ALKBH4 is a novel enzyme that promotes translation through modified uridine regulation

Received for publication, January 11, 2023, and in revised form, July 17, 2023 Published, Papers in Press, July 26, 2023,
https://doi.org/10.1016/j.jbc.2023.105093

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Reviewed by members of the JBC Editorial Board. Edited by Karin Musier-Forsyth

Epitranscriptomics studies the mechanisms of acquired RNA modifications. The epitranscriptome is dynamically regulated by specific enzymatic reactions, and the proper execution of these enzymatic RNA modifications regulates a variety of physiological RNA functions. However, the lack of experimental tools, such as antibodies for RNA modification, limits the development of epitranscriptomic research. Furthermore, the regulatory enzymes of many RNA modifications have not yet been identified. Herein, we aimed to identify new molecular mechanisms involved in RNA modification by focusing on the AlkB homolog (ALKBH) family molecules, a family of RNA demethylases. We demonstrated that ALKBH4 interacts with small RNA, regulating the formation and metabolism of the (R)-5-carboxyhydroxymethyl uridine methyl ester. We also found that the reaction of ALKBH4 with small RNA enhances protein translation efficiency in an *in vitro* assay system. These findings indicate that ALKBH4 is involved in the regulation of uridine modification and expand on the role of tRNA-mediated translation control through ALKBH4.

RNA undergoes a variety of posttranscriptional modifications, and more than 170 modifications have been identified in various species. The recent development of antimodified RNA antibodies and next-generation sequencing revealed that RNA modifications, cumulatively known as the epitranscriptome, have important physiological functions, including the maintenance of stem cell properties and sex determination, regulation of circadian rhythms, and stability of the RNA of viruses, such as HIV (1–3). Acquired RNA modification is dynamically regulated by specific enzymatic reactions, wherein the enzymes that introduce modifications are known as writers, enzymes that remove modifications are known as erasers, and proteins that recognize and bind to RNA modifications are known as readers (3, 4). When these enzymatically regulated RNA modifications function correctly, they regulate RNA stability, subcellular localization, and splicing. However, studies on RNA modifications for which there are no available antibodies, or for modifications that are difficult to study using chemical approaches, have been limited (5, 6). Furthermore, for many RNA modifications, regulatory enzymes have not yet been identified, and their physiological functions remain unknown. Therefore, methods other than antibody and next-generation sequencing need to be developed to elucidate the biochemical mechanisms that regulate RNA modifications and their biological functions.

AlkB homolog (ALKBH) family molecules are human homologs of AlkB, an *Escherichia coli* protein which oxidatively demethylates DNA/RNA, consisting of nine family molecules (ALKBH1–8, fat mass and obesity-associated gene (FTO)). In *E. coli*, AlkB plays a role as a repair enzyme for nucleic acid alkylation damage; however, the ALKBH family has also been implicated in other complex biological functions, such as the regulation of RNA stability and efficiency of protein translation (7–10). The ALKBH family molecules use various substrates, including DNA (ALKBH1–4, FTO) (11), RNA (ALKBH1, ALKBH3, ALKBH7, ALKBH8, and FTO) (12), and proteins (ALKBH1, ALKBH4) (13, 14). By catalyzing oxidation reactions and acting as erasers of acquired modifications, the ALKBH family contributes to the functional diversification of various biomacromolecules. RNA is subject to a great variety of modifications; however, only the ALKBH and ten-eleven translocation families have been reported to act as erasers (15–18). However, although most of the ALKBH family molecules use RNA as a substrate, ALKBH2, ALKBH4, and ALKBH6 have not been reported to react enzymatically with RNA.

Among the various RNAs, tRNAs are the most frequently and diversely modified RNAs (19). tRNAs are the substrates of the ALKBH family, and we have reported that ALKBH3 improves the efficiency of protein translation (20). ALKBH3 enzymatic activity has been reported to cause the demethylation of 1-methyladenosine (m1A), 6-methyladenosine (m6A), and 3-methylcytidine (m3C), and more recently, that of 1,N6-dimethyladenosine (21). Furthermore, as an ALKBH family member that reacts with tRNA, previous studies have revealed that ALKBH8, the only ALKBH family molecule with both an RNA-binding domain and a methylation domain, is involved in 5-methoxycarbonylmethyluridine (mcm5U) and 5-carboxyhydroxymethyluridine (mchm5U) modifications. The ALKBH8 methylation domain utilizes 5-carboxymethyl uridine as a substrate to produce mcm5U (22–24). mcm5U

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in the wobble position of tRNA enhances binding to mRNA codons and stabilizes the ribosomal A site, thereby positively regulating the efficiency and integrity of protein translation (25, 26). Moreover, ALKBH8 converts mcm5U to (S)-mchm5U *via* 2-oxoglutaric acid (2-OG)- and Fe(II)-dependent oxygenase domains (23).

Therefore, in this study, we aimed to explore the possibility of ALKBH2, ALKBH4, and ALKBH6 using modified RNAs as substrates and behaving as regulators of acquired modifications, thereby representing a new molecular mechanism of RNA modification. To achieve this, we established a highly sensitive quantification method for modified nucleosides using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and an ionization technique called uniSpray. To explore new reactions involved in RNA modification, we investigated the enzymatic activity against the ALKBH family RNA using the RNA modification measurement method, which has not been reported to use RNA as a substrate. The findings of this study suggest that ALKBH4 is involved in the regulation of modified uridine, thereby suggesting the existence of a novel translational regulation system.

Results

Enzymatic activity of recombinant ALKBH4 and its role in uridine modification

We investigated the enzymatic activity of quality-confirmed recombinant proteins to investigate further reactions catalyzed by ALKBH2, ALKBH4, and ALKBH6 (Fig. S1*A*). As RNA modifications vary in amount from cell to cell and some modifications may be present in specific sequences, we considered that it may not be possible to detect the changes in modification when RNA from a single cell line reacts with the recombinant protein of ALKBH. Therefore, in this study, we mixed RNA extracted from UMUC-2, UMUC-3, A549, NCI-H520, PANC-1, MIA Paca-2, 786-O, ACHN, human embryonic kidney (HEK) 293, and HeLa cells with the ALKBH recombinant protein to examine enzyme activity (Fig. S1*B*). Initially, the recombinant protein was mixed with the RNA substrate, (2-OG), and Fe(II), which are necessary for enzyme activity. The reaction conditions were determined to allow the recombinant protein ALKBH3 to demethylate the methylated RNA modifications, m3C and m1A (Fig. S2). The mRNA, rRNA, and small RNA prepared from each cell line were mixed with recombinant ALKBHs, incubated at 37 °C for 2 h, and the RNA modifications were evaluated using UHPLC-MS/MS. The incubation of the different RNAs with recombinant ALKBH4 showed that the level of (R)-mchm5U in the small RNA fraction decreased after the reaction with recombinant ALKBH4 (Figs. 1, *A* and *B* and S3)). In this study, we detected (R)-mchm5U, a known tRNA modification, only in the small RNA fraction, which contains tRNA. In contrast, the levels of (S)-mchm5U, the optical isomer of (R)-mchm5U, remained unchanged. Among the RNAs reacted with ALKBH2, ALKBH4, and ALKBH6 in this study, only ALKBH4 led to a decrease in (R)-mchm5U levels in the small RNA fraction, suggesting that this is an ALKBH4-specific enzymatic reaction (Fig. 1*C*).

Influence of Fe(II) and 2-oxoglutarate on the regulation of RNA modification by ALKBH4

The decrease in (R)-mchm5U levels in the presence of ALKBH4 identifies (R)-mchm5U as a potential new substrate for ALKBH4. We subsequently sought to confirm that the decrease was due to the enzymatic activity of ALKBH4. When reacting small RNA with heat-denatured recombinant ALKBH4, the previously observed decrease in (R)-mchm5U levels was lost (Fig. 2*A*). (S)-mchm5U levels also showed no change (Fig. 2*B*). Since ALKBH4 has 2-OG- and Fe(II)-dependent oxygenase domains, we examined whether ALKBH4 exhibits 2-OG- and Fe(II)-dependent enzyme activities, similar to the other ALKBH family members. By varying the concentrations of 2-OG and Fe(II) in the recombinant ALKBH4 and RNA reaction solution, we demonstrated that (R)-mchm5U levels decrease in a 2-OG- and Fe(II)-dependent manner (Fig. 2, *C* and *D*), indicating that ALKBH4 reduces (R)-mchm5U levels in small RNA *via* 2-OG- and Fe(II)-dependent oxygenase activitives.

A previous study reported that ALKBH8 hydroxylates mcm5U at wobble positions in certain tRNAs to produce (S)-mchm5U (23). We hypothesized that ALKBH4 could also catalyze such a reaction, and therefore monitored mcm5U levels. We found that recombinant ALKBH4 also reduced mcm5U in small RNA (Figs. 3, *A* and *B* and S4)) in a 2-OG- and Fe(II)-dependent manner, indicating that ALKBH4 also catalyzes the conversion of mcm5U to (R)-mchm5U. However, the final RNA modification products produced by ALKBH4 catalyzing these uridine modifications have not been identified and will require further analysis. (Fig. 3*C*).

Role of ALKBH4 in the regulation of (R)-mchm5U and mcm5U levels in living cells

To investigate whether ALKBH4 regulates intracellular uridine modifications, we manipulated ALKBH4 expression in HEK293 cells. The successful introduction of ALKBH4 into HEK293 cells by an ALKBH4 expression vector was confirmed by quantitative PCR (qPCR) and Western blotting (Fig. 4, *A* and *B*). Small RNA was extracted from HEK293 cells with upregulated ALKBH4 expression, enzymatically digested to nucleosides, and analyzed for modified nucleosides by UHPLC-MS/MS. We found that the upregulation of ALKBH4 expression significantly decreased (R)-mchm5U levels and led to a decreasing trend in mcm5U levels (Fig. 4, *C* and *D*). Successful suppression of ALKBH4 expression by RNAi in HEK293 cells was confirmed by qPCR and Western blotting (Fig. 4, *E* and *F*). We found that the suppression of ALKBH4 expression increased mcm5U levels; however, (R)-mchm5U levels decreased (Fig. 4, *G* and *H*). These results indicate that ALKBH4 exhibits enzymatic activity in living cells and catalyzes the reaction of mcm5U to (R)-mchm5U.

Influence of the regulation of uridine modification by ALKBH4 on protein translation efficiency

We used small RNAs that were smaller than 200 bases and consisted of approximately 80% tRNAs. As mcm5U and

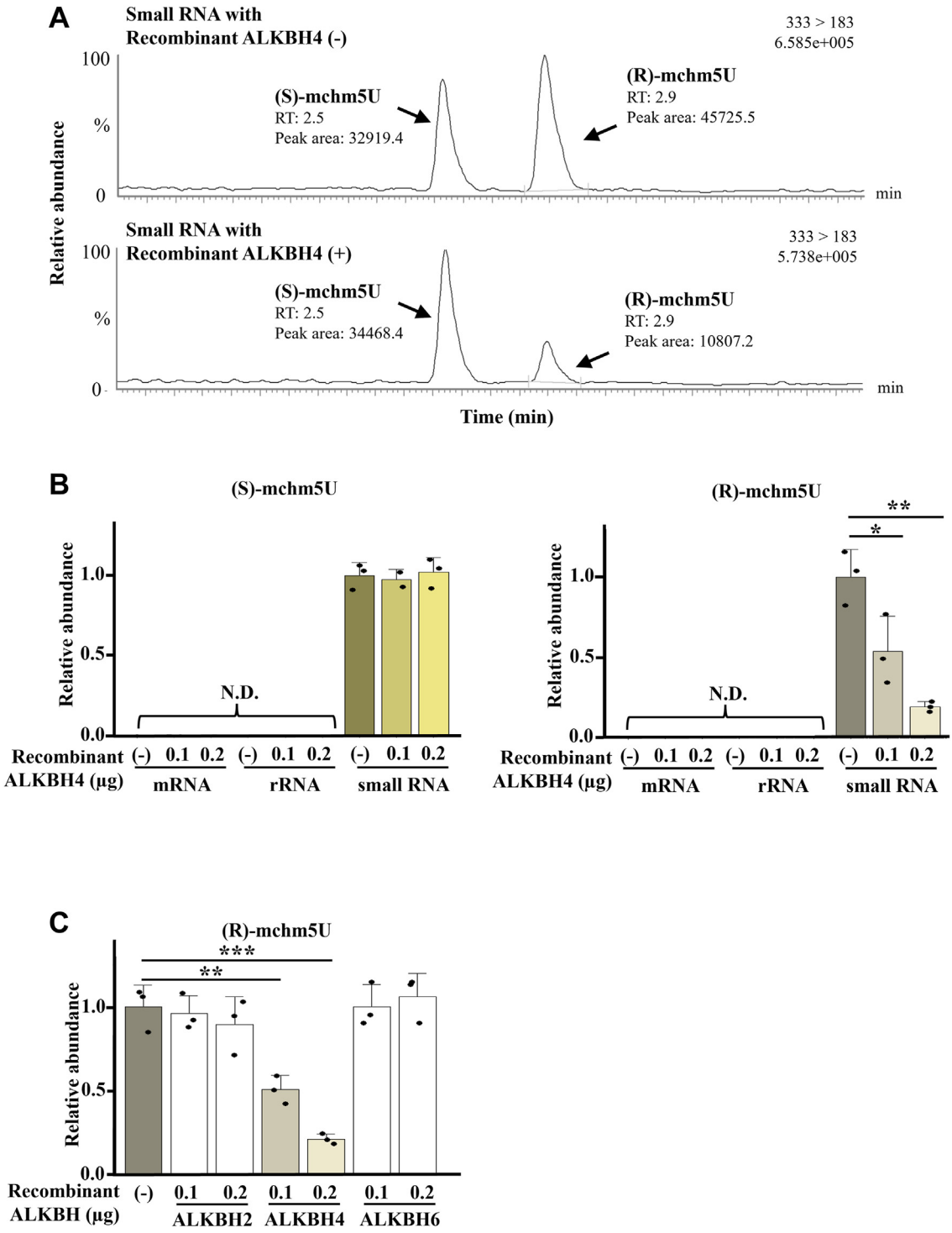

Figure 1. ALKBH4 enzymatic activity in (R)-mchm5U and (S)-mchm5U modulation. *A*, UHPLC-MS/MS tracks of (S)-mchm5U and (R)-mchm5U from the *in vitro* oxidation reaction of ALKBH4 recombinant protein with purified small RNA. Small RNA purified from UMUC-2, UMUC-3, A549, NCI-H520, PANC-1, MIA Paca-2, 786-O, ACHN, HEK293, and HeLa cells were mixed and incubated with either ALKBH4 recombinant or buffer containing only 2-OG and Fe (II). *B*, quantitative UHPLC-MS/MS analysis of (S)-mchm5U and (R)-mchm5U after reaction of ALKBH4 recombinant protein with mRNA, rRNA, and small RNA prepared from an RNA mixture of cell lines. Quantitative UHPLC-MS/MS analysis of (R)-mchm5U after the reaction of ALKBH4 recombinant protein with several types of mRNA, rRNA, and small RNA prepared from an RNA mixture of cell lines. *C*, quantification of (R)-mchm5U after the reaction of ALKBH2 and ALKBH6 recombinant proteins with small RNA prepared from an RNA mixture of cell lines. The RNA modification levels were normalized to ALKBH recombinant (–) values. Data are presented as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the ALKBH recombinant protein (–) values, one-way ANOVA followed by Bonferroni post hoc test, n = 3. 2-OG, 2-OG, 2-oxoglutaric acid; ALKBH, AlkB homolog; HEK, human embryonic kidney; N.D., not detected; mchm5U, 5-carboxyhydroxymethyluridine; UHPLC-MS/MS, ultra-high performance liquid chromatography tandem mass spectrometry.

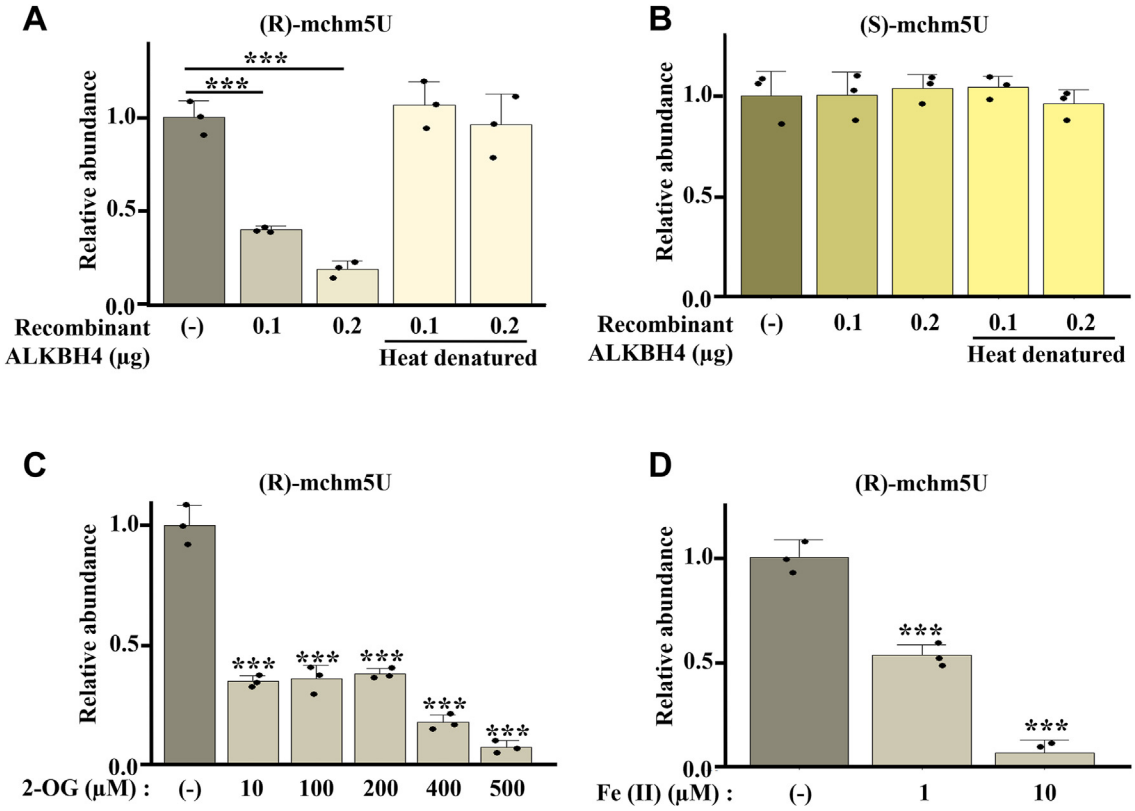

Figure 2. ALKBH4 activity follows the 2-oxoglutarate- and Fe(II)-dependent dioxygenases domain manner. *A*, quantification of (R)-mchm5U after reaction of small RNA with thermally denatured ALKBH4 recombinant protein. *B*, quantification of (S)-mchm5U after reaction of small RNA with thermally denatured ALKBH4 recombinant protein. *C*, quantification of (R)-mchm5U after reaction of small RNA with ALKBH4 recombinant protein at different 2-OG concentrations. *D*, quantification of (R)-mchm5U after reaction of small RNA with ALKBH4 recombinant protein at different Fe(II) concentrations. Data are presented as mean ± SD, ***$p < 0.001$ compared with the negative control groups without recombinant protein or cofactor, one-way ANOVA followed by Bonferroni post hoc test, n = 3. ALKBH, AlkB homolog; mchm5U, 5-carboxyhydroxymethyluridine.

(R)-mchm5U are found on uridine in the anticodon-loop position of tRNA (20), we hypothesized that ALKBH4 regulates uridine modification on tRNA. We purified the tRNA fraction by agarose gel electrophoresis of small RNA from HEK293 cells, wherein ALKBH4 expression was suppressed and approximately cut out a 70-bp band containing tRNA. The tRNA fraction was then nucleoside-degraded and analyzed by UHPLC-MS/MS. The analysis of the tRNA fraction of small RNA from HEK293 cells wherein ALKBH4 expression was suppressed, showed increased mcm5U levels (Fig. S5*A*), decreased (R)-mchm5U levels, and unchanged (S)-mchm5U levels (Fig. S5, *B* and *C*), suggesting that ALKBH4 regulates tRNA uridine modification. We evaluated whether ALKBH4-mediated modification of mcm5U and (R)-mchm5U affects protein translation efficiency using an *in vitro* protein translation system (Fig. 5*A*) and found that translated protein levels were found to be increased in a time-dependent manner (Fig. 5*B*). Furthermore, small RNA extracted from HEK293 cells with upregulated ALKBH4 expression exhibited increased protein translation (Fig. 5*C*). These findings suggest that ALKBH4 regulates tRNA uridine modification, thereby increasing the efficiency of protein translation. However, these are *in vitro* experiments, and it is unclear whether this reaction occurs in living cells. It is also unclear whether mcm5U, (R)-mchm5U, or the modifications finally generated by ALKBH4 affect protein translation, thereby suggesting that further analysis is required.

Discussion

In mammals, posttranscriptional RNA modifications occur in several types of RNAs, including mRNA, rRNA, and noncoding RNAs, such as miRNA, and the appropriate modification of individual RNAs can contribute to the maintenance of homeostasis. The greatest variation and frequency of modifications are found on tRNAs, with approximately 20% of tRNA being chemically modified. These modifications are found on the anticodon loop, D-loop, and T-loop and affect the tRNA function (19). Among anticodon-loop modifications, uridine modification at the wobble position affects codon binding, and modification at position 34 affects codon–anticodon binding; and the modifications on the D-loop and T-loop affect the tRNA conformation, stability, and translation processes. Understanding the physiological functions of chemical tRNA modifications is important for understanding complex networks *in vivo* (19).

ALKBH4 was previously shown to regulate actin–myosin interactions and actomyosin-dependent processes by demethylating $lysine_{84}$ of actin (14). Recently, ALKBH4 was reported to exhibit demethyltransferase activity toward DNA

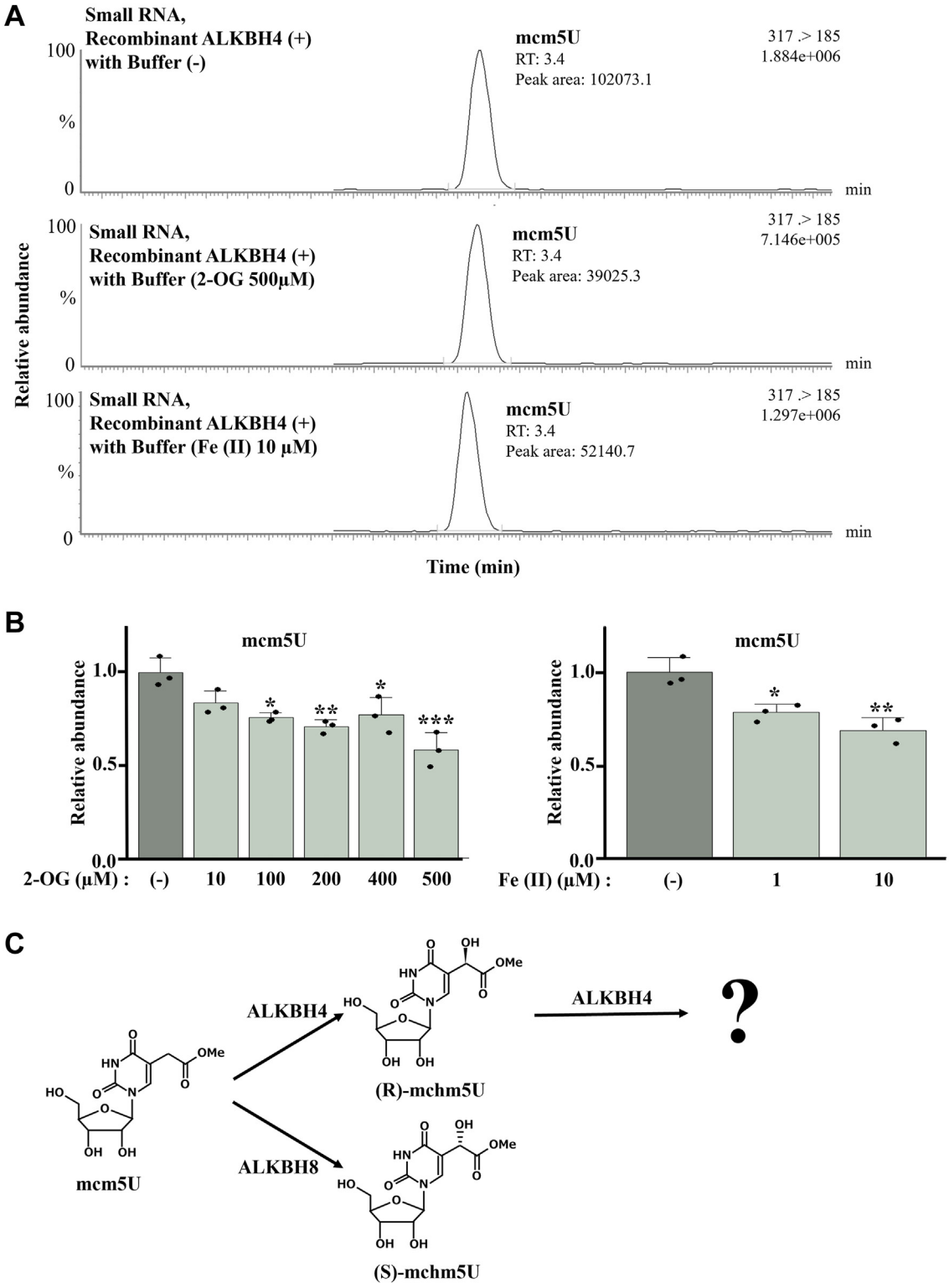

Figure 3. Optimization of buffer conditions suggesting that ALKBH4 may also contribute to mcm5U oxidation. *A*, ultra-high performance liquid chromatography tandem mass spectrometry tracks of mcm5U from the *in vitro* oxidation reaction of ALKBH4 recombinant protein with purified small RNA. Tracks for changing the 2-OG concentration in the reaction buffer from 200 to 500 μM and the concentration of Fe (II) from 40 to 10 μM. Quantification of mcm5U after the reaction of small RNA with ALKBH4 recombinant protein (*B*) at different 2-OG concentrations and different Fe(II) concentrations. *C*, reaction pathway of ALKBH4 suggested by the experimental results. ALKBH4 generates (R)-mchm5U rather than (S)-mchm5U from mcm5U and may actively modulate (R)-mchm5U. Data are presented as mean ± SD, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the negative control groups without recombinant protein or cofactor, one-way ANOVA followed by Bonferroni post hoc test, n = 3. 2-OG, 2-oxoglutaric acid; ALKBH, AlkB homolog; mcm5U, 5-methoxycarbonylmethyluridine; mchm5U, 5-carboxyhydroxymethyluridine.

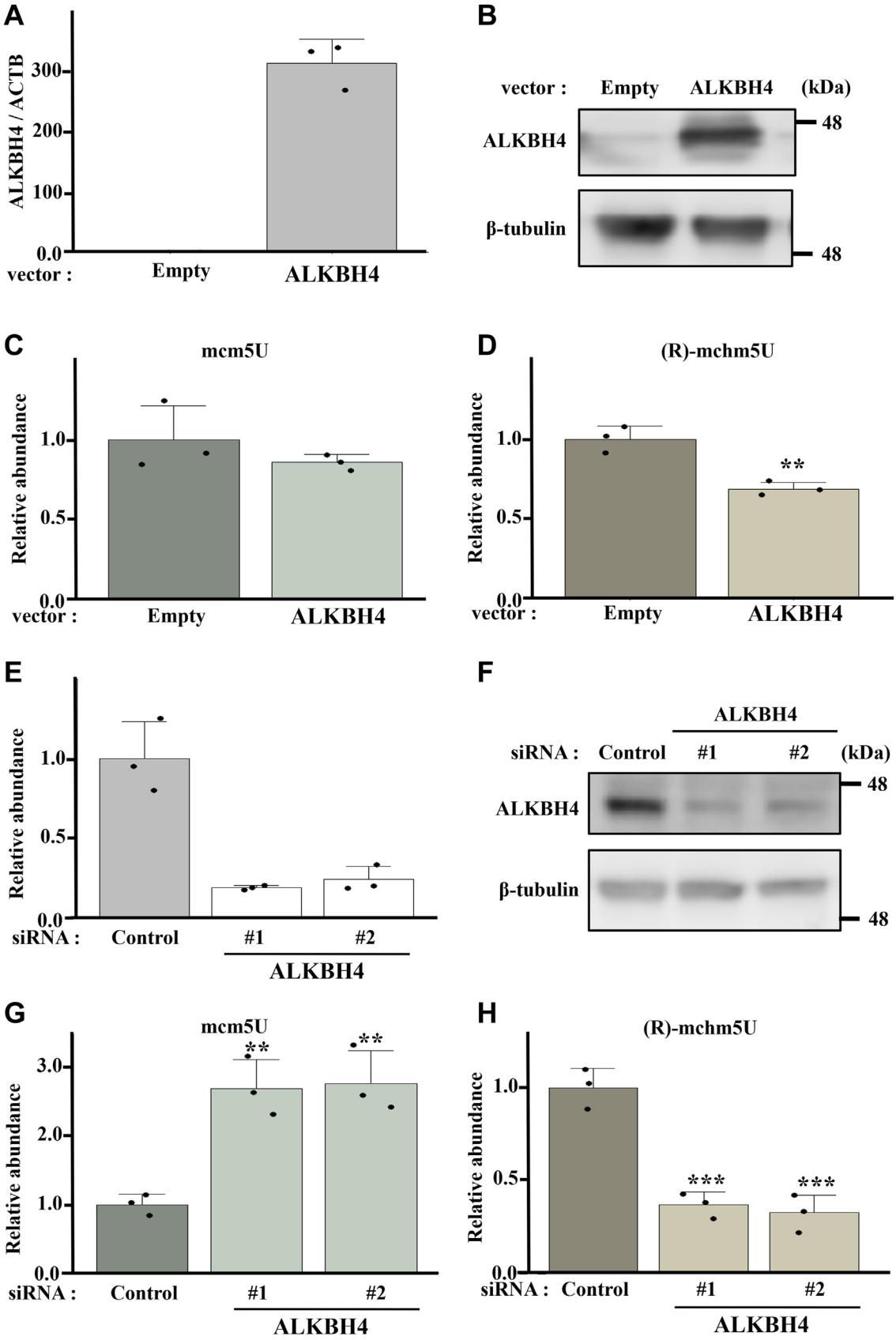

Figure 4. Role of ALKBH4 in the regulation of uridine modification in living cells. *A*, quantitative PCR analysis of ALKBH4 mRNA expression in HEK293 cells transfected with ALKBH4 expression vector. *B*, Western blot analysis of ALKBH4 protein expression in HEK293 cells transfected with ALKBH4 expression vector. *C*, comparison of mcm5U levels in HEK293 cells with upregulated ALKBH4 expression. *D*, levels of (R)-mchm5U levels in HEK293 cells with upregulated ALKBH4 expression. *E*, quantitative PCR analysis of ALKBH4 mRNA expression in HEK293 cells transfected with ALKBH4 siRNA. *F*, Western blot

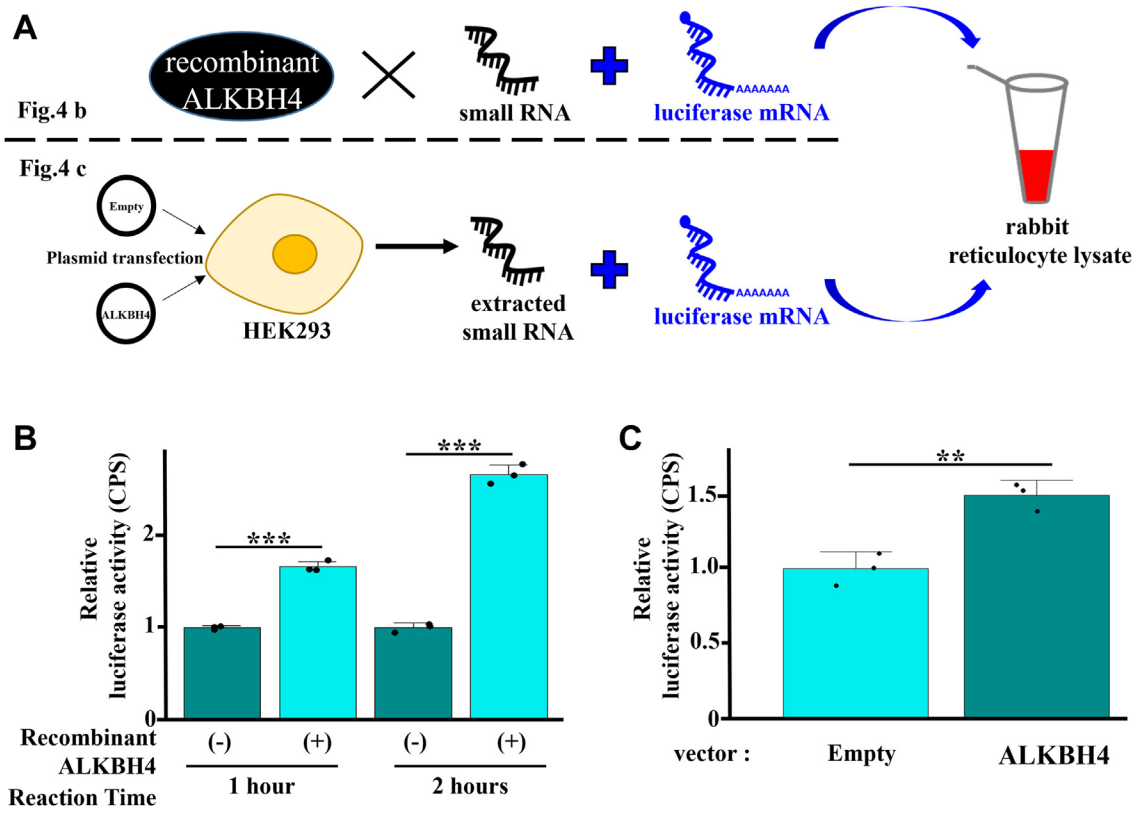

Figure 5. Effect of reaction of RNA with ALKBH4 on protein translation efficiency. *A*, schematic of *in vitro* translation experiments. Small RNA reacted with ALKBH4 recombinant protein or small RNA derived from HEK293 cells with induced ALKBH4 expression was used. The luciferase gene is translated in a translation system prepared by rabbit erythrocyte lysate, and the luciferase activity is then quantified and compared. *B*, measurement of synthetic luciferase activity when small RNA reacts with ALKBH4 recombinant protein in an *in vitro* translation experimental system. Small RNAs from HEK293 cells reacted with ALKBH4 recombinant protein for 1 and 2 h were reacted with a mixture of luciferase mRNA and rabbit erythrocyte lysate. The degree of protein translation is evaluated by the degree of luciferase activity. *C*, measurement of synthetic luciferase activity when small RNA extracted from HEK293 cells reacts with upregulated ALKBH4 expression in an *in vitro* translation experimental system. Data are presented as mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the groups without ALKBH4 recombinant protein and student's *t* test, n = 3 (*B*) and compared with empty vector transfectant groups and student's *t* test, n = 3 (*C*). ALKBH, AlkB homolog; HEK, human embryonic kidney.

m6A, thereby playing a role in the epigenetic regulation of gene expression (11). Our study revealed that ALKBH4 also exhibits enzymatic activity toward RNA and identified a novel role of ALKBH4 in the enzymatic conversion of tRNA mcm5U to (R)-mchm5U and the modification of (R)-mchm5U, indicating a novel RNA reaction pathway.

Furthermore, we showed that ALKBH4 affects the efficiency of protein translation, suggesting that the regulation of uridine modification by ALKBH4 functions as part of a system that controls gene expression. However, several factors still require elucidation. Firstly, the residues produced by the reaction of ALKBH4 with (R)-mchm5U have not yet been identified. One hypothesis is that ALKBH4 catalyzes the oxidation of the methoxycarbonyl hydroxymethyl group of (R)-mchm5U to another functional group, such as a carboxyl or hydroxymethyl group. However, no 5-carboxyluridine or 5-hydroxymethyluridine residues have been identified. Secondly, the exact location of the tRNA uridine modifications regulated by ALKBH4 has not yet been determined. Previous reports have shown that (R)-mchm5U is located in the uridine (UCG) anticodon of the $tRNA^{Arg}$ (23). In this study, we showed that ALKBH4 contributes to protein translation efficiency, but whether this is related to the regulation of the modification of this particular tRNA or whether a similar reaction mechanism exists in tRNAs encoding other amino acids needs to be elucidated. Thirdly, it is necessary to identify which protein expression is affected by the ALKBH4-mediated regulation of modified uridine. The regulation of modified uridine by ALKBH8 influences the expression of selenium-containing proteins (23, 26), indicating that tRNA modifications can regulate the expression of particular proteins. The regulation of modified uridine by ALKBH4 may therefore play a role in regulating the uptake of specific amino acids for a specific protein. Additionally, the detailed role of each modifier, such as mcm5U and (R)-mchm5U will also need to be clarified in this regard.

analysis of ALKBH4 protein expression in HEK293 cells transfected with ALKBH4 siRNA. *G*, comparison of mcm5U levels in HEK293 cells with suppressed ALKBH4 expression. *H*, levels of (R)-mcm5U in HEK293 cells with suppressed ALKBH4 expression. Data are presented as mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the empty vector transfectant groups and student's *t* test (*D*), or the control siRNA transfectant groups, one-way ANOVA followed by Bonferroni post hoc test (*G* and *H*), n = 3. ALKBH, AlkB homolog; HEK, human embryonic kidney; mcm5U, 5-methoxycarbonylmethyluridine; mchm5U, 5-carboxyhydroxymethyluridine.

There are several reports on the biological functions of ALKBH4, including its involvement in spermatogenesis (27), and its elevated expression in non–small cell lung cancer cells, contributing to cancer cell survival (28, 29). Previous studies have investigated the molecular biological functions of ALKBH4, including its role in the demethylation of the DNA m6A, and identified proteins that interact with ALKBH4 (30, 31). The results of this study suggest that the regulation of the modified RNA by ALKBH4 regulates biological phenomena. It is important to elucidate the biological functions and regulatory mechanisms of ALKBH4 through molecular biological analysis.

In conclusion, we investigated the RNA-directed enzymatic activity of the ALKBH family molecules using a previously established highly sensitive method by quantifying modified nucleosides *via* UHPLC-MS/MS and uniSpray ionization. Our results revealed that ALKBH4 regulates modified uridine and enhances protein translation efficiency, thereby expanding our knowledge of RNA reaction and translation. Future studies should aim to elucidate novel reaction pathways involving modified RNAs and their physiological functions.

Experimental procedures

Reagents and culture

The HEK293 cell line was obtained from RIKEN BRC (RIKEN BioResource Research Center) and grown at 37 °C under 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Fujifilm Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific), 2 mM L-Glutamine (Thermo Fisher Scientific), and 100 μg/ml kanamycin (Fujifilm Wako Pure Chemical Industries). The human cervical cancer cell lines HeLa, PANC-1, 786-O, and ACHN were obtained from the American Type Culture Collection, and UMUC-2 and UMUC-3 were supplied by Nara Medical University. These cell lines were grown at 37 °C under 5% CO_2 atmosphere in RPMI 1640 medium (Fujifilm Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific) and 100 μg/ml kanamycin (Fujifilm Wako Pure Chemical Industries).

RNA isolation and analysis of purified RNAs

Total larger RNA fractions, excluding RNAs less than 200 nts in length, were isolated from UMUC-2, UMUC-3, A549, NCI-H520, PANC-1, MIA Paca-2, 786-O, ACHN, HEK293, and HeLa cells using the miRNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. The small RNA fraction, enriched RNAs less than 200 nts in length, was isolated from UMUC-2, UMUC-3, A549, NCI-H520, PANC-1, MIA Paca-2, 786-O, ACHN, HEK293, and HeLa cells with the miRNeasy Mini Kit and RNeasy MinElute Cleanup Kit (Qiagen), according to the manufacturer's protocol. The Magnosphere UltraPure mRNA Purification Kit (Takara) was used to purify mRNA from large RNA. After heat-denatured RNA solution reacted with oligo(dT)30 immobilized magnetic particles, the supernatant was collected as the rRNA fraction and used for experiments. Purified RNA was analyzed with an Experion automated electrophoresis system using the RNA StdSens Analysis Kit (Bio-Rad).

In vitro *reaction of recombinant ALKBH protein and RNAs*

Recombinant ALKBH2, FLAG-His-tagged ALKBH3, FLAG-tagged ALKBH4, and FLAG-tagged ALKBH6 were expressed in silkworm pupae and purified *via* column chromatography (Sysmex). The purity of the recombinant protein was confirmed using Experion automated electrophoresis system using the Protein Analysis Kit (Bio-Rad). Purity was automatically calculated based on peak areas using Experion software (https://www.bio-rad.com/ja-jp/applications-technologies/experion-automated-electrophoresis-system?ID=LUSR04KG4). For enzyme inactivation, the recombinant ALKBH4 was thermally denatured at 100 °C for 10 min. In cases wherein ALKBH reacts with modifications present in specific RNA sequences, we anticipated the possibility that the changes caused by the enzymatic reaction would not be detectable by LC-MS/MS, if those RNA expression levels were low. Therefore, we used a mixture of RNAs extracted from multiple cell lines in this experiment. Equal amounts of mRNA, rRNA, and small RNA mixtures extracted from UMUC-2, UMUC-3, A549, NCI-H520, PANC-1, MIA Paca-2, 786-O, ACHN, HEK293, and HeLa cells were used as substrates. Subsequently, 200 ng of RNA was mixed and reacted with the ALKBH recombinant protein, whose quantity is shown in the Figure legend. Reactions were performed in 50 mM 3-morpholinopropanesulfonic acid buffer, and cofactors were required for enzyme activity. Unless otherwise listed in the Figure legend, 1 mM ascorbic acid (Fujifilm Wako Pure Chemical Industries), 0.2 mM 2-OG (Fujifilm Wako Pure Chemical Industries), 40 μM FeSO4 (Fujifilm Wako Pure Chemical Industries), 2 mM tris(2-carboxyethyl)phosphine (Thermo Fisher Scientific), and 0.04% NP-40 substrate (Fujifilm Wako Pure Chemical Industries) were added to a total solution of 250 μl with or without recombinant ALKBH proteins and incubated at 37 °C for 2 h. Unless otherwise stated, 200 ng recombinant ALKBH protein was used. RNA was then purified by ethanol precipitation and further examined. The concentration of each reagent was appropriately varied to evaluate the effects on recombinant ALKBH4 activity.

In vitro *translation reaction and luciferase assay*

Flexi Rabbit Reticulocyte Lysate System (Promega) was used in the experiment with some modifications to the manufacturer's protocol. For each experiment, 10 μl reaction mixture containing 7 μl Flexi rabbit reticulocyte lysate, 0.1 μl 1 mM amino acid mixture without leucine, 0.1 μl 1 mM amino acid mixture without methionine, 0.28 μl 2.5 M potassium chloride, 0.1 μg luciferase control RNA, and 0.1 or 0.2 μg small RNA was incubated for 10 min at 30 °C. To prevent the composition

of the reaction solutions from varying among samples, the reaction mixture was premixed and dispensed in equal volumes. In the assays of silkworm recombinant ALKBH4 and mock control–treated small RNAs, 0.1 μg small RNA was used. In the assay of small RNA derived from HEK293 cells with upregulated ALKBH4 expression, 0.2 μg small RNA was used. The luciferase activity was measured using an EnVision Multilabel Reader (PerkinElmer).

Digestion of RNA into nucleosides for mass spectrometry

Five microliters 0.1 M CH_3COONH_4 (pH 5.3) and 0.5 units nuclease P1 (Fujifilm Wako Pure Chemical Industries) were added to 200 ng purified RNA in 30 μl H_2O and incubated for 2 h at 45 °C. Subsequently, 0.05 units of bacterial alkaline phosphatase (Takara) were added and incubated for 2 h at 37 °C. Then, 60 μl H_2O and 20 μl chloroform (Fujifilm Wako Pure Chemical Industries) were added to the mixture. The sample was vortexed, and the resulting suspension was centrifuged for 5 min at 5000*g*. The aqueous layer was collected and evaporated. The resulting nucleoside residues were redissolved in ultrapure water (Fujifilm Wako Pure Chemical Industries).

UHPLC-MS/MS conditions

This procedure was performed in accordance with our previously developed method (32). UHPLC-UniSpray-MS/MS analyses were conducted on a Waters ACQUITY UPLC system (Waters) coupled to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters). Liquid chromatography separations were conducted on an ACQUITY UPLC CSH C18 (1.7 μm, 2.1 × 100 mm) column (Waters) at 42 °C and a flow rate of 0.4 ml min^{-1}. The mobile phase consisted of solvent A (5 mM CH_3COONH_4 in H_2O) and solvent B (5 mM CH_3COONH_4 in MeOH), beginning with 99% solvent A/1% solvent B, followed by a 6 min linear gradient of 1% to 32% solvent B, a 0.5 min linear gradient of 32% to 99% solvent B, 4 min at 99% solvent B, and 4.5 min re-equilibration with the initial mobile phase. Data for one run were acquired for 15 min. Samples were analyzed in a random order to avoid systemic bias. To improve detection sensitivity, a UniSpray ion source (Waters) was used for nucleoside ionization. The UniSpray ionization parameters were as follows: source temperature, 125 °C; desolvation temperature, 500 °C; desolvation gas flow, 800 L h^{-1}; and cone gas flow, 150 L h^{-1}. The Xevo TQ-XS triple quadrupole mass spectrometer (Waters) was operated in positive ion mode to monitor four major nucleosides, 42 modified nucleosides, and dG15N5 (IS). Ion transitions, cone voltage, and collision energy were determined using MassLynx V4.2 IntelliStart software (Waters; https://www.waters.com/waters/en_US/MassLynx-MS-Software/nav.htm?cid=513662&lset=1&locale=en_US&changedCountry=Y). The multiple reaction monitoring transitions (*m/z* of precursor and product ion) and parameters (cone voltage and collision energy) for the nucleosides are listed in Table S1 1. Data acquisition and analysis were conducted using MassLynx V4.2 and TargetLynx XS (Waters).

Nucleosides standard for UHPLC-MS/MS analysis

The full names of nucleosides are listed in Table S1 1. A, C, G, and I were obtained from Fujifilm Wako Pure Chemical Industries; S4U was obtained from Abcam; hm5C was obtained from Berry & Associates; t6A was obtained from Biolog; m1A, m6A, Im, m1G, ac4C, f5C, mcm5U, m5Um, and Y were obtained from Carbosynth; i6A, ho5U, m3Um, D, and m5D were obtained from Granlen; m7G and mcm5S2U were obtained from Santa Cruz Biotechnology; m2G and U were obtained from Sigma-Aldrich; Am, Gm, m5C, Cm, m5U, and Um were obtained from Tokyo Chemical Industry; m6, 6A, m6Am, m1I, m22G, m3C, and S2U were obtained from Toronto Research Chemicals; dG15N5 was obtained from Cambridge Isotope Laboratories; and m6t6A, cm5U, ncm5U, nchm5U, (R),(S)-mchm5U, mnm5S2U, mcm5Um, and ncm5Um were synthesized by the Graduate School of Pharmaceutical Sciences, Osaka University.

ALKBH4 knockdown by siRNA transfection

HEK293 cells were seeded at 10 × 10^5 cells/well in a 12-well plate and transfected either with 5 nM control siRNA (Thermo Fisher Scientific/4390844), ALKBH4 siRNA#1 (Thermo Fisher Scientific/s29432), or ALKBH4 siRNA#1 (Thermo Fisher Scientific/s29434) using 3 μl Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. At 24 h after transfection, the cells were replated in 6-well plates and incubated for another 48 h.

ALKBH4 overexpression by vector transfection

Empty or ALKBH4 expression p3 × FLAG-CMV-14 (Sigma-Aldrich) vectors were transfected into HEK293 cells seeded at 10 × 10^5 cells/well in 12-well plates using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) and P3000 Enhancer Reagent (Thermo Fisher Scientific), according to the manufacturer's protocol. At 24 h after transfection, the cells were replated in 6-well plates and incubated for another 48 h.

Western blot analysis

Cell lysates were resolved on 10% SDS-polyacrylamide gel (Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Merck Millipore). Membranes were blocked with 5% skim milk (Morinaga Milk Industry) at room temperature (20–25 °C) for 30 min and subsequently incubated overnight with anti-ALKBH4 antibody (NBP2-14737; Novus Biologicals) or anti-β-tubulin antibody (T4026; Sigma-Aldrich) at 4 °C. This was followed by incubation with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G (Santa Cruz Biotechnology) for 1 h. Bound horseradish peroxidase conjugates were visualized using the ECL Prime Western Blotting System (GE Healthcare) and captured using Amersham Imager 680 (GE Healthcare).

Quantitative real-time PCR

Reverse transcription of 500 ng large RNA was performed using the PrimeScript RT-PCR Kit (Takara), and quantitative real-time PCR was performed using THUNDERBIRD SYBER qPCR mix (TOYOBO) with the CFX96 Real-Time System (Bio-Rad), according to the manufacturer's protocol. Thermal cycling conditions included an initial step at 95 °C for 10 s, 40 cycles at 95 °C for 5 s, and 60 °C for 20 s. The ALKBH4- and ACTB-specific primers were as follows: ALKBH4 forward: 5′-TGATGCTGATCGAGGACTTTGTG-3′, reverse: 5′-AAGCCCTCGGTCTTTAGCTTCTG-3′; ACTB forward: 5′-GGCACCCAGCACAATGAA-3′, reverse: 5′-CTAAGTCA TAGTCCGCCTAGAAGCA-3′.

Quantification and statistical analyses

No statistical methods or criteria were used to estimate sample size or to include or exclude samples. Statistical information, such as n and error calculations, is provided in the figure legends. Cultured cell data were collected from three biological replicates. Three immunoblots were performed on biologically independent samples and similar results were obtained. For all other experiments in this study, at least three biologically independent replicates were used, and data are presented as mean ± SD. The two-tailed Student's *t* test was used to evaluate the statistical significance of differences between two groups, whereas multiple comparisons were made by one-way ANOVA, followed by Bonferroni's multiple comparison test. Microsoft Excel (Microsoft; https://www.microsoft.com/en-us/microsoft-365/excel) and RStudio (R-Tools Technology; https://posit.co/download/rstudio-desktop/) were used for data graphing. GraphPad Prism (GraphPad; https://www.graphpad.com/features) software was used for all statistical analyses.

Data availability

All other data are available from the corresponding author upon reasonable request. This session contains all data availability information in this study.

Supporting information—This article contains supporting information.

Acknowledgments—We would like to thank Editage (http://www.editage.com) for editing and reviewing this manuscript for English language. This research was partially supported by the Research Support Project for Life Science and Drug Discovery (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]) from AMED (Grant Number JP22ama121054) and supported by JSPS KAKENHI (Grant Number 18K14653).

Author contributions—T. K. investigation; T. K. conceptualization; H. H. writing-review and editing; H. H. funding acquisition; H. H., Y. U., K. J., and K. T. supervision; M. T., A. T., and K. K. resources; K. T. project administration.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: 2-OG, 2-oxoglutaric acid; ALKBH, AlkB homolog; FTO, fat mass and obesity-associated gene; HEK, human embryonic kidney; LC, liquid chromatography; m1A, 1-methyladenosine; m3C, 3-methylcytidine; m6A, 6-methyladenosine; mchm5U, 5-carboxyhydroxymethyluridine; mcm5U, 5-methoxycarbonylmethyluridine; qPCR, quantitative PCR; UHPLC-MS/MS, ultra-high performance liquid chromatography tandem mass spectrometry.

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