) at its 3' end. The UMI allows robust duplicate removal, and the fixed sequence provides an anchor point to correctly identify the first nucleotide of the mRNA. The ligation reaction was inactivated by heating at 65 °C for 10 minutes and then immediately used in a reverse transcription reaction. 3 pmol of ReCappable-seq RT primer was annealed to the 3' adapter of RNA fragments by heating to 65 °C for 5 minutes and cooling to 25 °C at a rate of 0.1 °C/sec. Reverse transcription was carried out at 55 °C for 45 minutes in a reaction containing 0.5 mM dNTPs, 5 mM DTT, 20 U RNaseOUT, 50 mM Tris-HCl pH 8.3, 75 mM KCl, and 300 U SuperScript III (ThermoFisher #18080044). Following heat inactivation, the reaction was cleaned using ethanol-AMPure XP and cDNA was resuspended in 21 µL. The final PCR was performed using 8 µL cDNA in a 40 µL reaction containing 1X Phusion HF master mix (NEB #M0531L) and 4 µL each of a unique i5 and i7 barcoded primer combination

for each sample (NEB #E7600S). Cycling conditions were typically 98 °C 2 minutes, then 11-13 cycles of 98 °C 15 seconds, 65 °C 30 seconds, 72 °C 30 seconds, with a final 5 minute 72 °C extension. The optimal number of cycles for each library was determined by performing a set of test cycles using 1 µL cDNA in a 20 µL reaction. PCR libraries were purified with 2 rounds of bead clean-up using 0.9X volume SPRIselect beads. Libraries were pooled at equimolar concentrations and sequenced in paired-end mode with 50-150 bp reads depending on the library on either an Illumina NovaSeq, NextSeq, or HiSeq (please refer to GEO accession for specific details for each library).

ReCappable-seq analysis

The beginning of read 1 is the UMI plus an ATAT spacer sequence, and the nucleotide directly following this is the TSS. Reads were first filtered to identify pairs with the correct UMI+ATAT sequence, then the UMI was added to FASTQ headers using UMI-tools v1.1.1⁵¹. ATAT sequence discarded. Adapters were trimmed using Cutadapt v3.4⁴⁸. Next, reads mapping to ribosomal RNA and small non-coding RNAs were filtered away by aligning to these sequences using bowtie2 v2.4.2⁵². Reads were then aligned to GRCh38 and m⁶Am standard sequences using HISAT2⁴⁹. The alignment results were deduplicated by UMI-tools (--paired --chimeric-pairs=discard --unpaired-reads=discard --method=unique). Only reads without 5' softclipping were used. A customized script based on Pysam⁵⁰ was used to extract the 5' ends from the BAM file. To annotate the sites by a gene, the 5' ends were firstly annotated by the nearest TSS within the 100 bp region. If multiple annotations were found, the annotation was selected by the priority of snRNA > snoRNA > mRNA > lncRNA > others. BEDtools v2.27.1⁵³ was used to find the nearest annotation. To more accurately estimate the expression levels of each TSN, we normalized the read counts using the "RUVg" function in RUVSeq package⁵⁴. To calculate the expression levels of TSNs in wide-type cells, we calculated the TPM values based on the normalized read counts. To calculate the differential expression between wild-type and *PCIF1* knockout cells, the normalized read counts were proceeded by DESeq2⁴⁵.

CROWN-seq library preparation

CROWN-seq uses the glyoxal-based guanosine protection protocol from GLORI²⁰ and a TSN enrichment protocol that is modified from ReCappable-seq¹⁸. In CROWN-seq, glyoxal protection is very important to prevent both internal G's from being converted into xanthosine, which can interrupt base pairing and cause mutations during reverse transcription⁵⁵. Because N7-methyl does not interrupt the interaction between glyoxal and N1 and N2 positions of guanosines, glyoxal protection is also very useful to prevent m⁷G from being converted, which can help 5' end enrichment. After glyoxal protection, A bases are deaminated into inosines by sodium nitrite. After deamination, the 5' end RNA fragments with a m⁷G cap were enriched by ReCappable-seq workflow, where the m⁷G caps were replaced by a 5' desthio-biotinylated cap for enrichment by streptavidin beads. 3' adapter and 5' adapter (with unique molecular indexes (UMIs)) were ligated to the enriched 5' RNA fragments, so that the library can be made by reverse transcription followed by indexing PCR. Detailed workflow is described below.

Conversion. 0.8-2.5 µg oligo(dT) selected RNA was used as input. RNA was first diluted in 14 µl water. To perform glyoxal protection, 6 µl 8.8 M glyoxal and 20 µl DMSO were then added to the diluted RNA and well mixed. The 40 µl mix was first incubated at 50 °C for 30 minutes, then 10 µl boric acid was added to the mix. The 50 µl mix was then incubated for an additional 30 minutes at 50 °C. After protection, the 50 µl protected RNA was mixed with 50 µl deamination buffer (25 µl 1500 mM NaNO₂, 4 µl 500 mM MES, pH 6.0, 10 µl 8.8 M glyoxal, and 11 µl water). The deamination reaction was performed at 16 °C for 8 hours. After deamination, the RNA was recovered by ethanol precipitation. To remove the glyoxal adduct from the RNA, the RNA pellet was dissolved in 50 µl deprotection buffer (500 mM TEAA pH=8.6, 47.5% deionized formamide) and was incubated at 95

°C for 10 minutes. After incubation, the reaction was brought to 250 µl with water. Converted RNA was purified by ethanol precipitation and eluted in 39 µl water for 5' end enrichment. The converted RNA was stored at -80 °C before 5' end enrichment.

Recapping. To eliminate the contamination of RNA with 5'-triphosphate and 5'-monophosphate, 5 µl 10X CutSmart buffer, 5 µl Quick CIP (5 U/µl) (NEB #M0525L), and 1 µl SUPERase-In RNase inhibitor (ThermoFisher #AM2696) were added to the 39 µl converted RNA to set up a dephosphorylation reaction. The dephosphorylation reaction was performed at 37 °C for 30 minutes. The reaction was cleaned up using Zymo RCC-5 column and the RNA was eluted in 42 µl water. To decap the m⁷G capped RNA, a 50 µl decapping reaction was set up by adding 5 µl 10X yDcpS buffer, 2 µl (200 U) yDcpS (NEB #M0463S), and 1 µl SUPERase-In to the 42 µl dephosphorylated RNA. The decapping reaction was performed at 37 °C for 1 hour. This unique decapping enzyme liberates m⁷GMP, resulting in mRNAs with a 5'-diphosphate. The reaction was cleaned and eluted as before. The reaction was cleaned up using Zymo RCC-5 column and the RNA was eluted in 33.5 µl water. The 5'-diphosphorylated mRNAs were recapped with desthiobiotin-GTP (DTB-GTP, NEB #N0761S) using vaccinia capping enzyme (VCE, NEB #M2080S) (5 µL VCE buffer, 0.5 µL inorganic pyrophosphatase (NEB #M0361S), 5 µL DTB-GTP (5 mM), 50 U VCE, 1 µl SUPERase-In) at 37 °C for 1 hour. The reaction was cleaned up using Zymo RCC-5 column and the RNA was eluted in 30 µl water. Now the RNA is ready for streptavidin enrichment.

5' enrichment. Streptavidin beads (NEB #S1421S) were washed in a high salt wash buffer (10 mM Tris-HCl pH 7.5, 2 M NaCl, 1 mM EDTA) and resuspended in the high salt buffer at 4 mg/mL. To enrich the RNA and tag the 5' and 3' end by the specific adapter, the 30 µl recapped RNA was first mixed with 30 µl streptavidin beads and incubated at room temperature for 45 minutes with agitation. Beads were washed twice in high salt buffer, twice in a lower salt buffer (10 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM EDTA), and twice in PNK wash buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.2% Tween). To remove 3' phosphates resulting from fragmentation during conversion, beads were resuspended in 50 µl PNK reaction without ATP (5 µl 10X PNK buffer, 1 µl T4 PNK (#M0201S), 1 µl SUPERase-In, 43 µl water), and incubate at 37 °C for 30 minutes with agitation. The beads were then washed once in PNK wash buffer, once in 2 M NaCl wash, and twice in PNK wash. Next, RNA was ligated to a 74 nt-long 3' adapter ([see Table S7](#)) in the following 40 µl 3' ligation mix: 4 µl T4 RNA ligase buffer, 2 µl T4 RNA ligase 2 truncated KQ (NEB #M0373L), 1 µl SUPERase-In, 2 µl extended-L7 adapter (20 µM stock), 16 µl of 50% PEG-8000) and incubated at 25 °C for 2 hours. After incubation, the reaction buffer was removed by washing once with high salt buffer and twice with PNK wash buffer. To remove the exceeded adapter, the beads were incubated in 50 µl adapter digestion reaction (40 µl water, 5 µl 10X RNA ligase buffer, 1 µl RecJf (NEB #M0264S), 1 5' Deadenylase (NEB #M0331S), 1 µl SUPERase-In) at 30 °C for 15 minutes then at 37 °C for 15 minutes. The beads were washed once with PNK wash, once with high salt buffer, and twice with low salt buffer. To elute the DTB labeled RNA, beads were then suspended with 30 µl low salt wash buffer containing 1 mM free D-biotin (ThermoFisher #B20656) and incubate at room temperature for 1 hour. The DTB-labeled RNA was purified with ethanol-AMPure XP (RNA:beads:ethanol=1:2:3) and eluted in 30 µl water. To increase stringency, the streptavidin bead enrichment was repeated omitting enzymatic steps and instead washing three times with high salt and then three times with lower salt wash, and the eluate was cleaned again by ethanol-AMPure XP.

5' adapter addition. The DTB-GTP cap was removed using 0.5 U/µl RppH (NEB #M0356S) in 1X ThermoPol buffer (NEB #M0356S) and incubating at 37 °C for 1 hour. The resulting 5'-monophosphate RNA fragments were purified by ethanol-AMPure XP and eluted in 10 µl water. 1 µl (10 pmol) reverse transcription primer ([see Table S7](#)) was pre-annealed to the templates by heating up to 75 °C for 5 minutes, then 37 °C for 15 minutes, 25 °C for 15 minutes, and chilled at 4 °C. 10 pmol of a 5' adapter ([see Table S7](#)) was ligated for 3 hours at 25 °C with 2 U/µl T4 RNA ligase 1 (NEB #M0437M). This RNA adapter contains an 8 nt- or 11 nt-long UMI followed by a fixed

sequence (AUAU) at its 3' end. The UMI allows robust duplicate removal, and the fixed sequence provides an anchor point to correctly identify the first nucleotide of the mRNA. 40 µl ligation product was used.

cDNA synthesis and PCR. Reverse transcription was carried out at 50 °C for 45 minutes in a 50 µl reaction containing 0.5 mM dNTPs, 5 mM DTT, 20 U RNaseOUT, 50 mM Tris-HCl pH 8.3, 75 mM KCl, and 300 U SuperScript III. To perform indexing PCR, 40 µl Phusion master mix (NEB #M0532L) was added to the reverse transcription product, along with 5 µl i5 indexing primer and 5 µl i7 indexing primer (NEB #E7600S). Cycling conditions were typically 98 °C 2 minutes, then 16 cycles of 98 °C 15 seconds, 65 °C 30 seconds, 72 °C 30 seconds, with a final 5 minute 72 °C extension. Two rounds of 0.9X AMPureXP bead purifications were performed to remove primers. Normally ~10 ng indexed library was obtained for each library. The libraries were mixed and sequenced by NovaSeq 6000 or NovaSeqX.

CROWN-seq data processing

The read pairs were firstly quality trimmed by Cutadapt⁴⁸, -m 32 -q 20 -e 0.25 -a AGATCGGAAGAGCACACGTC. For the 8 nt-long 5' adapter, -A ATATNNNNNNNAGATCGGAAGAGCGTCGTG was used; for the 11 nt-long adapter, -A ATATNNNNNNNNNAGATCGGAAGAGCGTCGTG was used. Then the UMI along with the fixed ATAT spacer sequences were extracted by UMI-tools⁵¹. The alignment process was modified from the previous RNA bisulfite alignment strategy⁴⁷. In brief, *in silico* converted read pairs (read1 A-to-G, read2 T-to-C) were aligned by HISAT2⁴⁹ against A-to-G converted (for positive strand) and T-to-C converted (for negative strand) reference genome and transcriptome first (key options: -k 5 -fr -rna-strandness FR -no-temp-splicesite -no-mixed). Then the unique alignments were extracted and the *in silico* converted reads were inverse-transformed to the original format. Since two sequences after conversion can be easily confused, we require the best alignment results can be well distinguished from the secondary alignments. Here, the alignment scores (AS tag in Hisat2 alignments, higher is better) of the best alignments should be higher than -10. Meanwhile, the difference between the best alignments and secondary alignments should be larger than 9. For paired-end alignments, the alignment scores of read1 and read2 were summed. Only read1 was used in the 5' end analysis. Only read1 reads without 5' end softclips were used. Pileup was performed to obtain the read coverages of every 5' end in the transcriptome. Non-conversion rates of the transcription start nucleotides were calculated by A counts over A and G counts.

To annotate the TSNs mapped in CROWN-seq, we used the TSSs in Gencode v45 as the reference TSS positions. We first calculated the distance between the mapped TSNs and the annotated TSSs by BEDtools⁵³. We then tried to assign a TSN to a gene if there was an annotated TSS <100 nt away. Because there can be multiple annotations available, we used the following priority in selecting gene annotations: snRNA > snoRNA > protein-coding > lncRNA > others. We also annotated TSNs which come from RNA highly similar to snRNA, snoRNA, or their pseudogenes. To do so, we first built a BLASTn database containing all snRNA, snoRNA, and their pseudogene sequences from Gencode v45. We then performed BLASTn (BLAST 2.9.0+⁵⁶) on the A-TSNs along with the first 50 nt downstream sequences to examine the similarity to the known snRNA, snoRNA, and pseudogenes. The following parameter was used: -qcov_hsp_perc 50 -perc_identity 50 -word_size 10. Sequences with bitscore ≥50 were considered as snRNA/snoRNA-like. We also annotated uORF and IRES elements based on ORFdb⁵⁷ and IRES atlas⁵⁸, respectively.

The related pipeline and scripts are available at <https://github.com/jhfoxliu/CROWN-seq>.

RT-qPCR

1 µg total RNA was used as input. The RNA was then mixed with 1 µl Oligo dT(18) (100 pmoles) (ThermoFisher #SO131), and 1 µl dNTP in 14.5 µl total volume. The mix was incubated at 65 °C for 5 minutes, then on ice for >30 seconds. After the incubation, 4 µl 5X RT mix (Maxima H-buffer, ThermoFisher #EP0751), 0.5 µl RNaseOUT (ThermoFisher #10777019), and 1 µl Maxima H-RTase were added to the mix. Reverse transcription was performed at 25 °C for 10 minutes, then 50 °C for 30 minutes. After reverse transcription, 1 µl cDNA was used for qPCR. In addition to the cDNA input, the qPCR buffer contains 10 µl Power SYBR Green PCR Master Mix (ThermoFisher #368577), 0.5 µl forward primer, 0.5 µl reverse primer, and 8 µl water. qPCR was performed based on the standard quantification program in QuantStudio™ 5 System.

Gene ontology analysis

Gene ontology analyses were performed with R package ClusterProfiler⁵⁹. P-value cutoffs were set to 0.05 and q-value cutoffs were set to 0.1. “Cellular Components” and “Biological Process” terms were analyzed. Importantly, corresponding gene sets, rather than all genes, were used as the backgrounds in term enrichment computation. Since the output terms were normally redundant, terms were de-redundancy by the “simplify” function in R package GOSemSim⁶⁰ (cutoff=0.7, by=“p.adjust”, select_fun=min).

Motif analysis

To search for the potential motifs for elements related to transcription initiation, we used “re” package in Python to match specific motifs, which are indicated in the figure legends.

To search for the transcription factor binding sites, we used FIMO⁶¹ to scan for motifs in HOCOMOCO v11 core motifs database⁶².

RNA secondary prediction and minimal free energy calculation

ViennaRNA package (version 2.5.1) was used to perform RNA secondary structure prediction⁶³. The RNAfold Python API “RNA” was used in the analysis. The folding temperature was set to 37°C. The minimum free energy of the predicted structure was used.

Quantification and statistical analysis

Quantitative and statistical methods are described above and in figure legends according to their respective technologies and analytic approaches. Statistical analysis and visualization were mainly performed with Python (version 3.8.7). R (version 4.2.2) was used in differential gene expression analysis and Gene Ontology analysis.

Versions of key Python packages: numpy (1.23.5); pandas (1.5.2); scipy (1.9.3); matplotlib (3.6.2); seaborn (0.12.1); matplotlib-venn (0.11.9).

Versions of key R packages: DESeq2 (1.38.1); clusterProfiler (4.6.0); enrichplot (1.18.3); GOSemSim (2.24.0); org.Hs.eg.db (3.16.0).

All boxplots and violin plot summary statistics show the median and IQR of the underlying data. Statistical tests are described in the appropriate figure legends. Student’s t-test was applied for two sample non-paired comparisons. One-sided or two-sided testing was performed according to figure legends. If possible, we omitted significance “stars” from figures; p-values (or equivalent) are instead reported.

Data and code availability

All sequencing data can be accessed from NCBI Gene Expression Omnibus: [GSE188510](#) for ReCappable-seq; [GSE233655](#) for CROWN-seq data.

Because of the size limit of supplemental tables, several large tables (e.g., the table for all mRNA m⁶Am sites quantified in this study) were not directly included in the Supplemental Tables. These large supplemental tables have been uploaded to Zenodo: <https://zenodo.org/records/12760731> (DOI: [10.5281/zenodo.12760731](https://doi.org/10.5281/zenodo.12760731)).

All original code has been deposited on GitHub:

- ReCappable-seq analysis: <https://github.com/jhfoxliu/ReCappable-seq>
- GLORI analysis: https://github.com/jhfoxliu/GLORI_pipeline
- CROWN-seq analysis: <https://github.com/jhfoxliu/CROWN-seq>

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Materials availability

This study did not generate new unique reagents.

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Additional information

Author contributions

J.F.L., B.R.H., and S.R.J. designed the experiments. J.F.L., B.R.H., and L.N. performed experiments. J.F.L. and B.R.H. analyzed experimental data. J.F.L. and S.R.J. wrote the manuscript.

Additional files

Supplementary figures and notes.

Table S1. Comparison of m⁶Am mapping methods.

Table S2. The design of m⁶Am standards.

Table S3. mRNA m⁶Am stoichiometry in different cell lines.

Table S4. Gene Ontology enrichment of genes with relatively low m⁶Am sites.

Table S5. Comparing A-transcription-start nucleotide expression between wild-type and PCIF1 knockout cells. [↗](#)

Table S6. m^6Am stoichiometry of A-TSN in snRNA, snoRNA, and pseudogenes. [↗](#)

Table S7. Oligos used in this study. [↗](#)

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- 59. Yu G., Wang L.-G., Han Y., He Q.-Y 2012) **clusterProfiler: an R package for comparing biological themes among gene clusters** *Omics: a journal of integrative biology* **16**:284–287
- 60. Yu G., Li F., Qin Y., Bo X., Wu Y., Wang S 2010) **GOSemSim: an R package for measuring semantic similarity among GO terms and gene products** *Bioinformatics* **26**:976–978
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- 62. Kulakovskiy I.V., Vorontsov I.E., Yevshin I.S., Sharipov R.N., Fedorova A.D., Rumynskiy E.I., Medvedeva Y.A., Magana-Mora A., Bajic V.B., Papatsenko D.A 2018) **HOCOMOCO: towards a complete collection of transcription factor binding models for human and mouse via large-scale ChIP-Seq analysis** *Nucleic acids research* **46**:D252–D259
- 63. Lorenz R., Bernhart S.H., Höner zu Siederdissen C., Tafer H., Flamm C., Stadler P.F., Hofacker I.L. 2011) **ViennaRNA Package 2.0** *Algorithms for molecular biology* **6**:1–14

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Reviewer #1 (Public review):

Summary:

In this manuscript, Liu et al. present CROWN-seq, a technique that simultaneously identifies transcription-start nucleotides and quantifies N6,2'-O-dimethyladenosine (m6Am) stoichiometry. This method is derived from ReCappable-seq and GLORI, a chemical deamination approach that differentiates A and N6-methylated A. Using ReCappable-seq and CROWN-seq, the authors found that genes frequently utilize multiple transcription start sites, and isoforms beginning with an Am are almost always N6-methylated. These findings are consistently observed across nine cell lines. Unlike prior reports that associated m6Am with mRNA stability and expression, the authors suggest here that m6Am may increase transcription when combined with specific promoter sequences and initiation mechanisms. Additionally, they report intriguing insights on m6Am in snRNA and snoRNA and its regulation by FTO. Overall, the manuscript presents a strong body of work that will significantly advance m6Am research.

Strengths:

The technology development part of the work is exceptionally strong, with thoughtful controls and well-supported conclusions.

Weaknesses:

Given the high stoichiometry of m6Am, further association with upstream and downstream sequences (or promoter sequences) does not appear to yield strong signals. As such, transcription initiation regulation by m6Am, suggested by the current work, warrants further investigation.

<https://doi.org/10.7554/eLife.104139.2.sa3>

Reviewer #2 (Public review):

Summary:

In the manuscript "Decoding m6Am by simultaneous transcription-start mapping and methylation quantification" Liu and co-workers describe the development and application of CROWN-Seq, a new specialized library preparation and sequencing technique designed to detect the presence of cap-adjacent N6,2'-O-dimethyladenosine (m6Am) with single nucleotide resolution. Such a technique was a key need in the field since prior attempts to get accurate positional or quantitative measurements of m6Am positioning yielded starkly different results and failed to generate a consistent set of targets. As noted in the strengths section below the authors have developed a robust assay that moves the field forward.

Furthermore, their results show that most mRNAs whose transcription start nucleotide (TSN) is an 'A' are in fact m6Am (85%+ for most cell lines). They also show that snRNAs and snoRNAs have a substantially lower prevalence of m6Am TSNs.

Strengths:

Critically, the authors spent substantial time and effort to validate and benchmark the new technique with spike-in standards during development, cross-comparison with prior techniques, and validation of the technique's performance using a genetic PCIF1 knockout. Finally, they assayed nine different cell lines to cross-validate their results. The outcome of their work (a reliable and accurate method to catalog cap-adjacent m6Am) is a particularly notable achievement and is a needed advance for the field.

Weaknesses:

No major concerns were identified by this reviewer.

Mid-level Concerns: All previous concerns were addressed in the revised version

<https://doi.org/10.7554/eLife.104139.2.sa2>

Reviewer #3 (Public review):**Summary:**

m6Am is an abundant mRNA modification present on the TSN. Unlike the structurally similar and abundant internal mRNA modification m6A, m6Am's function has been controversial. One way to resolve controversies surrounding mRNA modification functions has been to develop new ways to better profile said mRNA modification. Here, Liu et al. developed a new method (based on GLORI-seq for m6A-sequencing), for antibody-independent sequencing of m6Am (CROWN-seq). Using appropriate spike-in controls and knockout cell lines, Liu et al. clearly demonstrated CROWN-seq's precision and quantitative accuracy for profiling transcriptome-wide m6Am. Subsequently, the authors used CROWN-seq to greatly expand the number of known m6Am sites in various cell lines and also determine m6Am stoichiometry to generally be high for most genes. CROWN-seq identified gene promoter motifs that correlate best with high stoichiometry m6Am sites, thereby identifying new determinants of m6Am stoichiometry. CROWN-seq also helped reveal that m6Am does not regulate mRNA stability or translation (as opposed to past reported functions). Rather, m6Am stoichiometry correlates well with transcription levels. Finally, Liu et al. reaffirmed that FTO mainly demethylates m6Am, not of mRNA but of snRNAs and snoRNAs.

Strengths:

This is a well-written manuscript that describes and validates a new m6Am-sequencing method: CROWN-seq as the first m6Am-sequencing method that can both quantify m6Am stoichiometry and profile m6Am at single-base resolution. These advantages facilitated Liu et al. to uncover new potential findings related to m6Am regulation and function. I am confident that CROWN-seq will likely be the gold standard for m6Am-sequencing henceforth.

Weaknesses:

Though the authors have uncovered a potentially new function for m6Am, they need to be clear that without identifying a mechanism, their data might only be demonstrating a correlation between the presence of m6Am and transcriptional regulation rather than causality.

<https://doi.org/10.7554/eLife.104139.2.sa1>

Author response:

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

Public Reviews:**Reviewer #1 (Public review):****Summary:**

In this manuscript, Liu et al. present CROWN-seq, a technique that simultaneously identifies transcription-start nucleotides and quantifies N6,2'-O-dimethyladenosine (m6Am) stoichiometry. This method is derived from ReCappable-seq and GLORI, a chemical deamination approach that differentiates A and N6-methylated A. Using ReCappable-seq and CROWN-seq, the authors found that genes frequently utilize multiple transcription start sites, and isoforms beginning with an Am are almost always N6-methylated. These findings are consistently observed across nine cell lines. Unlike prior reports that associated m6Am with mRNA stability and expression, the authors suggest here that m6Am may increase transcription when combined with specific promoter sequences and initiation mechanisms. Additionally, they report intriguing insights on m6Am in snRNA and snoRNA and its regulation by FTO. Overall, the manuscript presents a strong body of work that will significantly advance m6Am research.

Strengths:

The technology development part of the work is exceptionally strong, with thoughtful controls and well-supported conclusions.

We appreciate the reviewer for the very positive assessment of the study. We have addressed the concerns below.

Weaknesses:

Given the high stoichiometry of m6Am, further association with upstream and downstream sequences (or promoter sequences) does not appear to yield strong signals. As such, transcription initiation regulation by m6Am, suggested by the current work, warrants further investigation.

We thank the reviewer for the insightful comments. We have softened the language related to m⁶Am and transcription regulation. We totally agree with the reviewer that future investigation is required to determine the molecular mechanism behind m⁶Am and transcription regulation.

Reviewer #2 (Public review):

Summary:

In the manuscript "Decoding m6Am by simultaneous transcription-start mapping and methylation quantification" Liu and co-workers describe the development and application of CROWN-Seq, a new specialized library preparation and sequencing technique designed to detect the presence of cap-adjacent N6,2'-O-dimethyladenosine (m6Am) with single nucleotide resolution. Such a technique was a key need in the field since prior attempts to get accurate positional or quantitative measurements of m6Am positioning yielded starkly different results and failed to generate a consistent set of targets. As noted in the strengths section below the authors have developed a robust assay that moves the field forward.

Furthermore, their results show that most mRNAs whose transcription start nucleotide (TSN) is an 'A' are in fact m6Am (85%+ for most cell lines). They also show that snRNAs and snoRNAs have a substantially lower prevalence of m6Am TSNs.

Strengths:

Critically, the authors spent substantial time and effort to validate and benchmark the new technique with spike-in standards during development, cross-comparison with prior

techniques, and validation of the technique's performance using a genetic PCIF1 knockout. Finally, they assayed nine different cell lines to cross-validate their results. The outcome of their work (a reliable and accurate method to catalog cap-adjacent m6Am) is a particularly notable achievement and is a needed advance for the field.

Weaknesses:

No major concerns were identified by this reviewer.

We thank the reviewer for the positive assessment of the method and dataset. We have addressed the concerns below.

Mid-level Concerns:

(1) In Lines 625 and 626, the authors state that “our data suggest that mRNAs initiate (mis-spelled by authors) with either Gm, Cm, Um, or m6Am.” This reviewer took those words to mean that for A-initiated mRNAs, m6Am was the ‘default’ TSN. This contradicts their later premise that promoter sequences play a role in whether m6Am is deposited.

We thank the reviewer for the comment. We have changed this sentence into “Instead, our data suggest that mRNAs initiate with either Gm, Cm, Um, or Am, where Am are mostly m⁶Am modified.” The revised sentence separates the processes of transcription initiation and m⁶Am deposition, which will not confuse the reader.

(2) Further, the following paragraph (lines 633-641) uses fairly definitive language that is unsupported by their data. For example in lines 637 and 638 they state “We found that these differences are often due to the specific TSS motif.” Simply, using ‘due to’ implies a causative relationship between the promoter sequences and m6Am has been demonstrated. The authors do not show causation, rather they demonstrate a correlation between the promoter sequences and an m6Am TSN. Finally, despite claiming a causal relationship, the authors do not put forth any conceptual framework or possible mechanism to explain the link between the promoter sequences and transcripts initiating with an m6Am.

(3) The authors need to soften the language concerning these data and their interpretation to reflect the correlative nature of the data presented to link m6Am and transcription initiation.

For (2) and (3). We have softened the language in the revised manuscript. Specifically, for lines 633-641 in the original manuscript, we have changed “are often due to” into “are often related to” in the revised manuscript, which claims a correlation rather than a causation.

Reviewer #3 (Public review):

Summary:

m6Am is an abundant mRNA modification present on the TSN. Unlike the structurally similar and abundant internal mRNA modification m6A, m6Am's function has been controversial. One way to resolve controversies surrounding mRNA modification functions has been to develop new ways to better profile said mRNA modification. Here, Liu et al. developed a new method (based on GLORI-seq for m6A-sequencing), for antibody-independent sequencing of m6Am (CROWN-seq). Using appropriate spike-in controls and knockout cell lines, Liu et al. clearly demonstrated CROWN-seq's precision and quantitative accuracy for profiling transcriptome-wide m6Am. Subsequently, the authors used CROWN-seq to greatly expand the number of known m6Am sites in various cell lines and also determine m6Am stoichiometry to generally be high for most genes.

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This is a well-written manuscript that describes and validates a new m6Am-sequencing method: CROWN-seq as the first m6Am-sequencing method that can both quantify m6Am stoichiometry and profile m6Am at single-base resolution. These advantages facilitated Liu et al. to uncover new potential findings related to m6Am regulation and function. I am confident that CROWN-seq will likely be the gold standard for m6Am-sequencing henceforth.

Weaknesses:

Though the authors have uncovered a potentially new function for m6Am, they need to be clear that without identifying a mechanism, their data might only be demonstrating a correlation between the presence of m6Am and transcriptional regulation rather than causality.

We thank the reviewer for the very positive assessment of the CROWN-seq method. We have softened the language which is related to the correlation between m⁶Am and transcription regulation.

Reviewer recommendations:

We thank the reviewers for their constructive suggestions. In the revised manuscript, we have corrected the errors and updated the requested discussions and figures.

Reviewer #1 (Recommendations for the authors):

(1) The prior work from the research group, "Reversible methylation of m6Am in the 5' cap controls mRNA stability" (PMID: 28002401), should be cited, even if the current findings differ from earlier conclusions-particularly in line 58 and the section titled "m6Am does not substantially influence mRNA stability or translation".

We thank the reviewer for this comment. We have added the citation.

(2) I wonder why the authors chose to convert A to I before capping and recapping, as RNA fragmentation caused by chemical treatment may introduce noise into these processes.

We thank the reviewer for this comment. This is a very good point. We have indeed considered this alternative protocol. There are two concerns in performing decapping-and-recapping before A-to-I conversion: (1) it is unclear whether the 3'-desthiobiotin, which is essential for the 5' end enrichment, is stable or not during the harsh A-to-I conversion; (2) performing decapping-and-recapping first requires more enzyme and 3'-desthiobiotin-GTP, which are the major cost of the library preparation. This is because the input of CROWN-seq (~1 µg mRNA) is much higher than that in ReCappable-seq (~5 µg total RNA or ~250 ng mRNA). In the current protocol, many 5' ends are highly fragmented and therefore are lost during the A-to-I conversion. As a result, less enzyme and 3'-desthiobiotin-GTP are needed.

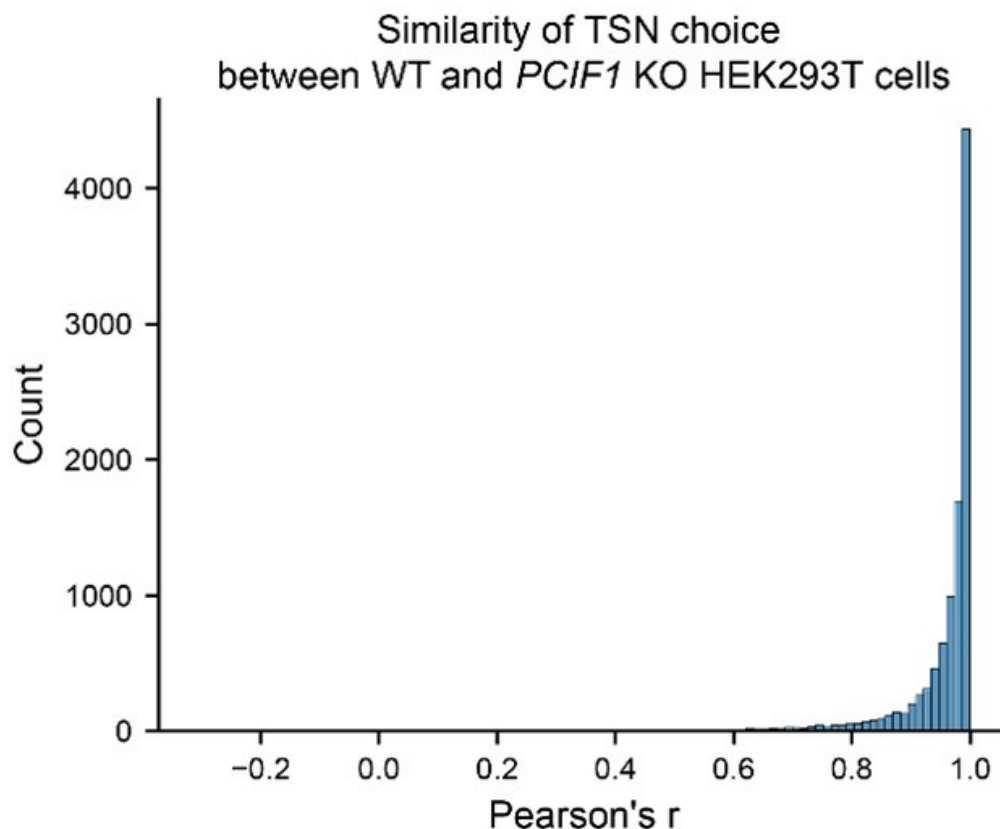
(3) During CROWN-seq benchmarking, the authors found that 93% of reads mapped to transcription start sites, implying a 7% noise level with a spike-in probe. This noise could lead to false positives in TSN assignments in real samples. It appears that additional filters (e.g., a known TSS within 100 nt) were applied to mitigate false positives. If so, I recommend that the authors clarify these filters in the main text.

We thank the reviewer for this comment. We think that the spike-in probes might lead to an underestimation of the accuracy of TSN mapping. The spike-in probes are made by in vitro transcription with m⁷Gpppm⁶AmG or m⁷GpppAmG analogs. We found that the in vitro transcription exhibits a small amount of non-specific initiation, which leads to spike-in probes with 5' ends that are not precisely aligned with the desired TSS. To better illustrate the mapping accuracy of CROWN-seq, we provided Figure 2H, which compares the non-conversion rates of newly found A-TSNs between wild-type and *PCIF1* knock cells. If the newly found A-TSNs are real, they should show high non-conversion rates in wild-type cells (i.e., high m⁶Am) and almost zero non-conversion rates (i.e., Am) in *PCIF1* knockout cells. As expected, most of the newly found A-TSNs are true A-TSNs since they are m⁶Am in wild-type and Am in *PCIF1* knockout. Thus, we think that CROWN-seq is very precise in TSS mapping. We have clarified this in the Discussion.

(4) I wonder if *PCIF1* knockout affects TSN choice and abundance. If not, this data should be presented. If so, how are these changes accounted for in Figure 2H and Figure S5?

We thank the reviewer for this comment. *PCIF1* KO does not really affect TSN choice. Here we calculate the correlation of relative TSN expression within genes between wild-type and *PCIF1* KO cells (shown using Pearson's r). It shows that most of the genes have similar TSN choices (with higher Pearson's r) in both wild-type and *PCIF1* KO cells. Thus, *PCIF1* KO does not alter global TSN expressions.

Author response image 1.



(5) The manuscript refers to Am as a rare modification in mRNA (e.g., introduction lines 101-102; discussion lines 574, 608; and possibly other locations) without specifying this only applies to transcription start sites. As this study does not cover entire mRNA sequences, these statements may not be misleading.

We thank the reviewer for this comment. We have clarified it.

Reviewer #2 (Recommendations for the authors):

(1) On line 122, the authors state that: "On average, a gene uses 9.5{plus minus}9 (mean and s.d., hereafter) TSNs (Figure 1A)." However, they do not discuss the dispersion apparent in the TSNs they observed. Figure panels 1A, B, and S1A, B show a range of 120 bases or less. What is the predominant range of distances between annotated TSNs and the newly identified ones?

1a) For example, what percentage of new TSNs fall within 20? 50? 75? bases of the annotated sites? Additional text describing the distribution of these TSNs would help readers better understand the diversity inherent in these novel 5' RNA ends. Notably, this additional text likely is best placed in the CROWN-Seq section related to Figure 2 or S2.

We thank the reviewer for this comment. We have updated Figure S2 to describe the newly found TSSs. Depending on the coverage in CROWN-seq, the TSSs with higher coverage tend to overlap with or locate proximally to known TSSs. In contrast, the TSSs with low coverage tend to be located further away from annotated TSSs.

1b) The alternate TSNs can have effects on splicing patterns and isoform identity. Providing a few sentences to explain how regularly this occurs would be helpful.

We thank the reviewer for this comment. It is a very interesting point. Different TSNs can indeed have different splicing patterns. Although the discovery of splicing patterns regulated by TSNs is out of the scope of this study, we have discussed this possibility in the revised Discussion section.

(2) On Lines 241 and 242, the authors mentioned that 1284 sites were excluded from the analysis based on low (under 20-explained in the figure legend) read count, distance from TSS, or false negatives (which are not explained). Although I agree that the authors are justified in setting these reads aside, the information could be useful to readers willing to perform follow-up work if their mRNAs of interest were included in these 1284 sites.

2a) An annotation of all of these sites (broken down by category, i.e. the 811, the 343, and the 130) as a supplementary table should be provided.

We thank the reviewer for this comment. We have added the categories to the revised Table S1.

(3) Although I have marked several typos/grammar mistakes in several parts of this review, others exist elsewhere in the text and should be corrected.

We thank the reviewer for this comment. We have corrected them.

(4) In lines 122 and 123 the authors say "Only ~9% of genes contain a single TSN (Figure 1A)." However, their figure shows 81% with a single TSN. Why is there a 10% discrepancy?

We thank the reviewer for this comment. We have corrected the plot in Figure 1A, to match the description.

(5) The first Tab of Table S2 is labeled 'Legend', but is blank. Is this intentional?

We thank the reviewer for this comment. We have updated the table legends.

(6) On lines 70 and 76 of the supplementary figure file pertaining to Figure S2, the legend labels for Figure S2E and S2F are not accurate, they need to be changed to G and H.

(7) In Figure 4A 'percentile' is misspelled.

(8) The color-coding legend for the 4 bases is missing from (and should be added to) Figure S4A.

(9) On Lines 984, 1163, and 1194 the '2s' should be properly sub-scripted where appropriate.

For (6) to (9). We thank the reviewer for finding these issues. We have now corrected them.

Reviewer #3 (Recommendations for the authors):

(1) The authors should discuss if their results can definitively distinguish between the SSCA+1GC motif promoting m6Am that, in turn, promotes transcription, versus the SCA+1GC motif promoting m6Am but also separately promoting transcription in a

m6Am-independent manner. The authors should also discuss this in light of recent findings by An et al. (2024 Mol. Cell), which support the former conclusion.

We thank the reviewer for the suggestion. We now have updated the Discussion to address that our paper and An et al. can support each other.

(2) Given that the authors showed m6Am promotes gene expression (Figure 5) but does not affect mRNA stability (Fig. S5), logic dictates that m6Am must regulate mRNA transcription. However, the authors should explain why this regulation focuses on the initiation aspect of transcription rather than other aspects of transcriptional e.g. premature termination, pause release, and elongation.

We thank the reviewer for this comment. In this study, we did not profile the 3' ends of nascent RNAs and thus we can only make conclusions about the overall transcription process but not a specific aspect. We have updated the revised Discussion section to mention that An et al. discovered that m⁶Am can sequester PCF11 and thus promote transcription, and therefore some of the effects we see could be related to differential premature termination.

(3) Authors should add alternative versions of Figure 1D but with 3 colours corresponding to Am vs. m6Am vs. Cm/Gm/Um for all the cells, they performed CROWN-seq on.

We thank the reviewer for this comment. We have updated Figure S5 as the corresponding figure showing the fraction of Am vs. m6Am vs. Cm/Gm/Um.

(4) Figure 2H (left): Please comment on the few outliers that still show high non-conversion even in PCIF1-KO cells.

We thank the reviewer for this comment. We have discussed the outliers in the main text. These outliers can be found in the revised Table S3.

(5) Line 254: "Second, if these sites were RNA fragments they would not contain m6Am." is missing a comma.

(6) S2G and S2H labelling in Figure S2 legends is wrong.

For (5) and (6). We thank the reviewer for these comments. We have corrected them.

(7) Figure 3D: Many gene names are printed multiple times (e.g. ACTB is printed 5 times). Is this correct; is each dot representing 1 cell line?

We thank the reviewer for this comment. These gene names represent different transcription-start nucleotides. We now clarify that each instance refers to a different start site.

(8) S5A-C: Even if there's no substantial difference, authors should still display the Student's T-test P-values as they did for S5D-G.

We thank the reviewer for this comment. We have updated the P-values.

(9) Figure 5C and S5E: Why are the authors not showing the respective analysis for C-TSN and U-TSN genes?

We thank the reviewer for this comment. Most mRNAs start with A or G. We therefore selected G-TSN as the control. Unlike G-TSNs which occur in diverse sequence and promoter contexts, C-TSNs and U-TSNs are unusual. Genes that mainly use C-TSNs and U-TSNs are the so-called “5' TOP (Terminal OligoPyrimidine)” genes. The 5' TOP genes are mostly genes related to translation and metabolism, and thus their expressions reflect the homeostasis of cell metabolism. Thus, we were concerned that any differential expression of the C-TSN and U-TSN genes between wild-type and PCIF1 knockout cells might reflect specific effects on TOP transcriptional regulation rather than the general effects of PCIF1 on transcription.

(10) Line 82, 470, 506, 676: *The authors should also cite Koh et al (2019 Nat. Comm.) in these lines that describe how snRNAs can also be m6Am-methylated and how FTO targets these same snRNAs for demethylation.*

We thank the reviewer for this comment. We have updated the citation.

<https://doi.org/10.7554/eLife.104139.2.sa0>