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## $N^6$ -Hydroperoxymethyladenosine: a new intermediate of chemical oxidation of $N^6$ -methyladenosine mediated by bicarbonate-activated hydrogen peroxide†

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$N^6$ -Methyladenosine ( $m^6A$ ) represents a relatively abundant modification in eukaryotic RNA. Because  $m^6A$  has similar properties to adenosine and a low reactivity, limited research has been focused on this nucleoside. In this study, we revealed an important intermediate in the oxidation of  $m^6A$  through the bicarbonate-activated peroxide system. Over the course of oxidation, we found a new mechanism in which  $N^6$ -hydroxymethyladenosine ( $hm^6A$ ),  $N^6$ -formyladenosine ( $f^6A$ ) and  $N^6$ -hydroperoxymethyladenosine ( $oxm^6A$ ) were intermediate products, and adenosine was the final product. In this study,  $oxm^6A$  was isolated using HPLC and characterized by mass spectrometry, NMR and diphenyl-1-pyrenylphosphine (DPPP) fluorescence detection. This study provides a new modified nucleoside and demonstrates oxidative demethylation of  $m^6A$  by reactive oxygen species at the nucleobase level and in RNA strands.

Received 9th February 2015

Accepted 10th March 2015

DOI: 10.1039/c5sc00484e

www.rsc.org/chemicalscience

$N^6$ -Methyladenosine represents the most abundant modification in the mRNA of higher eukaryotes, present at a frequency of approximately three sites on each mRNA.<sup>1</sup>  $m^6A$  is also present on tRNA, rRNA and lncRNA.<sup>2</sup> This modification plays an important role in the regulation of gene expression.<sup>3</sup> Since its discovery last century,<sup>4</sup>  $m^6A$  has been the object of relatively few studies. Recently, fat mass- and obesity-associated proteins (FTO)<sup>5</sup> and AlkBH5<sup>6</sup> were found to be  $m^6A$  demethylases, indicating a novel regulatory mechanism in mammalian cells. Two new modifications,  $N^6$ -hydroxymethyladenosine ( $hm^6A$ ) and  $N^6$ -formyladenosine ( $f^6A$ ), have been found to participate in the FTO-mediated demethylation process, which may influence RNA-protein interactions and regulate gene expression.<sup>7</sup> In addition, transcriptome-wide profiling of  $m^6A$  in mRNA and lncRNA has revealed new insights into the role of RNA modification.<sup>8</sup> These developments have renewed interest in the investigation of this particular, distinctive modification. Therefore, we aspire to use a chemical method to differentiate  $m^6A$  from A.

Hydrogen peroxide is a widely used oxidant with a high content of active oxygen,<sup>9</sup> but its relatively slow oxidizing rate limits its usage. Bicarbonate is present in cells and serum at high concentrations, ranging from 14.7–25 mM,<sup>10</sup> and plays an important role in biological oxidation.<sup>11</sup>  $H_2O_2$  and  $NH_4HCO_3$  are environmentally friendly reagents;  $H_2O_2$  produces only water as a by-product, and  $NH_4HCO_3$  easily decomposes to  $NH_3$ ,  $CO_2$ , and  $H_2O$ . The reaction conditions are mild at natural pH values.

Owing to its high reactivity towards secondary amines, we considered whether the oxidant could react with  $m^6A$ . Surprisingly, instead of producing  $N$ -oxides, demethylated adenosine was produced, and the presence of several intermediates in the reaction system suggested a potential mechanism in the chemical reaction (Scheme 1). These results suggest that  $H_2O_2$ /bicarbonate can act as a reactive oxygen species (ROS) for demethylation. In this study, we determine a key intermediate in the demethylation process, and we investigate the underlying mechanism.

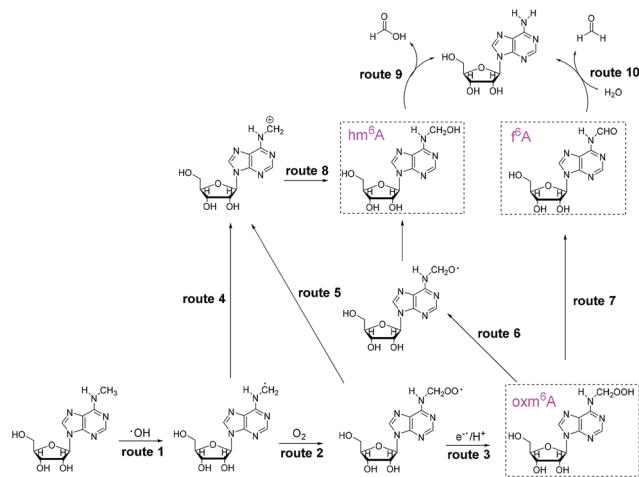
To investigate the demethylation process, we used high-performance liquid chromatography (HPLC) to monitor the reaction (UV detector at 260 nm). When a 2 mM aliquot of  $m^6A$  was treated with 200 mM  $H_2O_2$  and 1 M  $NH_4HCO_3$  at 37 °C for one hour, four products were formed: A,  $hm^6A$ ,  $oxm^6A$  and  $f^6A$  (Fig. 1). The LC-MS data showed masses corresponding to A (267.9),  $hm^6A$  (297.8),  $oxm^6A$  (313.8) and  $f^6A$  (295.9), successively in the positive-ion mode (Fig. S1†). Product A was further characterized by  $^1H$  and  $^{13}C$  NMR (see ESI†). To confirm the

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† Electronic supplementary information (ESI) available: Methods, figures and spectra are included. See DOI: 10.1039/c5sc00484e

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**Scheme 1** Proposed mechanism of the demethylation process, and structures of oxidation products.

occurrence of  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$ , these compounds were synthesized according to reported procedures.<sup>7</sup> An equilibrium reaction between adenosine and formaldehyde produced  $\text{hm}^6\text{A}$  (Scheme 1, Route 9). Further HPLC analysis indicated that the synthesized  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  have the same retention times as the reported  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$ , respectively (Fig. 1b–d). We found that  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  were unstable and could decompose to A (adenosine) during HPLC analysis (Fig. 1c and d).  $\text{N}^6$ -Hydroperoxymethyladenosine ( $\text{oxm}^6\text{A}$ ) was found to be a new intermediate, in addition to  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$ , during the demethylation of  $\text{m}^6\text{A}$  (Fig. 1b). When we incubated the  $\text{m}^6\text{A}$  with bicarbonate or  $\text{H}_2\text{O}_2$  alone, no reaction was observed (Fig. S2 and S3†).

Diphenyl-1-pyrenylphosphine (DPPP), as a fluorescent reagent, can be used for hydroperoxide determinations.<sup>12</sup> When we incubated the intermediate with DPPP in the presence of butylated hydroxytoluene (BHT) at 37 °C for 1 h, the fluorescence increased, indicating the formation of a hydroperoxide intermediate (Fig. 2). Further characterization of  $\text{oxm}^6\text{A}$  was achieved using high-resolution mass spectrometry,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and TOCSY (ESI, Fig. 3, S4 and S5†), with the corresponding chemical structures shown in Scheme 1. To confirm the chemical shifts of the protons in N–H and O–H,  $^1\text{H}$  NMR was performed in  $\text{DMSO}-d_6$  and in  $\text{D}_2\text{O}$ . In the  $\text{DMSO}-d_6$  solution, the chemical shifts of the protons were 8.61 ppm (–N–H) and 11.71 ppm (–OOH) (Table 1). To confirm our hypothesis, we changed the solution to  $\text{D}_2\text{O}$ , where deuterons can be incorporated at the N–H and O–H positions because of hydrogen-deuterium (H/D) exchange behavior. As we expected, these two protons disappeared in the  $\text{D}_2\text{O}$  solution (Fig. 3). We then used total correlation spectroscopy (TOCSY) to show the H–H correlation; the TOCSY spectrum was acquired using a 600 MHz Bruker Avance II spectrometer equipped with a 5 mm triple resonance cryoprobe. The pulse sequence was DIPSI2ETGP. The relaxation delay was 1 s, with 8 acquisitions per increment, and a spectral width of  $8 \times 8$  ppm and time domain of  $2k \times 176$  were used. In the spectrum, the NH proton had a cross peak with  $\text{CH}_2$

at  $\delta$  (8.66, 5.26 ppm), further confirming the  $\text{oxm}^6\text{A}$  structure. When we analysed the reaction mixture using LC-MS, we detected a relatively small mass signal of 311.8; this finding may indicate the generation of another intermediate,  $\text{N}^6$ -carboxyladenosine, in a relatively low yield (Fig. S1c†). Meanwhile, our control experiments indicated that adenosine, uridine, cytidine and guanosine were stable in the  $\text{H}_2\text{O}_2$ /bicarbonate solution at concentrations of 200 mM  $\text{H}_2\text{O}_2$  and 1 M  $\text{NH}_4\text{HCO}_3$  (Fig. S6†) after one hour.

Because our goal was to fully investigate the mechanism of  $\text{m}^6\text{A}$  demethylation, we extended the reaction time to 24 hours. After 24 hours, we found that only A (primary product) and a small amount of  $\text{oxm}^6\text{A}$  were present (Fig. 1e), whereas  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  disappeared. This result suggested that  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  were converted into A (Scheme 1, Routes 9 and 10).

To investigate the behaviour of  $\text{oxm}^6\text{A}$ , it was separated from the reaction mixture, incubated in HEPES buffer (50 mM, pH 7.4) at 37 °C and then analysed by HPLC every 2 h. We found that the amount of A increased at the expense of  $\text{oxm}^6\text{A}$  (Fig. S7 in the ESI†), and it had a half-time of approximately 8.5 h (Fig. S8†), which was more stable than  $\text{hm}^6\text{A}$  and  $\text{f}^6\text{A}$  (approximately 3 h).

In the  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  system, the hydroxyl radical was trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) to give a signal using Electron Paramagnetic Resonance (EPR) (Fig. S9†). In the reaction system, the addition of DMSO, a hydroxyl radical scavenger, dramatically decreased the chemical demethylation level of  $\text{m}^6\text{A}$  (Fig. S10†). We speculate that the reaction underwent a hydroxyl radical mechanism. A hydroxyl radical abstracted a hydrogen atom from a methyl group to yield a carbon radical, which could then bind with  $\text{O}_2$  to form  $\text{oxm}^6\text{A}$  (Scheme 1, Routes 2 and 3) or bind with  $\cdot\text{OH}$  to form  $\text{hm}^6\text{A}$  (Scheme 1, Routes 4 and 8), parallel to the decomposition mechanism for 5'-hydroperoxymethyluracil and 5'-hydroperoxymethylcytosine, as proposed by Richard Wagner's group.<sup>13</sup> To confirm the possibility of the  $\cdot\text{OH}$  radical mechanism, we used Fenton-type reagents to react with  $\text{m}^6\text{A}$ . The formation of  $\text{hm}^6\text{A}$ ,  $\text{oxm}^6\text{A}$ ,  $\text{f}^6\text{A}$  and A was also observed using LC-MS analysis, confirming the reaction mechanism (Fig. S18†). Under identical experimental conditions but with the addition of a small amount of  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$  in the  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  reaction mixture, the reaction rate markedly increased (Fig. S11†). As the reaction is based on the hydroxyl radical mechanism, and  $\text{Fe}^{2+}$  as well as  $\text{Cu}^{2+}$  have great influences on the reaction, we therefore used Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) to investigate the presence of iron(II) and copper(II) in the  $\text{H}_2\text{O}_2$ /bicarbonate reaction system. No signals were observed, and both the concentration of  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$  were lower than  $10 \text{ ng mL}^{-1}$ , indicating the reaction could proceed with just a bicarbonate-activated peroxide system (the optimized operating conditions are shown in Table S1†). In the demethylation process, two pathways are shown. A hydroxyl radical attacks the methyl radical to form  $\text{hm}^6\text{A}$  (Scheme 1, Routes 4 and 8) and  $\text{O}_2$  attacks the methyl radical to form  $\text{oxm}^6\text{A}$  (Scheme 1, Routes 2 and 3). The  $\text{oxm}^6\text{A}$  and its peroxide radical can decompose to  $\text{hm}^6\text{A}$  (Scheme 1, Routes 5, 8 and 6) and  $\text{f}^6\text{A}$  (Scheme 1, Route 7), and



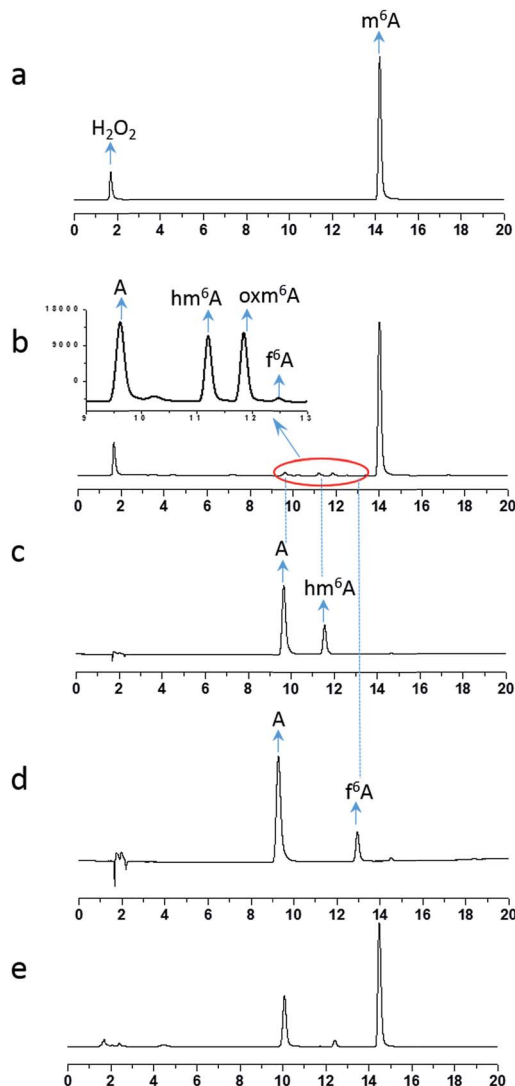


Fig. 1 HPLC chromatograph of 2 mM  $m^6A$  incubated with 200 mM  $H_2O_2$  and 1 M  $NH_4HCO_3$  at 37 °C for 0 h (a), 1 h (b) and 24 h (e). As shown in the HPLC profiles, when the reaction proceeded for 1 h, one major product (A) was produced, accompanied by three intermediates ( $hm^6A$ ,  $f^6A$ ,  $oxm^6A$ ). The synthesized  $hm^6A$  (c) and  $f^6A$  (d) standards have the same retention time as two of the new peaks in the reaction mixture. Because  $hm^6A$  and  $f^6A$  are unstable, they can coexist with A during HPLC analysis.

we propose that the new route in the demethylation process would improve the efficiency of the demethylation reaction compared to just attacking the methyl radical by a hydroxyl radical.

Next, because  $m^6A$  is preferentially present in the consensus sequence  $RRm^6ACH$  (R is A/G and H is A/C/U),<sup>14</sup> to examine whether the reaction occurs in RNA oligos, we prepared a 9-mer oligoribonucleotide (5'-CUGGm<sup>6</sup>ACUGG-3') containing one  $m^6A$  site and treated it with 10 mM  $H_2O_2$  and 100 mM bicarbonate at 37 °C for 48 h. Because RNA may decompose in a high concentration of  $H_2O_2$ , we decreased the concentration of  $H_2O_2$  and  $NH_4HCO_3$ . After the reaction, the oligo RNA was analysed using MALDI-TOF mass spectrometry as shown in Fig. S12.† We

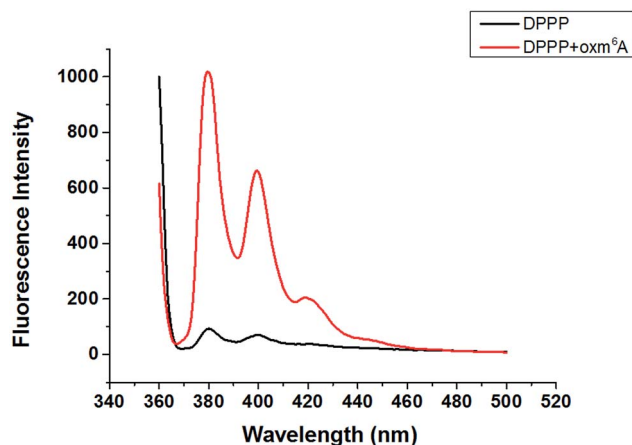


Fig. 2 Fluorescence emission spectra ( $\lambda_{ex}$  = 352 nm) of DPPP in the presence of (a) and in the absence of  $oxm^6A$  (b) after incubation with BHT at 37 °C for 60 min.

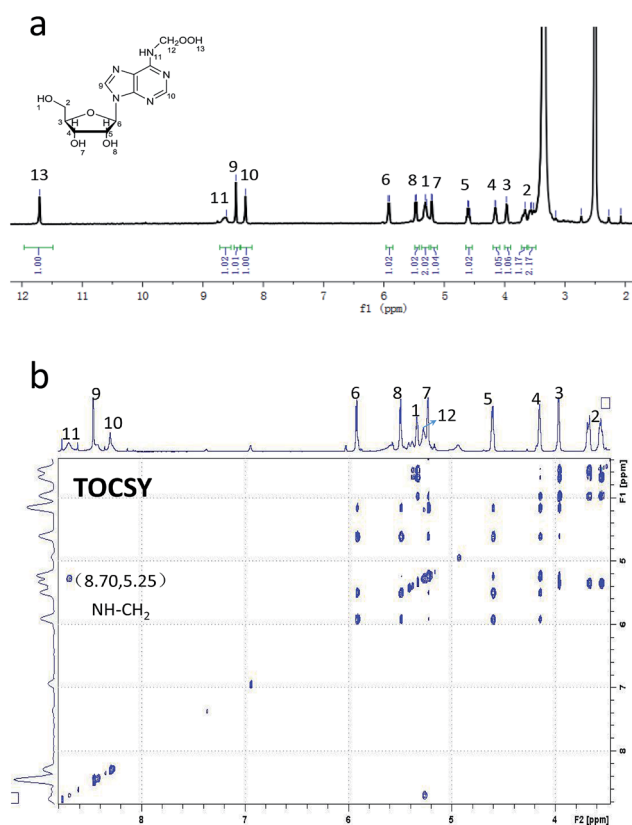


Fig. 3  $^1H$  NMR spectrum (a) and TOCSY spectrum (b) of  $oxm^6A$  in  $DMSO-d_6$ .

found a  $m^6A$  -14 Da peak, representing a demethylation product, as well as a +14 Da peak and a +17 Da peak, which may correspond to  $N^6$ -formyladenosine and  $N^6$ -hydroxymethyladenosine intermediates in the demethylation pathway, respectively. At natural pH levels (pH 7.4),  $hm^6A$ ,  $oxm^6A$  and  $f^6A$  were relatively stable, but an alkaline phosphate digestion may accelerate their decomposition. Therefore, to verify the

Table 1  $^1\text{H}$  chemical shifts ( $\delta$ , ppm) of oxm<sup>6</sup>A in DMSO- $d_6$  at room temperature

Number	Chemical shift ( $\delta$ , ppm)	Number	Chemical shift ( $\delta$ , ppm)	Number	Chemical shift ( $\delta$ , ppm)
1	5.33	6	5.91	11	8.66
2	3.68	7	5.23	12	5.26
3	3.96	8	5.50	13	11.72
4	4.14	9	8.46		
5	4.61	10	8.31		

presence of hm<sup>6</sup>A, oxm<sup>6</sup>A and f<sup>6</sup>A in the oligo RNA after the reaction, we used RNase T1 followed by nuclease P1 to digest the oligo RNA,<sup>7</sup> then analysed the reaction using LC-MS. In this analysis, RNase T1 can selectively digest the phosphodiester bond after G. We successfully detected the formation of A, hm<sup>6</sup>A, oxm<sup>6</sup>A, and f<sup>6</sup>A in the digested nucleoside, similar to our proposed mechanism for a single nucleoside (Fig. S13†).

To explore the reaction kinetics of the oxidation, two micrograms of oligo RNA were incubated with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 300  $\mu\text{M}$   $\text{NH}_4\text{HCO}_3$  at 37  $^\circ\text{C}$  for 30 h in six parallel experiments, followed by digestion with nuclease P1 and alkaline phosphate. The amount of A generated from m<sup>6</sup>A was quantified using LC-MS every 3 hours (the calibration curve is shown in the ESI, Fig. S14†). As depicted in Fig. S15,† the A content exhibited a strong linear relationship with reaction time over a period of 30 hours. After adding  $\text{Fe}^{2+}$  to the  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  mixture and incubating it with oligo RNA, HPLC analysis of the enzymatically digested nucleosides in RNA showed the presence of demethylated adenosine with a decreased level of m<sup>6</sup>A after oxidation for 1 h (Fig. S16†).

Although FTO-mediated oxidation of m<sup>6</sup>A may decrease the level of m<sup>6</sup>A *in vitro*, no *in vitro* experiments have been reported in which a chemical reagent was used to demethylate m<sup>6</sup>A. We explored whether m<sup>6</sup>A in genomic RNA is a substrate of  $\text{H}_2\text{O}_2/\text{NH}_4\text{HCO}_3$  *in vitro*. Total RNA was extracted from Hela cells using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Four micrograms of genomic RNA was incubated with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 1 mM  $\text{NH}_4\text{HCO}_3$  at 37  $^\circ\text{C}$  for 12 h. After digestion with nuclease P1 and alkaline phosphatase, the solution was analysed by LC-MS. The results showed a decrease in the m<sup>6</sup>A/A ratio by 10% in the genomic RNA (Fig. S17†), indicating that the reagents demethylated m<sup>6</sup>A *in vitro*.

## Conclusions

In conclusion, we reported a new chemical method for the oxidative demethylation of m<sup>6</sup>A and determined an important intermediate in the reaction system. Three intermediates, N<sup>6</sup>-hydroxymethyladenosine (hm<sup>6</sup>A), N<sup>6</sup>-formyladenosine (f<sup>6</sup>A), and N<sup>6</sup>-hydroperoxymethyladenosine (oxm<sup>6</sup>A), were characterized, and the mechanism underlying the decomposition was illustrated. We also determined that the reaction could occur in oligo RNA and genomic RNA *in vitro*.  $\text{H}_2\text{O}_2$  is a reactive oxygen species that is endogenously produced during normal metabolism<sup>15</sup> and immune responses,<sup>16</sup> and a high concentration of

bicarbonate is found in cells and serum. Thus, this route may occur *in vivo* and play a role in cells. ROS have been proven to directly react with genomic DNA in a chemical reaction.<sup>17</sup> Recently, reports have shown that ROS can induce the oxidative conversion of 5mC to 5hmC in a TET dioxygenase-dependent manner,<sup>18</sup> indicating ROS regulate the enzymatic catalytic reaction. We propose that the oxm<sup>6</sup>A was formed through direct oxidation by ROS *in vivo*, just like the nucleoside analogues formed in RNA induced by Fenton-type reagents.<sup>17b</sup> Further study is in progress to study the presence and biological function of oxm<sup>6</sup>A *in vivo*. The discovery of the new intermediate oxm<sup>6</sup>A and the chemical route for the demethylation of m<sup>6</sup>A to A may offer new insight into the study of m<sup>6</sup>A.

## Acknowledgements

This work was supported by the National Basic Research Program of China (973 Program) (2012CB720600, 2012CB720603), the National Science Foundation of China (nos 91213302, 21432008, 91413109) and the National Grand Program on Key Infectious Disease (2012ZX10003002-014). The oligo RNA was kindly offered by He's Group at the University of Chicago. The TOCSY spectrum was collected by Prof. Maili Liu and Dr Xiongjie Xiao at Wuhan Institute of Physics and Mathematics. We thank Prof. Bin Hu and Dr Man He for the ICP-OES service.

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