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Transcriptome-wide profiling of N^6 -methyladenosine via a selective chemical labeling method†

Yalun Xie,^{‡a} Shaoqing Han,^{‡a} Qiming Li,^b Zhentian Fang,^a Wei Yang,^a Qi Wei,^a Yafen Wang,^a Yu Zhou,^b Xiaocheng Weng^{‡a} and Xiang Zhou^{‡*a}

Studies of chemical modifications on RNA have ushered in the field of epitranscriptomics. N^6 -Methyladenosine (m^6A) is the most typical RNA modification and is indispensable for basic biological processes. This study presents a chemical pulldown method (m^6A -ORL-Seq) for transcriptome-wide profiling of m^6A . Moreover, chemical labeling results in a specific reverse transcription (RT) truncation signature. This study has identified four thousand high-confidence m^6A sites at single-base resolution in the human transcriptome. Unlike previously reported methods based on m^6A -antibody or m^6A -sensitive enzymes, the antibody/enzyme-free chemical method provides a new perspective for single-base m^6A detection at the transcriptome level.

Introduction

More than 163 chemical modifications have been identified in RNA, these modifications bearing varied chemical groups regulate gene expressions in multiple ways.¹ Among them, N^6 -methyladenosine (m^6A) is the most abundant post-transcriptional modification in messenger RNA (mRNA) and noncoding RNA (ncRNA) in eukaryotes.² The discovery of m^6A -associated proteins (m^6A writers, readers, and erasers) has revealed that m^6A is a crucial regulator in mRNA splicing, export, translation, decay, and structure.^{3–11} Furthermore, aberrant levels of m^6A have been closely associated with tumor development.^{12,13} To interpret further the biological functions of m^6A , detection and precise location of m^6A in transcriptome is essential.

Combined with m^6A specific antibodies and RNA-Seq, two independent works (m^6A -Seq or MeRIP-Seq) reported the map of m^6A methylomes among mammals and determined a highly conserved m^6A motif (DRACH, D = G/A/U; R = G/A; H = A/C/U) that was enriched in 3' UTRs and near stop codons.^{14,15} However, this most used approach has several fated drawbacks, including low resolution (>200 nt), binding to particular RNA sequences, large amounts of starting samples (>100 μ g total RNA), and abundant false positives (limited specificity). Therefore,

antibody-dependent or antibody-free enzyme-dependent methods have been subsequently developed. Crosslinking immunoprecipitation-based methods (PA-Seq,¹⁶ m^6A -CLIP,¹⁷ and miCLIP¹⁸) improve resolution. The antibody-free enzymatic approaches (MAZTER-Seq,¹⁹ m^6A -REF-Seq,²⁰ DART-Seq,²¹ m^6A -label-Seq,²² m^6A -SEAL-Seq²³ and m^6A -SAC-Seq²⁴) have also been developed for higher resolution and quantitative profiling. Meanwhile, third-generation sequencing techniques including single-molecule real-time (SMRT) sequencing²⁵ and nanopore sequencing,^{26,27} enable long-read and single-molecule sequencing of m^6A . However, the sequencing data derived from enzymatic approaches also contains abundant false positives resulting from sequence bias or RNA structure. MAZTER-Seq and m^6A -REF-Seq use an m^6A -sensitive RNA endoribonuclease (MazF) to facilitate m^6A sequencing at single-base resolution. On the other hand, Luo *et al.*²⁸ confirmed that a CCACAG motif and RNA secondary structure affects MazF cleavage, leading to widespread false positives. Although third-generation sequencing enables the direct detection of RNA modification, the high error probability limits its application to practical samples.²⁹ Hence, an antibody/enzyme-free and selective chemical labeling method is urgently necessary to solve these problems.

Due to the inert reactivity of methyl group in m^6A , chemical discrimination of m^6A and A is a great challenge for chemist. To date, no antibody/enzyme-free chemical approach was developed for selective and efficient labeling of m^6A in RNA. It is worth mentioning that Gaunt *et al.*³⁰ recently reported a method based on metallophotocatalyst-assisted chemistry can label N^6 -methyladenosine (6mdA) in DNA. However, when this method was applied to the labeling of a 13 nt RNA oligo containing m^6A , the yield was as low as 5% together with considerable RNA degradation, thus this method is difficult to be used for the

^aCollege of Chemistry and Molecular Sciences, Key Laboratory of Biomedical Polymers of Ministry of Education, Wuhan University, Wuhan, China. E-mail: xzhou@whu.edu.cn

^bCollege of Life Science, Wuhan University, Wuhan, Hubei, China

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‡ These authors contributed equally to this work.



study of m⁶A transcriptomics at the current stage. In this article, we report an unprecedented chemical method for selective labeling and transcriptome-wide profiling of m⁶A *via* three steps of chemical reactions, oxidation, reduction, functional labeling (m⁶A-ORL-Seq, Fig. 1).

Coincidentally, Hili *et al.*³¹ and Helm *et al.*³² reported sodium nitrite-based chemical methods for m⁶A detection. However, these two methods are not applicable to transcriptome-wide studies at the current stage and are also powerless for locating m⁶A sites of low modification level unless the combination with m⁶A immunoprecipitation. The detailed discussion compared to the method in this study is stated in ESI.† Meanwhile, Wu *et al.*³³ reported sodium nitrite-based chemical methods for genomic 6 mA profiling.

Experimental

Synthesis of RNA oligo and model containing m⁶A

The RNA phosphoramidites were purchased from Wuhu Hua- ren Science and Technology Co., Ltd. 12/15/60 nt m⁶A-modified RNA was synthesized on 12-column DNA synthesizer (PolyGen). Universal CPG (39.5 μmol g⁻¹, 1000 Å, Beijing DNACHEM Biotechnology Co. Ltd.) bound RNA was firstly treated with a mixture of 28% aqueous ammonia and 40% aqueous methylamine (200 μL, 1 : 1), then concentrated *in vacuo* and incubated with a mixture of DMSO and Et₃N·3HF (200 μL, 1 : 1) at 60 °C for 2.5 h. At last RNA oligonucleotides were purified using ethanol precipitation.

N-Nitrosation (oxidation) of m⁶A to Nm⁶A, deamination of A/ G/C to I/X/U in ribonucleosides

A 1.5 mL tube was mixed with 50 μM C, G, A, and m⁶A in 200 μL sodium acetate buffer (1.0 M, pH 4.5). In an ice bath, 3.00 scem

NO bubbling into the bottom of the tube (lid is open) for 3.0 min through a 22 gauge needle (this treatment must be handled in a standard laboratory chemical hood). Then cover the lid, the solution was standing at 4 °C for 4–30 h. After incubation, the solution was directly injected into HPLC and analyzed. A: 3% CH₃CN in TEAA (100 mM, pH 7.5); B: 90% CH₃CN in H₂O; 35 °C, 1.0 mL min⁻¹, 260 nm, 0–10–15–25 min, 0–0–5–100% B.

N-Nitrosation (oxidation) of m⁶A to Nm⁶A in oligo/model RNA/total RNA/poly (A)⁺ RNA

A 1.5 mL tube was mixed with RNA in 200 μL sodium acetate buffer (3.0 M, pH 4.5, containing 2 mM EDTA). In an ice bath, 3.00 scem NO bubbling into the bottom of the tube (lid is open) for 6.0 min through a 22 gauge needle (this treatment must be handled in a standard laboratory chemical hood). Then cover the lid, the solution was standing at 4 °C for 20 h. Then RNA was purified using ethanol precipitation or RNA Clean & Concentrator-5 (RCC, Zymo).

TDO reduction of Nm⁶A to Am⁶A

For ribonucleosides: 100 μM Nm⁶A, 100 mM TDO, 300 mM Na₂CO₃/NaHCO₃ (pH 10.0), 50 °C, 1000 rpm, 5 min. For RNA oligo, 5.0 μg oligo, 100 mM TDO, 300 mM Na₂CO₃/NaHCO₃, 2 mM EDTA, pH 10.0, 50 °C, 1000 rpm, 10 min, total volume 50 μL. For total RNA and poly (A)⁺ RNA, 100 mM TDO, 300 mM Na₂CO₃/NaHCO₃, 2 mM EDTA, pH 10.0, 50 °C, 1000 rpm, 10 min, total volume 30 μL.

Biotinylation *via* probe BA labeling, Am⁶A to Bm⁶A in RNA

Oligo RNA/model RNA/total RNA/poly (A)⁺ RNA, 2.0 mM BA, 100 mM MES, 10% DMF, pH 5.5, 37 °C, 1000 rpm, 1.0 h, total volume 50 μL.

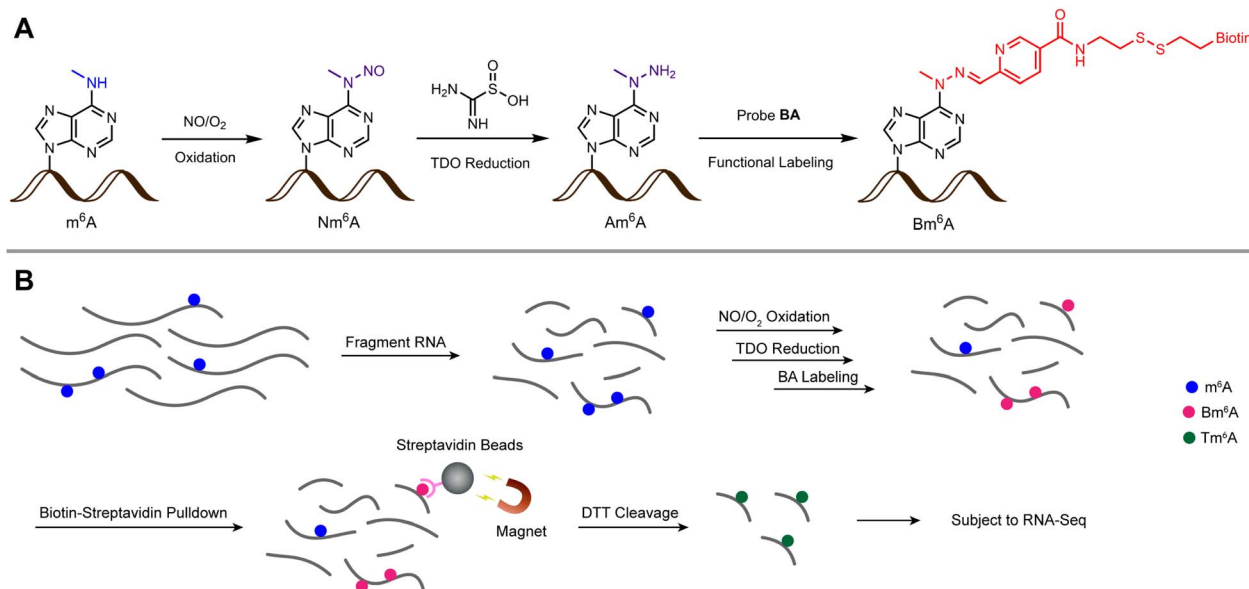


Fig. 1 A chemical method to selective label and locate m⁶A in transcriptomes. (A) RNA m⁶A was stepwise converted into Nm⁶A, Am⁶A, and Bm⁶A through a three-step chemical labeling method. (B) A schematic illustration of m⁶A-ORL-Seq for transcriptome-wide profiling of m⁶A. Nm⁶A: N⁶-nitroso m⁶A; Am⁶A: N⁶-amino m⁶A; Bm⁶A: biotinylated m⁶A; Tm⁶A: thiolated m⁶A; TDO: thiourea dioxide; BA: biotinylated aryl aldehyde probe.



Enzymatic digestion and LC-HRMS analysis

500 ng RNA oligonucleotide was digested with 1 U of nuclease P1 (Sigma-Aldrich) in digestion buffer (2 mM ZnCl₂, 10 mM NaCl, 25 μL) at 37 °C for 2 h, followed by incubating with 1 U rSAP (NEB) in freshly prepared 100 mM NH₄HCO₃ at 37 °C for 2 h. The digested samples were centrifuged at 12 000 rpm for 10 min, and 20 μL of the solution was loaded into LC-HRMS (Thermo Fisher Scientific LTQ Orbitrap Elite) analysis.

Human cell culture

HEK-293T cells were cultured in DMEM medium without sodium pyruvate (Dulbecco's Modified Eagle Medium, HyClone) supplemented with 10% FBS (Cegrogen) and 1% penicillin/streptomycin (Genview). The cells were maintained at 37 °C under a humidified atmosphere containing 5% CO₂.

Isolation of total RNA and poly (A)⁺ RNA from cells

Total RNA was isolated from HEK-293T with TRIzol (Invitrogen) according to the manufacturer's protocol. Then genomic DNA was removed by TURBO DNase (Invitrogen). Poly (A)⁺ RNA was isolated by subjecting total RNA to oligo (dT) enrichment using oligo d(T)₂₅ Magnetic Beads (NEB) according to the manufacturer's protocol.

RNA fragmentation

Total RNA or poly (A)⁺ RNA was fragmented using RNA fragmentation reagents (Thermo) at 70 °C. For 60–200 nt segments (m⁶A-ORL-Seq), using 15.0 min as incubating time; for 100–300 nt segments (MeRIP-Seq), using 8.0 min as incubating time.

Dot blot assay

RNA samples were pipetted and dotted on a tailored Amersham Hybond-N+ membrane (GE Healthcare). After drying naturally, the samples were fixed on the membrane by UV light (254 nm) cross-linking at room temperature and washed with 1× TBST (0.1% Tween-20) three times. Next, the membrane was blocked with 5% BSA at 70 rpm, 37 °C for 2 h and washed with 1× TBST five times. After incubation with streptavidin-horseradish peroxidase (1 : 1500) (Thermo Scientific) at 70 rpm, 37 °C for 1 h and washing with 1× TBST four times, the biotinylated samples in the membrane were visualized by enhanced chemiluminescence (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific) using a ChemiDoc XRS+ Imaging System (Bio-Rad). At last, the membrane was washed twice with 1× TBST and stained with methylene blue.

Protocol for m⁶A-ORL-Seq

5.0 μg model RNA (MALAT1-2577-m⁶A)/30.0 μg total RNA/5.0 μg poly (A)⁺ RNA was stepwise oxidized (*N*-nitrosation), TDO reduced and BA labeled. After each chemical treatment, RNA was purified using RCC. Purified biotinylated RNA (500 ng purified biotinylated model RNA), mixing with 50 μg Yeast tRNA (Thermo) and subjected to pulldown procedure. Non-treated RNA or biotinylated RNA was used as input. 25 μL Dynabeads

MyOne Streptavidin C1 (Thermo) was washed twice by 200 μL solution A (100 mM NaOH, 50 mM NaCl) to remove RNase contamination, then washed twice with RNase-free (RNF) H₂O. The beads were resuspended in 100 μL binding solution containing 10 μL of high-salt wash buffer (100 mM Tris, 10 mM EDTA, 1.0 M NaCl, pH 7.5, 0.05% Tween 20) and 90 μL RNF H₂O, and incubated with the biotinylated RNA for 1 h. The beads with biotinylated RNA were washed three times with 1.0 mL high salt wash buffer. Enriched RNA was cleaved from beads using 50 μL of 100 mM DTT at 37 °C, 1000 rpm for 15 min. After collecting the supernatant, the second 50 μL of 100 mM DTT cleavage was performed at 50 °C for 5 min. The twice-eluted RNA was combined and purified using RCC, subsequently quantified by Qubit 4. 50 ng RNA was used for the next library construction (detailed in part 10). Libraries were sequenced on the Illumina HiSeq X-Ten platform with a paired-end model (PE150).

MeRIP-seq

MeRIP-Seq (10.0 μg poly (A)⁺ RNA) was performed using Epi-Mark® N⁶-Methyladenosine Enrichment Kit (NEB) according to the manufacturer's protocol.

Data preprocessing

The human hg38 genome and list of human transcripts v31 were downloaded from Gencode (<https://www.gencodegenes.org/>). Note that we include a barcode of random decamer (NNNNNNNNNN) ligated to the fragments during library construction. Raw FASTQ reads were trimmed to remove adaptor contamination, random hexamer and aligned to the reference genome using cutadapt (v1.18)³⁴ and STAR 2.7.5a,³⁵ respectively. Reads less than 30 in length were removed, and only the proper pair and uniquely mapped alignments were persisted for the downstream pipelines. Then aligned reads were used for peak calling by exomePeak. Overlapped peaks between different samples were found by the BEDtools intersect function. MetaPlotR package³⁶ was used for creating metagene plots. HOMER was used to detect the sequence motif.

Candidate m⁶A sites at base resolution were determined according to the following steps: (i) scanned the motif DRACH in the peaks, counting the number of DRACH at the head or terminal of a sequence read. (ii) Each DRACH motif on the reference genome must be no less than 5 truncated reads. (iii) The stop rate at A must be greater than 0.1.

SELECT validation³⁷

The FTO demethylation (FTO+) was performed in a 50 μL scale reaction containing 1.0 μg total RNA or poly (A)⁺ RNA, purified FTO protein, 80 μM (NH₄)₂Fe(SO₄)₂, 2 mM L-ascorbic acid, 300 μM 2-ketoglutarate, 2 U/μL RNase inhibitor, 50 μg mL⁻¹ BSA, 50 mM HEPES, pH 7.0. For the control reaction (FTO-EDTA), EDTA was added to 5 mM before the addition of the enzyme. The reactions were incubated at 37 °C for 1 h and subsequently quenched by EDTA (0.5 M, 2.0 μL). Then RNA was purified using RCC for next use.



100 ng RNA sample (FTO+, FTO-EDTA) were added with Up primer (800 nM, 1.0 μ L), down primer (800 nM, 1.0 μ L) and dTTP (100 μ M, 1.0 μ L) in 17 μ L 1 \times CutSmart buffer (NEB). The RNA and primers were annealed by incubating mixture at a temperature gradient: 90 $^{\circ}$ C for 1 min, 80 $^{\circ}$ C for 1 min, 70 $^{\circ}$ C for 1 min, 60 $^{\circ}$ C for 1 min, 50 $^{\circ}$ C for 1 min, and then 40 $^{\circ}$ C for 6 min. Subsequently, adding 0.01 U Bst 2.0 DNA polymerase (NEB), 0.5 U SplintR ligase (NEB), and 10 nmol ATP to the former mixture to the final volume of 20 μ L. The reaction mixture was incubated at 40 $^{\circ}$ C for 20 min, denatured at 80 $^{\circ}$ C for 20 min and kept at 4 $^{\circ}$ C. Then 20 μ L qPCR reaction consisted of 2 \times Hieff qPCR SYBR Green Master Mix (Yeasen) 200 nM qPCR primer, 200 nM qPCR primer and 2.0 μ L reaction mixture was performed on the following parameters: 95 $^{\circ}$ C, 5 min; (95 $^{\circ}$ C, 10 s; 60 $^{\circ}$ C, 35 s; data collection) \times 40 cycles; 4 $^{\circ}$ C, hold.

Reverse transcription (RT) reactions for ODN-X

ODN-X (2 μ L, 4 μ M) was mixed with 1 μ L of 2 μ M 12 nt RT primer 5'-HEX-AGAATCATCGAA and denatured at 70 $^{\circ}$ C for 5 min, then cooled down slowly to 4 $^{\circ}$ C (1 $^{\circ}$ C per 10 s) and hold at 4 $^{\circ}$ C. After adding 5 U M-MuLV reverse transcriptase (NEB) or 5 U SS III reverse transcriptase (Invitrogen), 2 μ L corresponding 5 \times buffer (NEB M-MuLV buffer or 5 \times FS buffer), 3 μ L of 100 μ M dNTPs and to the RNA-primer mix (For ODN-Tm⁶A, additional 1 μ L of 100 mM DTT was added), the RT reactions were carried out at 42 $^{\circ}$ C for 30 min. Then 30 μ L deionized formamide was immediately added to the reaction mixture and heated to 90 $^{\circ}$ C for 10 min, followed by 20% denaturing PAGE analysis.

Results

Chemical labeling at the nucleoside level

Inspired by the design of *N*-nitrosation reactivity-based fluorescent probes,^{38,39} we noticed that m⁶A could transform into *N*-nitrosamine (Nm⁶A) via *N*-nitrosation. After attempts of several nitrosating reagents,^{40–42} the most direct reagent, nitric oxide (NO) aqueous solution, was eventually chosen to facilitate the *N*-nitrosation of m⁶A at a mild condition (4 $^{\circ}$ C, sodium acetate buffer, pH 4.5). While NO can also have the deaminating ability of other bases under aerobic conditions, which converts adenosine (A) into inosine (I), guanosine (G) into xanthosine (X), and cytosine (C) into uridine (U).^{43,44} Under optimized conditions, the conversion rate of m⁶A to Nm⁶A is 88%, A to I is 31%, G to X is 56%, C to U is 9.8% (Fig. 2A). Other abundant modifications in human transcriptome,⁴⁵ pseudouridine (Ψ), 5-methylcytidine (m⁵C), *N*¹-methyladenosine (m¹A), *N*⁴-acetylcytidine (ac⁴C), and *N*⁷-methylguanosine (m⁷G) were also tested in the same manner (ESI Fig. S30[†]); m⁵C was similarly converted into m⁵U, and m⁷G was similarly converted into m⁷X. Next, a green reductant, thiourea dioxide (TDO),⁴⁶ was screened out to reduce Nm⁶A into *N*⁶-amino m⁶A (Am⁶A, HPLC yield: 60%, Fig. 2B, ESI Fig. S11[†]) and m⁶A in a Na₂CO₃/NaHCO₃ buffered solution (300 mM, pH = 10.0) at 50 $^{\circ}$ C. Then, 2-pyridinecarboxaldehyde was used to test the reactivity with Am⁶A, which was efficiently transformed to the hydrazone in water without any catalyst (ESI,† part 2).

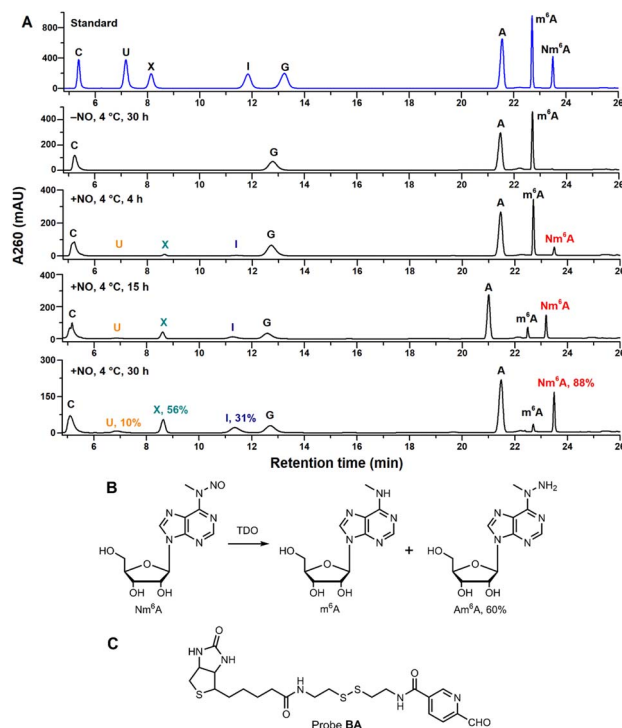


Fig. 2 (A) HPLC analysis of A/m⁶A/G/C ribonucleosides under NO/O₂ incubation. Conversion rates were determined by HPLC quantification at 260 nm using external standards. See ESI[†] for detailed conditions. The shifted retention times of A, m⁶A, and Nm⁶A in the fourth HPLC trace (15 h incubation) may be caused by the instability of instruments. (B) Nm⁶A nucleoside was converted into m⁶A and Am⁶A under TDO reduction. Reaction condition: 100 μ M Nm⁶A, 100 mM TDO, 300 mM Na₂CO₃/NaHCO₃, pH = 10.0, 50 $^{\circ}$ C, 5 min. (C) Chemical structure of probe BA.

Thus, a functional probe, biotinylated aryl aldehyde bearing a disulfide linkage (probe BA, Fig. 2C), was chemically synthesized, and Am⁶A was quantitatively labeled with a biotin molecule (Bm⁶A) via bioorthogonal hydrazone ligation.⁴⁷

Test the feasibility of this chemical approach into RNA labeling

A 12 nt RNA oligo (12nt-m⁶A) containing only U and m⁶A was synthesized. Optimization determined that 12nt-m⁶A was quantitatively converted into Nm⁶A modified oligo (12nt-Nm⁶A) through prior NO bubbling into an acidic buffered solution (ESI, Fig. S4[†]). 1 nmol of 12nt-Nm⁶A was converted into 12nt-Am⁶A via TDO reduction under heat and alkaline condition with the yield of 46% (ESI, Fig. S6[†]). After HPLC purification, almost 90% of the purified 12nt-Nm⁶A was converted into biotinylated product (12nt-Bm⁶A) labeling with probe BA (Fig. 3A, ESI, Fig. S8[†]). Meanwhile, we applied this chemical approach to labeling another 15 nt RNA oligo containing A/U/G/C/m⁶A (15nt-m⁶A). Denaturing polyacrylamide gel electrophoresis (PAGE) analysis indicated that almost 50% of 15nt-m⁶A was converted into biotinylated product (Fig. 3B). In addition, the modified oligo after each chemical incubation step was enzymatically digested into ribonucleosides as further confirmed by LC-HRMS (ESI, Fig. S10–S12[†]).



Before applying this method to the profiling of m⁶A methylomes, a comprehensive test was performed. Firstly, the optimal condition for *N*-nitrosation (the central step) was investigated by controlling the NO bubbling time and incubation time. Denaturing PAGE analysis and RNA quantification confirmed that the optimal *N*-nitrosation and TDO reduction conditions do not cause considerable RNA degradation (ESI, Fig. S13†). Then, total RNA was extracted from human embryonic kidney 293T (HEK-293T) and fragmented into 60–200 nt, followed by *N*-nitrosation. Then, the purified RNA was digested into ribonucleosides and further quantified using LC-MS/MS analysis (ESI, Fig. S14†), which determined 20 h as the optimal incubating time for the high ratio of Nm⁶A/I (avoiding excessive mutation of A) and sufficient Nm⁶A concentration (high labeling efficiency). As proof of concept, m⁶A-ORL was conducted to a 60 nt model RNA modified with an m⁶A modification (*MALAT1*-2577-m⁶A). After chemical labeling and purification, the dot blot assay proved the selective biotinylation of m⁶A in model RNA (Fig. 3C). Meanwhile, labeled model RNA was mixed with yeast tRNA and performed with a pulldown procedure. Afterward, the enriched RNA was subjected to RNA-Seq (Fig. 1B). Notably, under SuperScript III mediated reverse transcription (RT), labeled m⁶A (a thiol residue modified m⁶A, Tm⁶A, site 32) was read as A (Fig. 3D), and a major mutation of A to G (6.8%) was observed, since I was known to be read as G during RT.⁴⁸ Dot blot assay further confirmed that m⁶A-ORL was capable of selective labeling m⁶A-containing RNA in HEK-293T total RNA (ESI, Fig. S16†). These results indicated m⁶A-ORL is promising for the transcriptome-wide analysis of m⁶A.

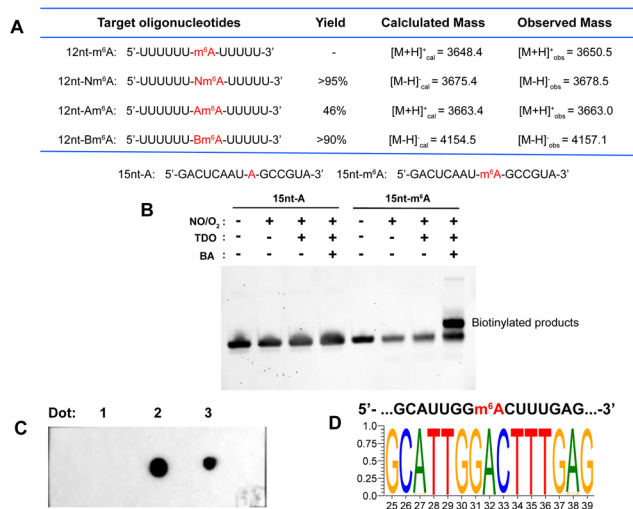


Fig. 3 (A) A table of results of 12 nt RNA oligo tests. Yields were calculated by HPLC at 260 nm. See ESI† for more details. (B) Denaturing PAGE analysis of 15 nt RNA oligo. (C) Dot blot assay confirmed selective biotinylation of m⁶A in *MALAT1*-2577-m⁶A. Dot 1: three-step labeling products of *MALAT1*-2577-A; Dot 2: three-step labeling products of *MALAT1*-2577-m⁶A; Dot 3: a mixture containing three-step labeling products of *MALAT1*-2577-A and 5'-biotinylated DNA. The methylene blue staining result was presented in ESI, Fig. S15.† (D) Motif for the sequence of model RNA (*MALAT1*-2577-m⁶A) after m⁶A-ORL-Seq procedure.

m⁶A-ORL-seq uncovers the transcriptome-wide m⁶A methylomes in HEK-293T

Next, m⁶A-ORL was performed on transcriptome-wide analysis of m⁶A in HEK-293T. First, 30 μg total RNA (m⁶A-ORL1) or 5.0 μg poly(A)⁺ RNA (m⁶A-ORL2) extracted from HEK-293T cells spiked with 0.01% spike-in RNAs (ESI,† page S29) were used to conduct three-step chemical reactions. The libraries were constructed following the procedure presented in ESI† and then subjected to high-throughput sequencing. Analyzing the count of spike-in RNAs confirmed m⁶A-ORL enable the enrichment of m⁶A fragment (ESI, Fig. S26†). A considerable amount of m⁶A peaks were identified in the human transcriptome with a canonical motif of DRACH (Fig. 4B). Remarkably, two prominent regions of m⁶A (*MALAT1*, *ACTB*) confirmed by miCLIP¹⁸ and m⁶A-REF-Seq²⁰ were also detected by m⁶A-ORL-Seq (Fig. 4A). The mean normalized reads were high specifically distributed near the center of m⁶A peaks, further verifying the low background enrichment of m⁶A segments and by m⁶A-ORL (Fig. 4C). A pie chart presents the fraction of m⁶A peaks in each of five non-overlapping transcript segments, the results were consistent with the previously reported distribution of m⁶A (Fig. 4D).^{14–25} Furthermore, a comparative analysis between m⁶A-ORL-Seq and the gold-standard MeRIP-Seq were conducted. The overall enrichment fold of m⁶A-ORL peaks is higher than MeRIP (ESI, Fig. S28†). Upon observation, nearly 50% of m⁶A-ORL peaks overlapped with MeRIP and a similar distribution of m⁶A peaks (Fig. 4E and F). These results indicated our method had the same level of reliability as MeRIP. Despite this, we focused on 50% of the peaks that did not overlap with MeRIP. The meta-gene profile of these unique peaks revealed the enrichment of 5' UTR and 3' UTR regions, signifying the plausible N⁶,2'-O-dimethyladenosine (m⁶Am) and m⁶A sites that MeRIP could not detect (Fig. 4H). Significant m⁶A and m⁶Am motifs (BCA,¹⁸ B = G/U/C) were searched out (Fig. 4I). These results further indicated the sequencing data was comprised of the thorough information for human m⁶A methylome with nearly null noise. Compared to the reported m⁶Am peaks derived from m⁶Am-Seq,⁴⁹ we found 24% of reported sites are overlapped with these unique peaks (Fig. 4J). Further base resolution analysis confirmed that considerable m⁶A and m⁶Am sites existed in m⁶A-ORL unique peaks (ESI, Fig. S17†). Meanwhile, m⁶A antibody was inclined to enrich >100 nt m⁶A-containing RNA fragments,^{14,15} while chemical labeling was more efficient to short fragments due to the steric hindrance. Overall, due to the novel chemistry's distinct attributes, m⁶A-ORL has a higher resolution (Fig. 4G) and provides a new perspective for m⁶A methylomes compared to MeRIP.

m⁶A-ORL detects m⁶A sites at single-base resolution

In the data analysis of m⁶A-ORL-Seq on model RNA, a specific signature of RT truncation was observed, marking the m⁶A site (site 32) at single-base resolution (Fig. 5A, ESI, Fig. S18†). Hence, it was speculated that the loss of hydrogen donor (replaced with a bulky group) at the N6 position caused an RT-stop (Fig. 5B). Then, several 15 nt RNA oligos containing N6-modified adenosine were synthesized using our recently



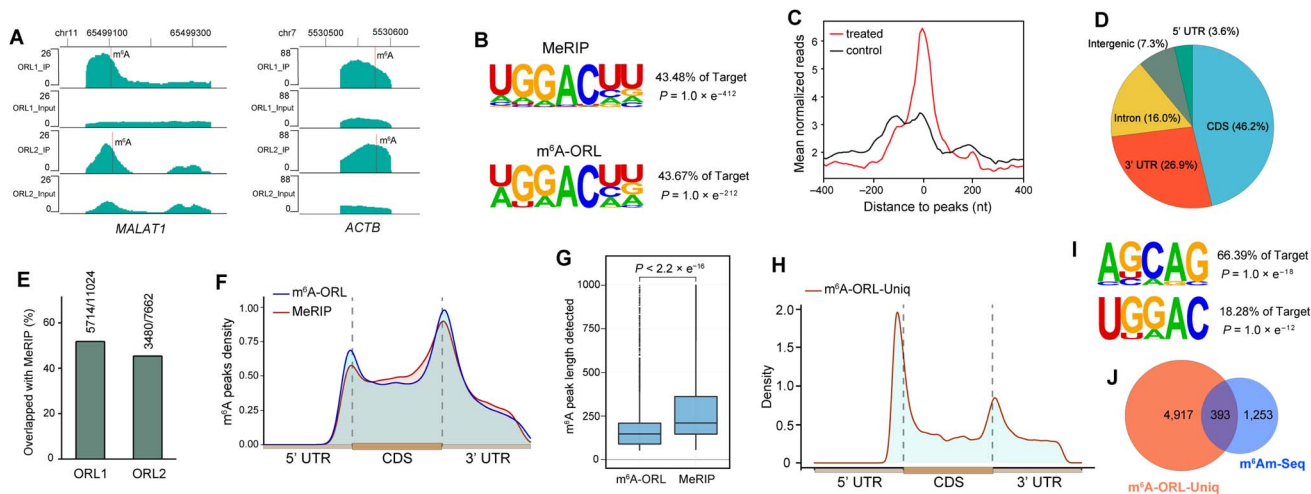


Fig. 4 m^6A -ORL-seq uncovers the transcriptome-wide m^6A methylomes in HEK-293T. (A) m^6A -ORL identified two known m^6A sites in the human transcriptome. *MALAT1* (chr11: 65499104) and *ACTB* (chr7: 5530572). (B) The motif search both yielded canonical DRACH in HEK-293T using MeRIP-Seq or m^6A -ORL-Seq. (C) A density plot of the distribution of mean normalized reads around the center of m^6A peaks. (D) Transcriptome-wide distribution of m^6A peaks. (E) Overlap of m^6A peaks identified by m^6A -ORL and MeRIP. (F) Metagene profile of the m^6A peaks identified by m^6A -ORL and MeRIP. (G) The length of m^6A peaks detected by m^6A -ORL and MeRIP. (H) Metagene profile of the m^6A -ORL-Uniq peaks. (I) Searched m^6A and m^6Am motif in the m^6A -ORL-Uniq peaks. (J) Overlap of m^6Am peaks between m^6A -ORL-Uniq peaks and m^6Am -Seq peaks.

developed method⁵⁰ (ESI,† part 13) to verify this hypothesis. RT reactions were subsequently performed using SuperScript III (SS III, extensively used for RNA-Seq library construction) and M-MuLV reverse transcriptase (Fig. 5C). Denaturing PAGE indicated that the RT reactions were smoothly extended encountering with A or m^6A while stopped at 12 nt where encountered an m^6A derivatives (m^6A , Bm^6A , Tm^6A). Based on RT truncation, a stringent threshold of stop rate (>10%) and truncated reads (>4) was set for high confidence. Then the DRACH motif was scanned in m^6A -ORL peaks, identifying 4001 sites that stopped at A. To reduce the false positives, the regions

of m^6A peaks in the input sample were detected referring to the same threshold, 18 sites were screened out to be false positives. The low rate of false positives (0.4%) indicated the high specificity of stop sites. The filtered 3983 sites were considered high-confidence m^6A sites identified by m^6A -ORL-Seq. A credible m^6A site (*YTHDF2*, chr1: 28743781) confirmed by miCLIP¹⁸ and m^6A -label-Seq²² was chosen as a representative example. Similarly, a significant truncation at A in the GGACG motif was revealed by m^6A -ORL-Seq (Fig. 5D). Statistics on frequency of sequence landscape revealed seven types of most frequent motif: GGACU (21%), GAACU (17%), AGACU (15.2%), UGACU (12.0%), AAACU

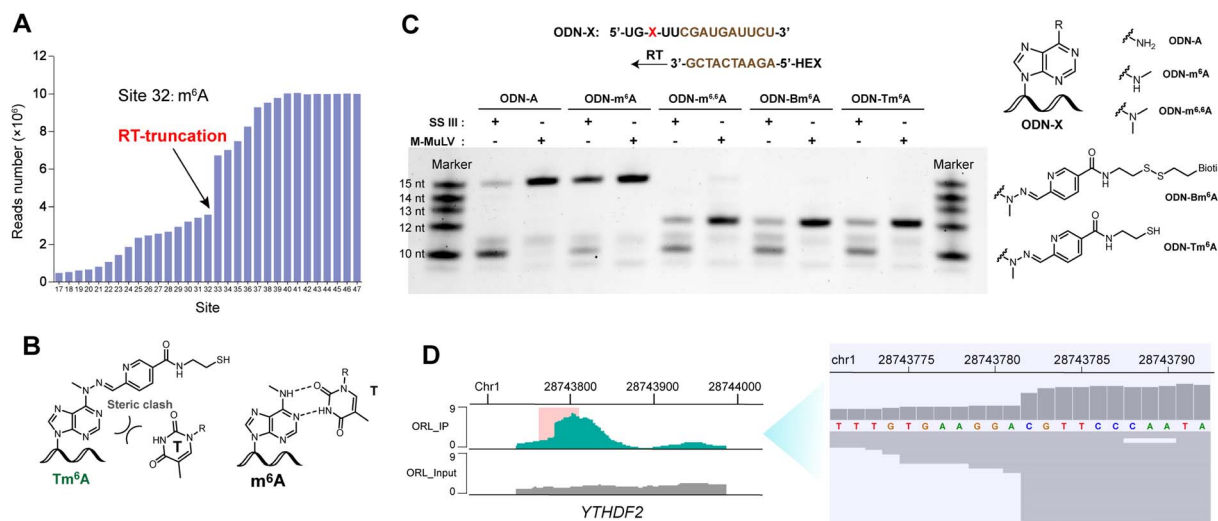


Fig. 5 m^6A -ORL results in a specific RT truncation signature. (A) Statistics on reads number of each site in model m^6A -RNA revealed a specific RT-truncation at site 32. (B) A schematic drawing indicated that m^6A pairs with T, Tm^6A may induce RT-stop. (C) RT experiments using several 15 nt oligos containing modified adenosine. (D) m^6A -ORL identified an m^6A site (*YTHDF2*, chr1: 28743781) at single-base resolution.



(8.4%), GGACA (7.6%), and GGACC (6.1%), which is consistent with the most widely used method miCLIP.

As belonging to chemical-assisted methods, the m⁶A sites identified by m⁶A-ORL-Seq were compared to m⁶A-label-Seq²² sites, m⁶A-SAC-Seq²⁴ sites, and m⁶A-SEAL-Seq²³ peaks (Fig. 6). It turns out m⁶A-ORL-Seq and m⁶A-SAC-Seq maintain higher consistency (Fig. 6A). The majority (77%) of m⁶A-ORL-Seq sites were located at m⁶A-SEAL-Seq peaks (Fig. 6B). To further assess the accuracy of these sites, a comprehensive comparison to the previously reported sites by different base resolution methods (miCLIP-CIMS,¹⁸ miCLIP-CITS,¹⁸ m⁶A-REF-Seq,²⁰ DART-Seq,²¹ m⁶A-label-Seq,²² m⁶A-SAC-Seq²⁴) was performed (ESI, Fig. S29†). The comparative results determined that the m⁶A sites identified by m⁶A-ORL had the highest overlapping rates except for DART-Seq, which indicates the m⁶A sites detected by m⁶A-ORL-Seq are the most reproducible and trustable. Altogether 2174 of 3983 high-confidence m⁶A were identified by these reported methods, and six sites derived from 1809 unique sites were further confirmed by SELECT (an independent m⁶A-sensitive ligation-based method)³⁶ validation (ESI, Fig. S23†). These results proved m⁶A-ORL-Seq is highly reliable for transcriptome-wide detection of m⁶A.

m⁶A-ORL can detects m⁶A sites of low methylation level

Counting spike-in RNAs in the sequencing data has indicated m⁶A-ORL-Seq can selectively enrich low m⁶A level fragments of low methylation level (ESI, Fig. S26†). To further confirm m⁶A-ORL can detects m⁶A sites of low methylation level, reported data which containing m⁶A stoichiometric information was referred to. MAZTER-Seq provided a feasible approach to m⁶A quantitative profiling (only for m⁶ACA motif).¹⁹ In their study, approximately 80 000 m⁶A sites were identified and relatively quantified in HEK-293T transcriptome. Therefore, identified m⁶ACA sites were extracted from the dataset of each method and were aligned with the dataset of MAZTER-Seq. Then, 775 of miCLIP-CIMS sites (27%), 214 of miCLIP-CITS sites (18%), 1742 of DART-Seq sites (22%), 783 of m⁶A-REF-Seq sites (18%), 46 of m⁶A-label-Seq sites (8%), and 148 of m⁶A-ORL-Seq sites (27%) were mapped to the dataset of MAZTER-Seq. Stoichiometry distribution (ESI, Fig. S24†) revealed that most sites identified by miCLIP-CIMS and DART-Seq were methylated at low levels, which was consistent with the distribution of global m⁶A

methylation levels.¹⁹ The methylation level distribution of sites detected by miCLIP-CITS and m⁶A-REF-Seq were relatively average. Considering the intrinsic characteristic of chemical labeling, m⁶A-label-Seq and m⁶A-ORL-Seq are apt to detect m⁶A of high methylation levels. However, m⁶A-label-Seq has no advantage in low methylation detection, m⁶A-ORL-Seq still provided considerable m⁶A sites of low methylation levels (20% of identified sites were methylated at the levels lower than 20%, ESI, Fig. S24†). m⁶A-SAC-Seq is a recently reported method which can also measure m⁶A methylation level.²⁴ 1043 of m⁶A-ORL-Seq sites (26.2%) was mapped to the dataset of m⁶A-SAC-Seq, indicating 52% of identified sites were methylated at the levels lower than 20% (ESI, Fig. S25†). These results confirmed that this chemical pulldown strategy enabled enrichment and detection of m⁶A sites at low methylation levels.

Discussion

Despite many methods being developed for m⁶A profiling, nature still cast a cloud over m⁶A code. The finding that little overlap between datasets seems worrisome (ESI, Fig. S29†), here we would share an opinion that technical noises and false positives resulting from the methodological flaws or sequencing depth are not the only major factor. (1) Aebersold *et al.*⁵¹ have revealed the effects of biological variation in HeLa cell lines across laboratories. Substantial heterogeneity was found between cell lines variants, and a progressive divergence was proceeded along with the times of cell passage. This variability could have a complex effect on the m⁶A methylation pattern. (2) m⁶A is a highly dynamic and extensive modification and the majority of m⁶A is methylated at low level, identified m⁶A sites might be a random subset of massive sites. Generally, all the current methods are limited in their ability to identify the low methylation sites in particular within lowly expressed genes. Meanwhile, many true methylated sites of low levels were probably filtered out for a high level of confidence. Schwartz *et al.*¹⁹ has revealed that many of m⁶A sites detected in only a single study are indeed methylated. Similar signs could also be found in DART-Seq. DART-Seq shows low overlap (<2.5%) with the methods listed in Fig. S29,† but has much higher overlap with MAZTER-Seq dataset. The majority of m⁶A sites identified by DART-Seq were methylated at low levels (ESI, Fig. S24†), which may cause the extremely low overlap with other methods. What's more, for m⁶A stoichiometry, a low correlation was found between two independent methods, MAZTER-Seq and m⁶A-SAC-Seq (ESI, Fig. S25†). This discrepancy could also be due to the biological variability and high complexity of m⁶A topologies.

The strengths and major concerns of the current base resolution methods for m⁶A profiling (miCLIP/m⁶A-CLIP, DART-Seq, MAZTER-Seq/m⁶A-REF-Seq, m⁶A-label-Seq) were listed (ESI, Table S6†). Compared to these antibody/enzyme-dependent methods, m⁶A-ORL is the only entirely chemical method, it is not expensive, easy to scale up. Most importantly, m⁶A-ORL provides the most reproducible m⁶A sites with high-confidence compared to all the current methods. However, m⁶A-ORL-Seq inevitably induced mutations (mainly, A to G

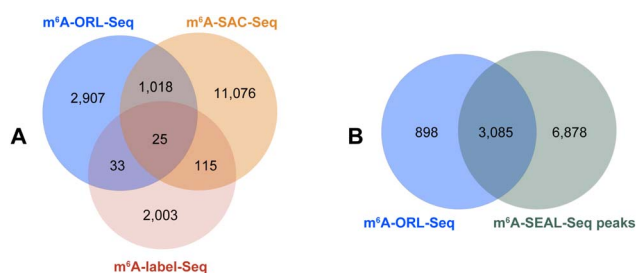


Fig. 6 Overlapping extents between the m⁶A datasets identified by different chemical-assisted methods. (A) Overlap between m⁶A-ORL-Seq, iodine-mediated m⁶A-label-Seq and m⁶A-SAC-Seq. (B) Overlap between m⁶A-ORL-Seq sites and m⁶A-SEAL-Seq peaks.



mutations) in bioinformatics analysis. Compared to the mutation rates (0.43–0.47%) and mapping rates (94–95%) of non-chemical treatment data, the mutation rates (2.4–3.2%) and mapping rates (82–90%) of m⁶A-ORL are acceptable. Meanwhile, the current protocol of m⁶A-ORL could be improved, for example, screening a more suitable nitrosating and reduction reagents were urgent for the lower mutation rates and higher labeling efficiency, thus the method could be further developed for relative and even absolute quantification of m⁶A levels (omitting the pulldown procedure). This unique chemistry enables the further application to chemical derivatization-assisted MS analysis⁵² for sensitive detection of m⁶A and other modifications. Similarly, m⁶A-ORL could also be used for the genomic profiling of 6mdA in DNA.

In principle other *N*-methyl modifications in RNA, such as *N*⁴-methylcytidine (m⁴C) and *N*²-methylguanosine (m²G), were probably *N*-nitrosated during chemical treatment. However, to the best of our knowledge, no reports confirmed the existence and abundance of m²G in the human cells were found, instead it was found in mice as a tRNA modification.^{53,54} m⁴C was found in human 12S rRNA,⁵⁵ while the abundance remains unknown. In general, these tRNA/rRNA modifications would not interfere with the m⁶A profiling in mRNA and lncRNA where researchers are more concerned with.

In summary, this study reported a novel chemical pulldown approach for transcriptome-wide profiling of m⁶A. This method can further detect high-confidence m⁶A sites at base resolution. Furthermore, since the principle of m⁶A-ORL is quite different from all the previously reported methods based on antibody/enzyme-dependent methods, m⁶A-ORL provides a new perspective for m⁶A detection at the transcriptome level and we believe it will help to obtain new insights into RNA methylation.

Data availability

Sequencing data have been deposited into the Gene Expression Omnibus (GEO). The accession number is GSE185753 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE185753>).

Code availability

All custom codes used in this study are available at <https://github.com/sqhan-whu/m6A-ORL-Seq>.

Author contributions

X. Z., X. W., and Y. Z. guided the research; Y. X. and X. Z. conceived the project; Y. X. designed and performed the experiments with the help of Z. F., W. Y., Q. W., and Y. W.; S. H. designed and performed the bioinformatics analysis with the help of Q. L.; Y. X. wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- 1 P. Boccaletto, M. A. Machnicka, E. Purta, P. Piatkowski, B. Baginski, T. K. Wirecki, V. de Crécy-Lagard, R. Ross, P. A. Limbach, A. Kotter, M. Helm and J. M. Bujnicki, *Nucleic Acids Res.*, 2018, **46**, D303–D307.
- 2 Y. Yue, J. Liu and C. He, *Genes Dev.*, 2015, **29**, 1343–1355.
- 3 B. S. Zhao, I. A. Roundtree and C. He, *Nat. Rev. Mol. Cell Biol.*, 2017, **18**, 31–42.
- 4 Y. Yue, J. Liu and C. He, *Genes Dev.*, 2015, **29**, 1343–1355.
- 5 B. Wu, L. Li, Y. Huang, J. Ma and J. Min, *Curr. Opin. Struct. Biol.*, 2017, **47**, 67–76.
- 6 J. Liu, Y. Yue, D. Han, X. Wang, Y. Fu, L. Zhang, G. Jia, M. Yu, Z. Lu, X. Deng, Q. Dai, W. Chen and C. He, *Nat. Chem. Biol.*, 2014, **10**, 93–95.
- 7 D. Theler, C. Dominguez, M. Blatter, J. Boudet and F. H. Allain, *Nucleic Acids Res.*, 2014, **42**, 13911–13919.
- 8 G. Jia, Y. Fu, X. Zhao, Q. Dai, G. Zheng, Y. Yang, C. Yi, T. Lindahl, T. Pan, Y. G. Yang and C. He, *Nat. Chem. Biol.*, 2011, **7**, 885–887.
- 9 S. D. Kasowitz, J. Ma, S. J. Anderson, N. A. Leu, Y. Xu, B. D. Gregory, R. M. Schultz and P. J. Wang, *PLoS Genet.*, 2018, **14**, e1007412.
- 10 J. M. Fustin, M. Doi, Y. Yamaguchi, H. Hida, S. Nishimura, M. Yoshida, T. Isagawa, M. S. Morioka, H. Kakeya, I. Manabe and H. Okamura, *Cell*, 2013, **155**, 793–806.
- 11 N. Liu, Q. Dai, G. Zheng, C. He, M. Parisien and T. Pan, *Nature*, 2015, **518**, 560–564.
- 12 S. Panneerdoss, V. K. Eedunuri, P. Yadav, S. Timilsina, S. Rajamanickam, S. Viswanadhappalli, N. Abdelfattah, B. C. Onyeagucha, X. Cui, Z. Lai, T. A. Mohammad, Y. K. Gupta, T. H. Huang, Y. Huang, Y. Chen and M. K. Rao, *Sci. Adv.*, 2018, **4**, eaar8263.
- 13 R. Su, L. Dong, C. Li, S. Nachtergaele, M. Wunderlich, Y. Qing, X. Deng, Y. Wang, X. Weng, C. Hu, M. Yu, J. Skibbe, Q. Dai, D. Zou, T. Wu, K. Yu, H. Weng, H. Huang, K. Ferchen, X. Qin, B. Zhang, J. Qi, A. T. Sasaki, D. R. Plas, J. E. Bradner, M. Wei, G. Marcucci, X. Jiang, J. C. Mulloy, J. Jin, C. He and J. Chen, *Cell*, 2018, **172**, 90–105.
- 14 D. Dominissini, S. Moshitch-Moshkovitz, S. Schwartz, M. Salmon-Divon, L. Ungar, S. Osenberg, K. Cesarkas, J. Jacob-Hirsch, N. Amariglio, M. Kupiec, R. Sorek and G. Rechavi, *Nature*, 2012, **485**, 201–206.
- 15 K. D. Meyer, Y. Saletore, P. Zumbo, O. Elemento, C. E. Mason and S. R. Jaffrey, *Cell*, 2012, **149**, 1635–1646.



- 16 K. Chen, Z. Lu, X. Wang, Y. Fu, G. Z. Luo, N. Liu, D. Han, D. Dominissini, Q. Dai, T. Pan and C. He, *Angew. Chem., Int. Ed.*, 2015, **54**, 1587–1590.
- 17 S. Ke, E. A. Alemu, C. Mertens, E. C. Gantman, J. J. Fak, A. Mele, B. Haripal, I. Zucker-Scharff, M. J. Moore, C. Y. Park, C. B. Vågbø, A. Kusnierzcyk, A. Klungland, J. E. Darnell Jr. and R. B. Darnell, *Genes Dev.*, 2015, **29**, 2037–2053.
- 18 B. Linder, A. V. Grozhik, A. O. Olarerin-George, C. Meydan, C. E. Mason and S. R. Jaffrey, *Nat. Methods*, 2015, **12**, 767–772.
- 19 M. A. Garcia-Campos, S. Edelheit, U. Toth, M. Safra, R. Shachar, S. Viukov, R. Winkler, R. Nir, L. Lasman, A. Brandis, J. H. Hanna, W. Rossmanith and S. Schwartz, *Cell*, 2019, **178**, 731–747.
- 20 Z. Zhang, L. Q. Chen, Y. L. Zhao, C. G. Yang, I. A. Roundtree, Z. Zhang, J. Ren, W. Xie, C. He and G. Z. Luo, *Sci. Adv.*, 2019, **5**, eaax0250.
- 21 K. D. Meyer, *Nat. Methods*, 2019, **16**, 1275–1280.
- 22 X. Shu, J. Cao, M. Cheng, S. Xiang, M. Gao, T. Li, X. Ying, F. Wang, Y. Yue, Z. Lu, Q. Dai, X. Cui, L. Ma, Y. Wang, C. He, X. Feng and J. Liu, *Nat. Chem. Biol.*, 2020, **16**, 887–895.
- 23 Y. Wang, Y. Xiao, S. Dong, Q. Yu and G. Jia, *Nat. Chem. Biol.*, 2020, **16**, 896–903.
- 24 L. Hu, S. Liu, Y. Peng, R. Ge, R. Su, C. Senevirathne, B. T. Harada, Q. Dai, J. Wei, L. Zhang, Z. Hao, L. Luo, H. Wang, Y. Wang, M. Luo, M. Chen, J. Chen and C. He, *Nat. Biotechnol.*, 2022, **40**, 1210–1219.
- 25 I. D. Vilfan, Y.-C. Tsai, T. A. Clark, J. Wegener, Q. Dai, C. Yi, T. Pan, S. W. Turner and J. Korlach, *J. Nanobiotechnology*, 2013, **11**, 8.
- 26 D. R. Garalde, E. A. Snell, D. Jachimowicz, B. Sipos, J. H. Lloyd, M. Bruce, N. Pantic, T. Admassu, P. James, A. Warland, M. Jordan, J. Ciccone, S. Serra, J. Keenan, S. Martin, L. McNeill, E. J. Wallace, L. Jayasinghe, C. Wright, J. Blasco, S. Young, D. Brocklebank, S. Juul, J. Clarke, A. J. Heron and D. J. Turner, *Nat. Methods*, 2018, **15**, 201–206.
- 27 A. Leger, P. P. Amaral, L. Pandolfini, C. Capitanchik, F. Capraro, V. Miano, V. Migliori, P. Toolan-Kerr, T. Sideri, A. J. Enright, K. Tzelepis, F. J. van Werven, N. M. Luscombe, I. Barbieri, J. Ule, T. Fitzgerald, E. Birney, T. Leonard and T. Kouzarides, *Nat. Commun.*, 2021, **12**, 7198.
- 28 Z. Zhang, T. Chen, H.-X. Chen, Y.-Y. Xie, L.-Q. Chen, Y.-L. Zhao, B.-D. Liu, L. Jin, W. Zhang, C. Liu, D.-Z. Ma, G.-S. Chai, Y. Zhang, W.-S. Zhao, W. H. Ng, J. Chen, G. Jia, J. Yang and G.-Z. Luo, *Nat. Methods*, 2021, **18**, 1213–1222.
- 29 L.-Y. Zhao, J. Song, Y. Liu, C.-X. Song and C. Yi, *Protein Cell*, 2020, **11**, 792–808.
- 30 M. Nappi, A. Hofer, S. Balasubramanian and M. J. Gaunt, *J. Am. Chem. Soc.*, 2020, **142**, 21484–21492.
- 31 Y. Mahdavi-Amiri, K. Chung Kim Chung and R. Hili, *Chem. Sci.*, 2021, **12**, 606–612.
- 32 S. Werner, A. Galliot, F. Pichot, T. Kemmer, V. Marchand, M. V. Sednev, T. Lence, J.-Y. Roignant, J. König, C. Höbartner, Y. Motorin, A. Hildebrandt and M. Helm, *Nucleic Acids Res.*, 2021, **49**, e23.
- 33 X. Li, S. Guo, Y. Cui, Z. Zhang, X. Luo, M. T. Angelova, L. F. Landweber, Y. Wang and T. P. Wu, *Genome Biol.*, 2022, **23**, 122.
- 34 M. Martin, *EMBnet J*, 2011, **17**, 10–12.
- 35 A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson and T. R. Gingeras, *Bioinformatics*, 2013, **29**, 15–21.
- 36 A. O. Olarerin-George and S. R. Jaffrey, *Bioinformatics*, 2017, **33**, 1563–1564.
- 37 Y. Xiao, Y. Wang, Q. Tang, L. Wei, X. Zhang and G. Jia, *Angew. Chem., Int. Ed.*, 2018, **57**, 15995–16000.
- 38 Z. Mao, H. Jiang, Z. Li, C. Zhong, W. Zhang and Z. Liu, *Chem. Sci.*, 2017, **8**, 4533–4538.
- 39 Z. Mao, H. Jiang, X. Song, W. Hu and Z. Liu, *Anal. Chem.*, 2017, **89**, 9620–9624.
- 40 M. P. V. Tato, L. Castedo and R. Riguera, *Chem. Lett.*, 1985, **14**, 623–626.
- 41 M. A. Zolfigol, *Synth. Commun.*, 1999, **29**, 905–910.
- 42 J. A. Hrabie, J. R. Klose, D. A. Wink and L. K. Keefer, *J. Org. Chem.*, 1993, **58**, 1472–1476.
- 43 D. A. Wink, K. S. Kasprzak, C. M. Maragos, R. K. Elespuru, M. Misra, T. M. Dunams, T. A. Cebula, W. H. Koch, A. W. Andrews, J. S. Allen and L. K. Keefer, *Science*, 1991, **254**, 1001–1003.
- 44 J. L. Caulfield, J. S. Wishnok and S. R. Tannenbaum, *J. Biol. Chem.*, 1998, **273**, 12689–12695.
- 45 D. Wiener and S. Schwartz, *Nat. Rev. Genet.*, 2021, **22**, 119–131.
- 46 P. Chaudhary, S. Gupta, P. Sureshbabu, S. Sabiah and J. Kandasamy, *Green Chem.*, 2016, **18**, 6215–6221.
- 47 R. K. Lim and Q. Lin, *Chem. Commun.*, 2010, **46**, 1589–1600.
- 48 C. Basilio, A. J. Wahba, P. Lengyel, J. F. Speyer and S. Ochoa, *Proc. Natl. Acad. Sci. U. S. A.*, 1962, **48**, 613–616.
- 49 H. Sun, K. Li, X. Zhang, J. Liu, M. Zhang, H. Meng and C. Yi, *Nat. Commun.*, 2021, **12**, 4778.
- 50 Y. Xie, Z. Fang, W. Yang, Z. He, K. Chen, P. Heng, B. Wang and X. Zhou, *Bioconjugate Chem.*, 2022, **33**, 353–362.
- 51 Y. Liu, Y. Mi, T. Mueller, S. Kreibich, E. G. Williams, A. Van Drogen, C. Borel, M. Frank, P.-L. Germain, I. Bludau, M. Mehnert, M. Seifert, M. Emmenlauer, I. Sorg, F. Bezrukov, F. S. Bena, H. Zhou, C. Dehio, G. Testa, J. Saez-Rodriguez, S. E. Antonarakis, W.-D. Hardt and R. Aebersold, *Nat. Biotechnol.*, 2019, **37**, 314–322.
- 52 Q.-Y. Cheng, J. Xiong, C.-J. Ma, Y. Dai, J.-H. Ding, F.-L. Liu, B.-F. Yuan and Y.-Q. Feng, *Chem. Sci.*, 2020, **11**, 1878–1891.
- 53 Y. Zhang, X. Zhang, J. Shi, F. Tuorto, X. Li, Y. Liu, R. Liebers, L. Zhang, Y. Qu, J. Qian, M. Pahima, Y. Liu, M. Yan, Z. Cao, X. Lei, Y. Cao, H. Peng, S. Liu, Y. Wang, H. Zheng, R. Woolsey, D. Quilici, Q. Zhai, L. Li, T. Zhou, W. Yan, F. Lyko, Y. Zhang, Q. Zhou, E. Duan and Q. Chen, *Nat. Cell Biol.*, 2018, **20**, 535–540.
- 54 Q. Chen, M. Yan, Z. Cao, X. Li, Y. Zhang, J. Shi, G.-h. Feng, H. Peng, X. Zhang, Y. Zhang, J. Qian, E. Duan, Q. Zhai and Q. Zhou, *Science*, 2016, **351**, 397–400.
- 55 L. V. Haute, A. G. Hendrick, A. R. D'Souza, C. A. Powell, P. Rebelo-Guiomar, M. E. Harbour, S. Ding, I. M. Fearnley, B. Andrews and M. Miniczuk, *Nucleic Acids Res.*, 2019, **47**, 10267–10281.

