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ALKBH8-mediated formation of a novel diastereomeric pair of wobble nucleosides in mammalian tRNA

Erwin van den Born^{1,*}, Cathrine B. Vågbo^{2,*}, Lene Songe-Møller^{3,*}, Vibeke Leihne¹, Guro F. Lien³, Grazyna Leszczynska⁴, Andrzej Malkiewicz⁴, Hans E. Krokan², Finn Kirpekar⁵, Arne Klungland^{3,6} & Pål Ø. Falnes^{1,3}

Mammals have nine different homologues (ALKBH1–9) of the *Escherichia coli* DNA repair demethylase AlkB. ALKBH2 is a genuine DNA repair enzyme, but the *in vivo* function of the other ALKBH proteins has remained elusive. It was recently shown that ALKBH8 contains an additional transfer RNA (tRNA) methyltransferase domain, which generates the wobble nucleoside 5-methoxycarbonylmethyluridine (mcm⁵U) from its precursor 5-carboxymethyluridine (cm⁵U). In this study, we report that (*R*)- and (*S*)-5-methoxycarbonylhydroxymethyluridine (mchm⁵U), hydroxylated forms of mcm⁵U, are present in mammalian tRNA^{Arg}_{UCG} and tRNA^{Gly}_{UCC}, respectively, representing the first example of a diastereomeric pair of modified RNA nucleosides. Through *in vitro* and *in vivo* studies, we show that both diastereomers of mchm⁵U are generated from mcm⁵U, and that the AlkB domain of ALKBH8 specifically hydroxylates mcm⁵U into (*S*)-mchm⁵U in tRNA^{Gly}_{UCC}. These findings expand the function of the ALKBH oxygenases beyond nucleic acid repair and increase the current knowledge on mammalian wobble uridine modifications and their biogenesis.

¹ Department of Molecular Biosciences, University of Oslo, PO Box 1041 Blindern, Oslo 0316, Norway. ² Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim 7489, Norway. ³ Centre for Molecular Biology and Neuroscience, Department of Molecular Microbiology, Institute of Medical Microbiology, Oslo University Hospital, Oslo, Norway. ⁴ Institute of Organic Chemistry, Technical University, Lodz 90-924, Poland. ⁵ Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M 5230, Denmark. ⁶ Institute of Basic Medical Sciences, University of Oslo, PO Box 1018 Blindern, Oslo 0315, Norway. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to A.K. (email: arne.klungland@rr-research.no) or to P.Ø.F. (email: pal.falnes@imbv.uio.no).

Transfer RNA (tRNA) modification is important both for correct folding and for optimal interaction with components of the protein synthesis machinery, such as aminoacyl transferases, messenger RNA (mRNA) and the ribosome. The nucleotide at the wobble position in the anticodon loop of tRNA interacts with the third nucleotide of the codon in mRNA, and wobble uridines are usually modified, both in bacteria and eukaryotes^{1,2}. Modification of wobble uridines ensures efficient decoding of both cognate and non-cognate codons^{3,4}, and prevents misreading of codons from 'split' boxes, where purine- and pyrimidine-ending codons encode different amino acids¹.

In eukaryotes, most wobble uridines carry the modification 5-methoxycarbonylmethyluridine (mcm⁵U), 5-carbamoylmethyluridine (ncm⁵U) or derivatives thereof (Fig. 1a). These modifications and their

biogenesis have been most closely studied in the yeast *Saccharomyces cerevisiae*. Here, tRNA isoacceptors that contain ncm⁵U decode in the 'family' codon boxes, in which all four codons encode the same amino acid, whereas tRNAs containing mcm⁵U usually decode in the split codon boxes. The Elongator complex has been shown to mediate an early step in mcm⁵U/ncm⁵U formation⁵, whereas the final methyl esterification reaction leading to the formation of mcm⁵U is catalysed by the methyltransferase (MT) Trm9 (ref. 6).

The 2OG/Fe(II) (2-oxoglutarate- and Fe²⁺-dependent) oxygenase superfamily encompasses enzymes involved in important processes, for example, generation of hydroxyproline in collagen, regulation of hypoxia-responsive genes, demethylation of histone proteins, nucleic acid repair and, as discovered recently, introduction of the epigenetic modification 5-hydroxymethylcytosine (5hmC) in DNA⁷⁻⁹. The

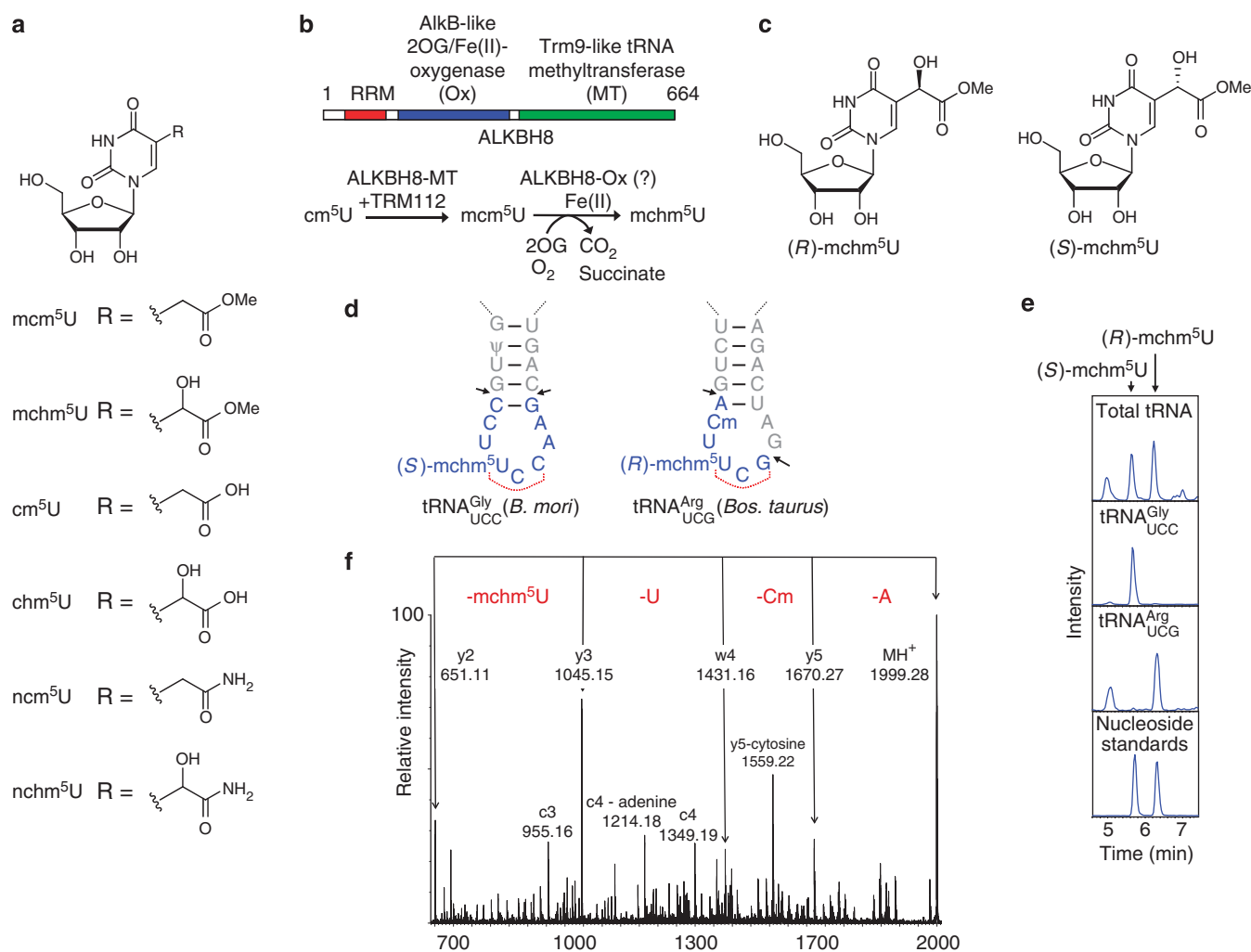


Figure 1 | Presence of two mcm⁵U diastereomers in the wobble position of mammalian tRNA. (a) Wobble uridine modifications described in the present work. mcm⁵U, 5-methoxycarbonylmethyluridine; mchm⁵U, 5-methoxycarbonylhydroxymethyluridine; chm⁵U, 5-carboxyhydroxymethyluridine; cm⁵U, 5-carboxymethyluridine; ncm⁵U, 5-carbamoylmethyluridine; nchm⁵U, 5-carbamoylhydroxymethyluridine. (b) Domain structure and proposed function of ALKBH8. RRM, RNA recognition motif. Together with the accessory protein TRM112, the MT domain of ALKBH8 converts cm⁵U into mcm⁵U. In the present work, the ALKBH8 oxygenase has been investigated for its ability to hydroxylate mcm⁵U into mchm⁵U. (c) Chemical structure of the two mchm⁵U diastereomers. (d) The anticodon stem-loop region of silkworm tRNA^{Gly}_{UCC} and calf tRNA^{Arg}_{UCG}. Anticodon (red line) containing products (blue) generated by RNase T1 cleavage (arrows), and analysed by MALDI-TOF MS, are indicated. (e) LC-MS/MS analysis of mchm⁵U nucleosides in calf liver total tRNA and isolated isoacceptors. (f) MALDI-quadrupole TOF tandem mass spectrometry of the RNase T1 product harbouring the anticodon of tRNA^{Arg}_{UCG}. Fragmentation pattern suggests the sequence A-Cm-U-mchm⁵U-C-G>p as indicated. c-ions contain the original 5'-end after cleavage of the phosphodiester bond between the phosphorous and the 5'-oxygen; y-ions contain the original 3'-end after cleavage of the phosphodiester bond between the phosphorous and the 5'-oxygen. w-ions contain the original 3'-end after cleavage of the phosphodiester bond between the 3'-carbon and the 3'-oxygen. The associated number gives the size of the fragment ion in nucleotide residues. >p denotes 2'-3' cyclic phosphate.

2OG/Fe(II) oxygenase AlkB from *Escherichia coli* is a repair enzyme that can demethylate lesions in DNA and RNA, such as 1-methyladenine and 3-methylcytosine^{10–12}. Bioinformatics analysis has identified a family of nine different mammalian AlkB homologues (ALKBH), including the obesity-associated protein FTO^{13,14}. Although *in vitro* repair activities have been observed for some ALKBH proteins^{10,13,15,16}, only ALKBH2 has been firmly established as a repair enzyme¹⁷, and non-repair functions have also been proposed^{18–20}. We and others recently showed that ALKBH8 contains a MT domain, which is the functional mammalian Trm9 homologue, and requires a small accessory protein, TRM112, for activity (Fig. 1b)^{21,22}.

As hydroxylation is the primary reaction catalysed by 2OG/Fe(II) oxygenases in animals, and as the MT domain of ALKBH8 is involved in mcm⁵U biogenesis, we were intrigued by a report documenting the presence of (S)-5-methoxycarbonylhydroxymethyluridine ((S)-mchm⁵U), a hydroxylated form of mcm⁵U, in tRNA_{UCC}^{Gly} from the silkworm *Bombyx mori*²³ (Fig. 1c,d). Mammalian and worm tRNA_{UCC}^{Gly} are highly similar, and the primary sequence of the anticodon loop is identical (Supplementary Fig. S1). This suggested that (S)-mchm⁵U may be present also in mammalian tRNA, and that the ALKBH8 oxygenase could be responsible for its formation (Fig. 1b). Indeed, whilst our manuscript was under preparation, Fu *et al.* independently reported that the AlkB domain of murine ALKBH8 can hydroxylate wobble mcm⁵U into (S)-mchm⁵U in a synthetic substrate resembling the anticodon stem-loop of tRNA_{UCC}^{Gly}²⁴.

In the present work, we demonstrate that (S)-mchm⁵U is found in the wobble position of mammalian tRNA_{UCC}^{Gly}, and that its diastereomer, (R)-mchm⁵U, is found in tRNA_{UCC}^{Arg}. Moreover, we show through studies of gene-targeted mice and recombinant enzymes that the MT domain of ALKBH8 provides the mcm⁵U precursor required for formation of mchm⁵U in tRNA_{UCC}^{Gly} and tRNA_{UCC}^{Arg}, whereas its AlkB domain represents an evolutionary conserved oxygenase that catalyses the hydroxylation of wobble mcm⁵U to (S)-mchm⁵U in tRNA_{UCC}^{Gly}.

Results

Analysis of mchm⁵U modifications in mammalian tRNA. To investigate the possible presence of mchm⁵U in mammalian tRNA, we analysed the modification status of nucleosides from bovine tRNA by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). In this analysis, a triple-quadrupole mass spectrometer was used to specifically select the relevant nucleoside ions, induce fragmentation and detect the resulting nucleobase fragment ions. Additional specificity was obtained by comparing chromatographic retention times with those of synthetic nucleoside standards. This allows highly specific, sensitive and simultaneous quantification of several nucleosides. Intriguingly, substantial amounts of both the *S* and *R* diastereomers of mchm⁵U were detected in total bovine tRNA nucleosides (Fig. 1e). We next isolated individual, wobble uridine-containing isoacceptors by hybridization to complementary, immobilized DNA oligonucleotides. We found, as expected, (S)-mchm⁵U to be present in tRNA_{UCC}^{Gly}, whereas tRNA_{UCC}^{Arg} contained (R)-mchm⁵U (Fig. 1e). We were unable to identify additional wobble uridine-containing tRNA isoacceptors carrying a mchm⁵U modification (data not shown). The presence of wobble (R)-mchm⁵U in tRNA_{UCC}^{Arg} was supported by matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF) mass spectrometry of an RNase T1 digest of the purified isoacceptor. Sequencing by MALDI-quadrupole-TOF tandem mass spectrometry confirmed the presence of mchm⁵U at the wobble position, and the fragmentation pattern also revealed ribose methylation of C32 (ref. 25), a very common tRNA modification (Fig. 1d,f)².

Analysis of wobble uridines in Alkbh8-targeted mice. To address the possible role of the ALKBH8 oxygenase in mchm⁵U biogenesis, we used a mouse model, in which essential exons of the *Alkbh8* gene

had been deleted (*Alkbh8*^{-/-}; ref. 22). Moreover, we developed two novel ‘knock-in’ (KI) mice in which an oxygenase-deficient *Alkbh8* transgene (*KI(MT⁺)*) or a MT-deficient *Alkbh8* transgene (*KI(Ox⁺)*) was expressed from the *Rosa26* or *Hprt* loci, respectively (Supplementary Figs S2–S5). These transgenes, or the combination of the two (*KI(MT⁺/Ox⁺)*), were introduced into the *Alkbh8*^{-/-} background (Fig. 2a). The expression of the transgenes was verified by reverse transcription and PCR (data not shown). All the mouse models were devoid of any obvious phenotype. First, we analysed by LC–MS/MS, total tRNA nucleosides from the different mice for the presence of the two mchm⁵U diastereomers. As expected, wild-type (WT) tRNA contained both (*R*)- and (*S*)-mchm⁵U (Fig. 2b). tRNA from the *Alkbh8*^{-/-} and *KI(Ox⁺)* mice, which lack functional MT activity, were devoid of both (*R*)- and (*S*)-mchm⁵U, whereas the *KI(MT⁺)* tRNA contained exclusively (*R*)-mchm⁵U. These data indicate that the formation of either mchm⁵U diastereomer requires the MT function, whereas the oxygenase is required for the formation of (*S*)-mchm⁵U only. This implies that a second unknown oxygenase, in the following referred to as OxX, is responsible for the hydroxylation of mcm⁵U to (*R*)-mchm⁵U. Furthermore, *KI(MT⁺/Ox⁺)* tRNA contained, like WT tRNA, both mchm⁵U stereoisomers (Fig. 2b), indicating that the MT and oxygenase activities of ALKBH8 can be uncoupled and provided by two individual proteins.

To study how manipulation of ALKBH8 activity affects the formation of modified uridines *in vivo*, we also measured the levels of various other modifications (Fig. 2c, Supplementary Figs S6 and S7). tRNA isolated from *KI(MT⁺)* mice displayed a strong accumulation of mcm⁵U, consistent with mcm⁵U being a direct substrate for the ALKBH8 oxygenase. In agreement with previous results²², we detected substantial amounts of 5-carboxymethyluridine (cm⁵U) and elevated levels of ncm⁵U in tRNA from the MT-deficient *Alkbh8*^{-/-} and *KI(Ox⁺)* mice. Notably, we also detected 5-carbamoylhydroxymethyluridine (nchm⁵U) and trace amounts of 5-carboxyhydroxymethyluridine (chm⁵U), the hydroxylated forms of ncm⁵U and cm⁵U, in tRNA from the MT-deficient mice (note that the *R* and *S* diastereomers of these nucleosides could not be separated chromatographically). This indicates that OxX and possibly also the ALKBH8 oxygenase will hydroxylate ncm⁵U, and to a lesser extent, cm⁵U, when mcm⁵U is absent.

Enzymatic activity of recombinant ALKBH8 on murine tRNA. To investigate whether the observed hypomodification of tRNA from gene-targeted mice could be reversed by recombinant enzymes, total tRNA from mouse liver was incubated with *E. coli*-expressed human ALKBH8, or combinations of its individual oxygenase (ALKBH8-Ox) and MT (ALKBH8-MT) domains (Fig. 2d,e). The results demonstrated that ALKBH8-Ox hydroxylates mcm⁵U into (S)-mchm⁵U, and corroborated the notion that the two ALKBH8 enzymatic domains can function independently (Fig. 2e; see Fig. 2c for samples not treated with enzyme; Supplementary Fig. S8). The latter was further substantiated by the observation that the oxygenase and MT functions of ALKBH8 could be selectively inhibited by the Fe(II) chelator EDTA and the MT inhibitor S-adenosylhomocysteine, respectively (Supplementary Fig. S8). Moreover, ALKBH8-Ox, but not a mutant with putative Fe(II) coordinating residues mutated, displayed the expected Fe(II)-dependent ability to convert 2OG to succinate (Supplementary Fig. S9), supporting the notion that ALKBH8 is a 2OG/Fe(II) oxygenase.

Modification status of tRNA_{UCC}^{Gly} from the Alkbh8-targeted mice. As our results strongly indicated that tRNA_{UCC}^{Gly} is the substrate of the ALKBH8 oxygenase, we also specifically investigated the modification status of this isoacceptor from the different gene-targeted mice. WT tRNA_{UCC}^{Gly} contained not only the expected (S)-mchm⁵U modification, but also some chm⁵U (Fig. 2f, Supplementary Fig. S10), possibly due to non-enzymatic hydrolysis of the labile ester

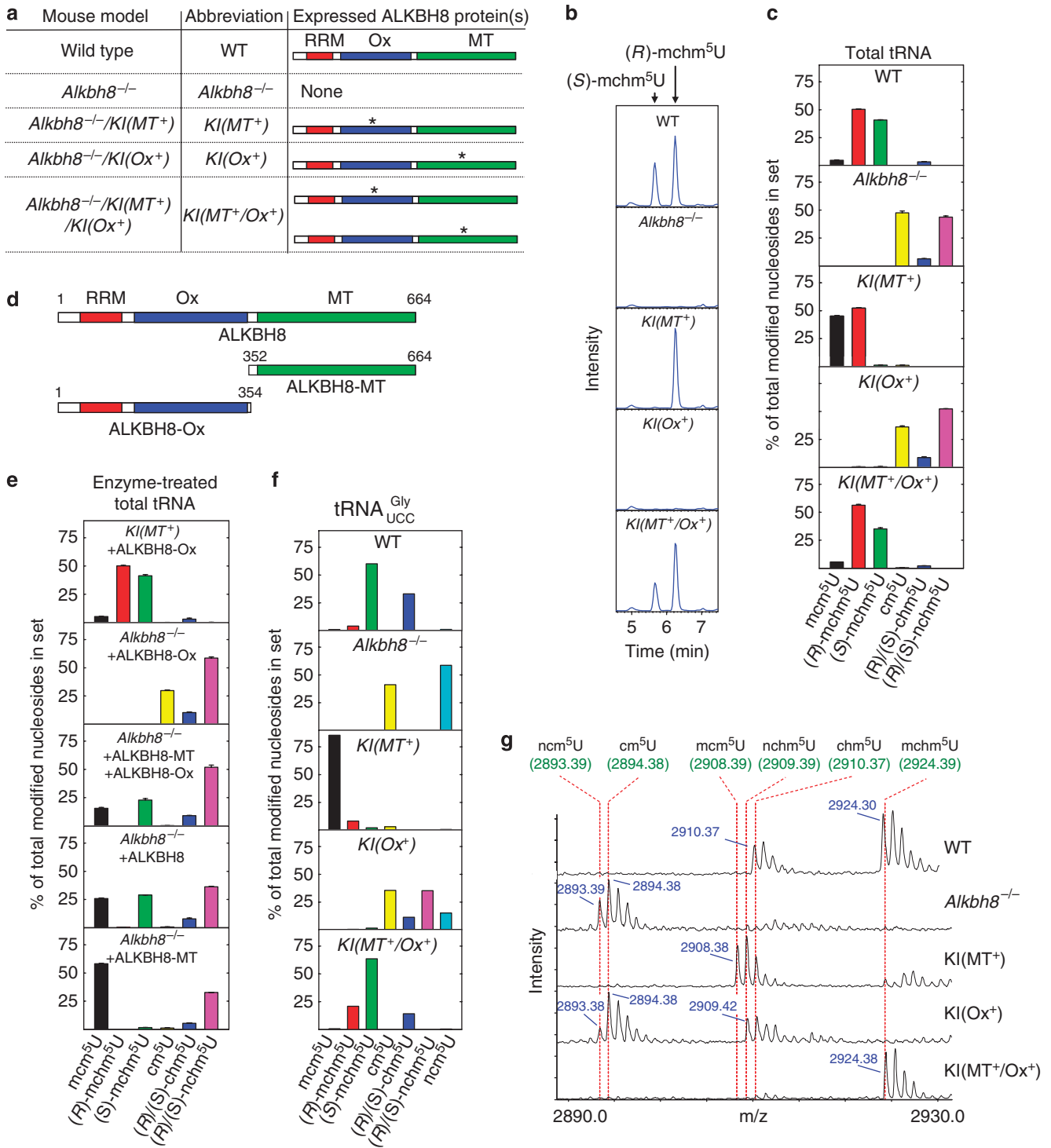


Figure 2 | Modification status of tRNA from *Alkbh8*-targeted mice. (a) Schematic representation of the ALKBH8 proteins expressed in the different mouse models. An asterisk indicates that the domain has been inactivated by point mutations. (b) Analysis of R and S diastereomers of mchm⁵U in total tRNA from gene-targeted mice. Murine tRNA was enzymatically degraded to nucleosides, which were analysed by LC-MS/MS. (c) Quantification of uridine modifications in total murine tRNA by LC-MS/MS. The level of each nucleoside is expressed as the molar percentage of the total amount of the modifications indicated in the figure. (d) Recombinant, *E. coli*-expressed ALKBH8 proteins, or individual domains, used for incubation with murine tRNA. (e) Modification status of murine tRNA treated with human ALKBH8 or its individual domains. ALKBH8 and ALKBH8-MT were associated with co-expressed, co-purified TRM112. (f) Modification status of murine tRNA^{Gly UCC} measured by LC-MS/MS. (g) MALDI-TOF analysis of the anticodon-containing RNase T1 product of tRNA^{Gly UCC}. The theoretical monoisotopic mass (green) as well as the measured mass (blue) of relevant modifications are indicated. Because of the natural isotope distribution, each modification gives rise to a cluster of peaks with 1.0 Da spacing where the leftmost peak corresponds to the monoisotopic mass. Overlapping isotope clusters from ncm⁵U and cm⁵U with clearly distorted peak patterns occur in the *Alkbh8*^{-/-} and *KI*(Ox⁺) samples. Error bars represent range between duplicate samples in a typical experiment.

bond in (S)-mchm⁵U, as previously reported²³. In agreement with previous findings²², *Alkbh8*^{-/-} tRNA^{Gly}_{UCC} contained ncm⁵U and cm⁵U, whereas *KI(Ox⁺)* tRNA^{Gly}_{UCC} contained nchm⁵U and chm⁵U in addition, indicating that ALKBH8-Ox, in the absence of its cognate mcm⁵U substrate can hydroxylate cm⁵U and ncm⁵U, albeit inefficiently. Moreover, *KI(MT⁺)* tRNA^{Gly}_{UCC} displayed the expected accumulation of mcm⁵U. We performed MALDI-TOF mass spectrometry of an RNase T1 digest of the isolated tRNA^{Gly}_{UCC} to verify its identity and to confirm the presence of mchm⁵U in its anticodon loop. Clearly, WT tRNA^{Gly}_{UCC} contained the expected ~2924.3 Da product corresponding to a (S)-mchm⁵U-modified anticodon loop (Figs 1d and 2g). Moreover, tRNA^{Gly}_{UCC} from the gene-targeted mice gave rise to alternative additional peaks, agreeing with the altered modification pattern observed by LC-MS/MS.

Evolutionary distribution of ALKBH8 and mchm⁵U modifications. On the basis of sequence homology searches, the ALKBH8 oxygenase appears to be present in most multicellular eukaryotes, such as mammals, bee (*Apis mellifera*), worm (*Caenorhabditis elegans*) and plant (*Arabidopsis thaliana*; Supplementary Fig. S11). Accordingly, we detected (S)-mchm⁵U in all these organisms (Fig. 3a),

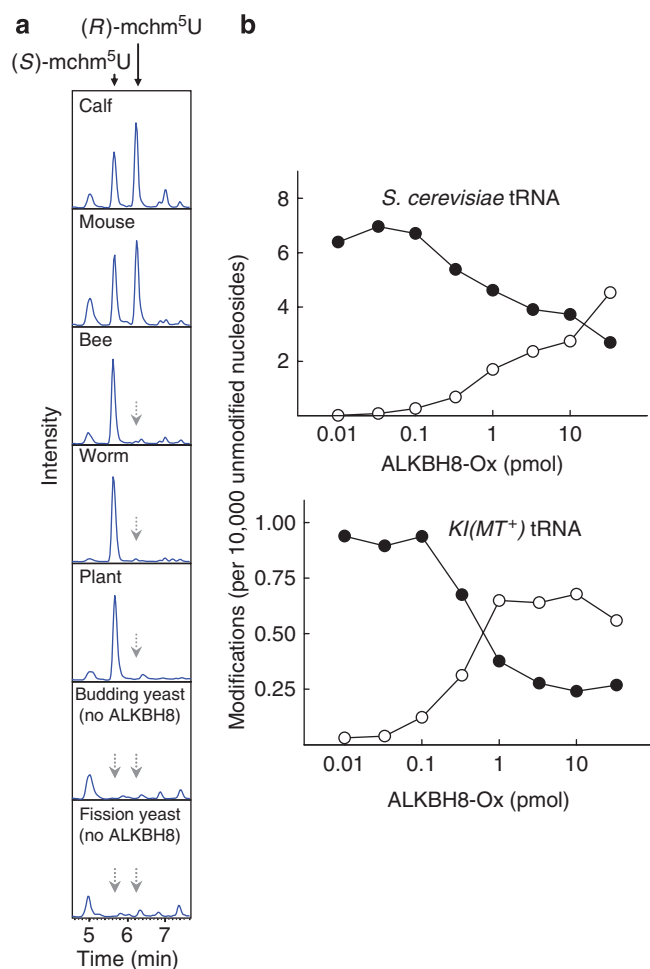


Figure 3 | Evolutionary conservation and cross-species activity of the ALKBH8 oxygenase. (a) mchm⁵U modifications in tRNA from various organisms, investigated by LC-MS/MS. (b) *In vitro* enzymatic activity of human ALKBH8-Ox on tRNA from the yeast *S. cerevisiae* (upper panel), and on tRNA from the *KI(MT⁺)* mice (lower panel). tRNA was incubated with different amounts of recombinant ALKBH8-Ox, and the levels of mchm⁵U (closed circles) and (S)-mchm⁵U (open circles) were analysed by LC-MS/MS.

but neither in the budding yeast *S. cerevisiae* nor in the fission yeast *Schizosaccharomyces pombe*, which both have mcm⁵U-modified tRNAs, but lack ALKBH8. Notably, (R)-mchm⁵U was detected only in the mammalian tissues tested (calf and mouse), and here at levels which represented ~350x the detection limit (0.003 (R)-mchm⁵U per 10,000 unmodified nucleosides). This indicates that if (R)-mchm⁵U is present in any of the other organisms, the level is at least two orders of magnitude lower than that in mammals. As was observed with the mcm⁵U-containing *KI(MT⁺)* tRNA, the majority of mcm⁵U in *S. cerevisiae* or *S. pombe* tRNA could be converted to (S)-mchm⁵U by incubation with ALKBH8-Ox (Fig. 3b, Supplementary Fig. S12). Taken together, the observed activity of human ALKBH8-Ox on tRNA from distantly related organisms and the co-occurrence of (S)-mchm⁵U and ALKBH8 suggest that hydroxylation of mcm⁵U into (S)-mchm⁵U is performed by ALKBH8 orthologues in a wide range of eukaryotes.

Wobble uridine modifications and protein translation. In yeast, inactivation of wobble uridine modification enzymes can impair translational decoding, as measured by decreased translation of relevant 'codon-runs'^{26,27}. To investigate the possible effects of the extensive wobble U mis-modification observed in the *Alkbh8*^{-/-} mice, we generated reporter plasmids containing both *Renilla* (Rluc; internal reference) and firefly (Fluc) luciferase genes, in which Fluc, but not Rluc, translation depends on in-frame translation with a codon-run of ten identical codons (Fig. 4a). We generated runs of A-ending codons matching the anticodons of several tRNAs containing mcm⁵U or derivatives, and corresponding non-cognate, G-ending codon-runs as controls. We then determined the Fluc/Rluc ratio on transfection of the constructs into primary fibroblasts from *Alkbh8*^{-/-} or WT mice (Fig. 4b). Clearly, different A-ending codon-runs affected translation to varying degrees, but the differences

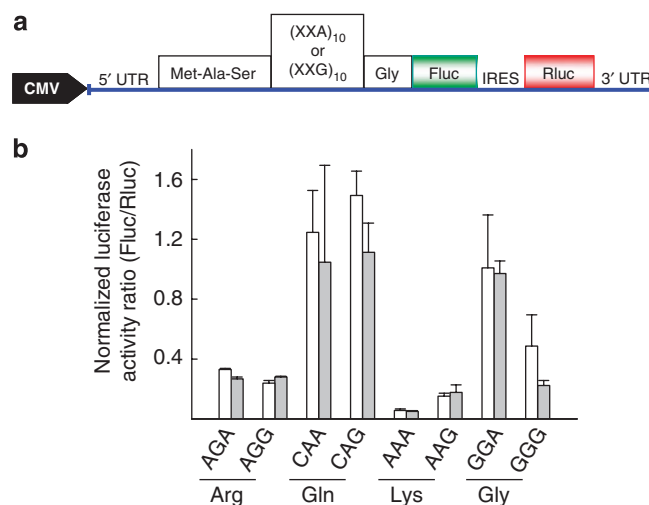


Figure 4 | Ability of *Alkbh8*^{-/-} tRNA to support translation. (a) Outline of the reporter construct used. The cytomegalovirus (CMV) promoter drives the expression of an mRNA (blue) encoding the protein elements indicated by boxes (drawing not to scale). (XXA)₁₀ and (XXG)₁₀ indicate runs of ten identical codons that end with A or G, respectively, and that are translated in frame with the firefly luciferase (Fluc) reporter. A *Renilla* luciferase (Rluc) reporter, which is translated from an internal ribosomal entry site (IRES) and independently of the codon run, serves as an internal reference. (b) Reporter constructs containing the indicated codon runs, encoding the indicated amino acids, were transfected into WT (white bars) and *Alkbh8*^{-/-} (grey bars) fibroblasts. The activity of the two reporter genes was analysed and used to calculate the efficiency of translation of the codon run. Error bars indicate the standard deviation of triplicate samples.

between *Alkbh8*^{-/-} and WT fibroblasts were generally small, showing that the altered wobble U modification pattern of *Alkbh8*^{-/-} tRNA has a negligible effect on translation efficiency in this experimental system.

Discussion

In this study, we have unravelled the biochemical function of the evolutionary conserved ALKBH8 oxygenase. Thus, tRNA modification is included in the broad range of reactions catalysed by 2OG/Fe(II) oxygenases, and the function of the ALKBH proteins is expanded beyond nucleic acid repair. Our data demonstrate how the MT and oxygenase activities of ALKBH8 act in a sequential manner, where the MT generates mcm⁵U, which in tRNA_{UCC}^{Gly} is further hydroxylated to (S)-mchm⁵U by the oxygenase. Furthermore, it is demonstrated how selective inactivation of the individual domains of ALKBH8 *in vivo* severely distorts the modification pattern of wobble uridines, and how the ALKBH8 oxygenase, in the absence of its cognate substrate mcm⁵U, will hydroxylate ncm⁵U and cm⁵U, albeit inefficiently (Fig. 5).

The present study reports the existence of two novel mammalian wobble uridine modifications, (R)- and (S)-mchm⁵U, and indicates that the evolution of more advanced eukaryotes has been accompanied by an increased complexity of such modifications. Most mcm⁵U-containing wobble nucleosides contain an additional, wobble restricting 2-thiolation (mcm⁵s²U), and the corresponding tRNAs decode in the split codon boxes. In contrast, tRNA_{UCC}^{Gly} and tRNA_{UCG}^{Arg}, which contain mchm⁵U, decode in family codon boxes. Therefore, one may speculate that the hydroxylation of mcm⁵U into mchm⁵U improves the reading of non-cognate codons; a typical feature of wobble uridine modifications found on family box tRNAs^{3,4}. This ability may be of particular importance in higher eukaryotes, for example, mammals, which consist of a wide diversity of specialized cells, such as elongated neurons. Here, protein translation often occurs at discrete locations²⁸, where low local concentrations of certain isoacceptors could necessitate the extensive use of non-cognate tRNAs in decoding.

Stereoisomers are frequently formed by epimerization, and one could envision (R)-mchm⁵U being formed by epimerization of (S)-mchm⁵U, rather than by hydroxylation of mcm⁵U. However, our presented data strongly argue against this notion, and predict an unknown oxygenase, OxX, for generation of (R)-mchm⁵U from mcm⁵U. First, the observation that (R)-mchm⁵U, but not (S)-mchm⁵U, is present in the *KI(MT⁺)* mice indicates that the former is generated independently of the latter. Second, in the *Alkbh8*^{-/-}

mice, in which the ALKBH8-Ox and ALKBH8-MT functions are both absent, total tRNA contained ncm⁵U (which is absent from WT tRNA). This presence of ncm⁵U may be explained by hydroxylation of accumulated mcm⁵U by the enzyme OxX, which normally catalyses the 2-hydroxylation of mcm⁵U into (R)-mchm⁵U. Future studies will investigate various oxygenases, including ALKBH proteins of unknown function, as candidates for the OxX function.

Recently, the mammalian Tet proteins (Tet1, Tet2 and Tet3) were shown to be DNA hydroxylases converting epigenetic 5-methylcytosine (5mC) modifications into 5hmC^{8,29}. Interesting analogies exist between ALKBH8 and the Tet proteins, as they all are 2OG/Fe(II) oxygenases acting on nucleic acids, and as they hydroxylate corresponding positions in mcm⁵U and 5mC, respectively. Conceivably, other ALKBH proteins may also catalyse reactions resulting in hydroxylation (rather than demethylation) of macromolecules.

Methods

Total tRNA and isoacceptor isolation. Total tRNA from *S. cerevisiae* and calf liver was purchased from Roche and Novagen, respectively. Total tRNA was purified from *Mus musculus* (liver), *A. mellifera* (head and thorax of worker bees), *C. elegans*, *A. thaliana* and *S. pombe* by using a RNA/DNA Maxi Kit (QIAGEN). Individual tRNA_{UCC}^{Gly} and tRNA_{UCG}^{Arg} isoacceptors were purified from total tRNA using 3'-biotinylated oligonucleotides (Supplementary Table S1), as previously described²².

Synthesis of mchm⁵U nucleoside standards. (R)- and (S)-mchm⁵U were obtained by a previously reported three-step procedure³⁰. The structure and homogeneity of these nucleoside derivatives were confirmed by nuclear magnetic resonance and mass spectrometry.

LC-MS/MS of nucleosides. The analysis was performed essentially as described²². Briefly, tRNA was enzymatically digested to nucleosides³¹, which were separated by reverse phase high-performance liquid chromatography on a Zorbax SB-C18 column, using a mobile phase consisting of 0.1% formic acid and a gradient of 5–50% methanol. Online mass spectrometry detection was performed using an Applied Biosystems/MDS Sciex 5000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex) with TurboIonSpray probe operating in positive electrospray ionization mode. The nucleosides were monitored by multiple reaction monitoring using the nucleoside to base ion mass transitions 317.2→185.1 (mcm⁵U), 333.2→201.1 (mchm⁵U), 303.2→171.1 (cm⁵U), 319.2→187.1 (chm⁵U), 302.2→170.1 (ncm⁵U), 318.2→186.1 (nchm⁵U), 268.2→136.1 (A), 244.2→112.1 (C), 284.2→152.2 (G) and 245.2→113.1 (U). Quantification was performed by comparison with pure nucleoside standards run in between the samples. Nucleoside standards for ncm⁵U and nchm⁵U were generated from mcm⁵U and mchm⁵U as previously described³², whereas cm⁵U and chm⁵U were generated by mild alkaline hydrolysis of mcm⁵U and mchm⁵U (ref. 22). The fragmentation pattern of the nucleoside standards was in accordance with theoretical predictions, and has been included in Supplementary Figure S13.

MALDI-TOF mass spectrometry. tRNA isoacceptors were digested with RNase T1 (Ambion) and samples prepared for mass spectrometry as previously described²². MALDI (matrix-assisted laser desorption/ionization) mass spectrometry was performed on a Perseptive Voyager STR instrument detecting positive ions in reflector TOF geometry. Spectrum processing was done with the MoverZ software (Genomic Solutions). MALDI tandem mass spectrometry was performed on a Waters QTOF Ultra MALDI instrument (Waters) with sample preparation as described above. The spectra were recorded in positive ion mode and processed with the supplied MassLynx software; additional details have been published previously³³.

Gene-targeted mice. Gene-targeting strategy and screening method to verify homologous recombination are presented in Supplementary Figures S2–S5.

Expression of recombinant proteins. Human ALKBH8 (aa 1–664), or its MT domain (ALKBH8-MT; aa 352–664), both in complex with human TRM112, and its AlkB domain (ALKBH8-Ox; aa 1–354) were purified as amino-terminal 6xHis-tagged protein from *E. coli* as previously described²².

Enzymatic treatment of tRNA. tRNA (5–10 µg) was incubated with recombinant protein (100 pmol, if not indicated otherwise) for 30 min at 37 °C in a 50 µl reaction mixture containing 25 µM S-adenosyl-L-methionine, 50 mM Tris-HCl pH 7.5, 25 mM KCl, 25 mM NH₄Ac, 0.5 mM MgCl₂, 2 mM ascorbic acid, 100 µM 2-oxoglutarate, 40 µM FeSO₄, 10 U RNase inhibitor and then precipitated with 1 volume of isopropanol in the presence of 1 M NH₄Ac and 10 µg of glycogen. Incubations with ALKBH8 and ALKBH8-MT were performed in the presence of the co-expressed and co-purified accessory protein TRM112. Pellets were washed with 70% EtOH, dried and dissolved in H₂O. The samples were subjected to nucleoside analysis by LC-MS/MS.

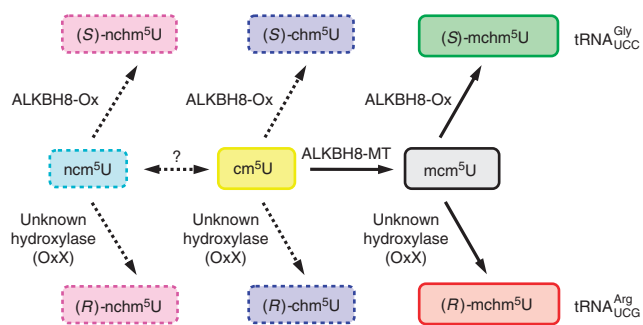


Figure 5 | Model for the generation of mchm⁵U modifications in tRNA_{UCC}^{Gly} and tRNA_{UCG}^{Arg}. The model depicts the roles of ALKBH8 and the putative oxygenase OxX in the formation of (S)-mchm⁵U and (R)-mchm⁵U in tRNA_{UCC}^{Gly} and tRNA_{UCG}^{Arg}, respectively (indicated by solid lines in arrows and borders). Dashed lines in arrows and borders indicate reactions and modifications, respectively, that occur in tRNA_{UCC}^{Gly} and tRNA_{UCG}^{Arg} when the ALKBH8 function is perturbed. The colour coding of borders corresponds to that of bar diagrams in Figure 2.

Codon-run reporter constructs. Short dsDNA fragments containing *Nco*I-specific DNA overhangs at both ends and harbouring ten successive, identical Lys, Gln, Arg or Gly codons were generated by annealing oligo pairs AAA₁₀-s/AAA₁₀-as, AAG₁₀-s/AAG₁₀-as, CAA₁₀-s/CAA₁₀-as, CAG₁₀-s/CAG₁₀-as, AGA₁₀-s/AGA₁₀-as, AGG₁₀-s/AGG₁₀-as, GGA₁₀-s/GGA₁₀-as or GGG₁₀-s/GGG₁₀-as (Supplementary Table S1), respectively, and inserted downstream of the translation initiation codon of the firefly luciferase gene in pDualLuc-IRES3 using its *Nco*I site³⁴. Subsequently, *Not*I-*Hind*III fragments from pDualLuc-IRES3 or derivatives containing a codon-run insertion were transferred to pCMV-Script (Stratagene). Following this strategy, pDualLuc-LysAAA₁₀, pDualLuc-LysAAG₁₀, pDualLuc-GlnCAA₁₀, pDualLuc-GlnCAG₁₀, pDualLuc-ArgAGA₁₀, pDualLuc-ArgAGG₁₀, pDualLuc-GlyGGA₁₀ and pDualLuc-GlyGGG₁₀ were constructed (Fig. 4a).

In vivo translation efficiency assay. Primary mouse embryonic fibroblasts were maintained in DMEM (BioWhittaker) supplemented with 15% fetal bovine serum (Saven & Werner) and 100 U per ml penicillin and streptomycin. Cells were grown to 90% confluency and transfected using a MicroPorator MP-100 (Digital Bio Technology, NanoEnTek). Cells (5 × 10⁵) were transfected with pDualLuc reporter plasmids using a pulse voltage of 1,350 V, 30 ms pulse width and a 100 μl Gold Tip according to the manufacturer's instructions, and immediately after electroporation transferred to pre-warmed 12-well dishes containing DMEM without antibiotics. Cells were collected at 24 h post-transfection and the intracellular Fluc and Rluc activities were subsequently measured using a Luminometer (Turner Designs TD-20/20), using the Dual-luciferase Reporter Assay according to the manufacturer's protocol (Promega).

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Author contributions

E.v.d.B., C.B.V., L.S.-M., V.L., G.F.L. and F.K. designed and performed experiments, and analysed data. G.L. synthesized nucleoside standards. H.E.K. and A.M. analysed data. A.K. and P.Ø.F. initiated the study, designed experiments, analysed data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional information

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