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Mammalian ALKBH8 Possesses tRNA Methyltransferase Activity Required for the Biogenesis of Multiple Wobble Uridine Modifications Implicated in Translational Decoding▽

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Uridines in the wobble position of tRNA are almost invariably modified. Modifications can increase the efficiency of codon reading, but they also prevent mistranslation by limiting wobbling. In mammals, several tRNAs have 5-methoxycarbonylmethyluridine (mcm^5U) or derivatives thereof in the wobble position. Through analysis of tRNA from *Alkbh8*$^{-/-}$ mice, we show here that ALKBH8 is a tRNA methyltransferase required for the final step in the biogenesis of mcm^5U. We also demonstrate that the interaction of ALKBH8 with a small accessory protein, TRM112, is required to form a functional tRNA methyltransferase. Furthermore, prior ALKBH8-mediated methylation is a prerequisite for the thiolation and 2′-*O*-ribose methylation that form 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) and 5-methoxycarbonylmethyl-2′-*O*-methyluridine (mcm^5Um), respectively. Despite the complete loss of all of these uridine modifications, *Alkbh8*$^{-/-}$ mice appear normal. However, the selenocysteine-specific tRNA ($tRNA^{Sec}$) is aberrantly modified in the *Alkbh8*$^{-/-}$ mice, and for the selenoprotein Gpx1, we indeed observed reduced recoding of the UGA stop codon to selenocysteine.

tRNAs are frequently modified at the wobble uridine, a feature that is believed to either promote or restrict wobbling depending on the type of modification. In the case of eukaryotes, the functions of wobble uridine modifications have been studied in the greatest detail in *Saccharomyces cerevisiae*. Here, the modifications 5-methoxycarbonylmethyluridine (mcm^5U), 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), and 5-carbamoylmethyluridine (ncm^5U) or its 2′-O-ribose-methylated form, ncm^5Um, are found in 11 out of 13 wobble uridine-containing tRNAs (22). mcm^5U and mcm^5s^2U are mostly found in "split" codon boxes, where the pyrimidine- and purine-ending codons encode different amino acids, while ncm^5U is found in "family" codon boxes, where all four codons encode a single amino acid. Early reports based on *in vitro* experiments suggested that wobble nucleosides, such as mcm^5U, ncm^5U, and their derivatives, may restrict wobbling (17, 37, 45), but the results of a recent comprehensive study performed *in vivo* in *S. cerevisiae* show that such modifications can improve the reading both of the cognate, A-ending codons and of the wobble, G-ending codons (22). This may suggest that the primary role of these modified nucleosides is to improve translational efficiency rather than to restrict wobbling.

The characterization of wobble uridine modifications in higher eukaryotes is very limited, and little is known about the enzymes that introduce them. In mammals, mcm^5s^2U has been found in the wobble position of $tRNA^{Glu(UUC)}$, $tRNA^{Lys(UUU)}$, and $tRNA^{Arg(UCU)}$ (40). Unlike yeast, mammals possess a specialized tRNA that is responsible for recoding the UGA stop codon to insert the 21st amino acid, selenocysteine (Sec). The mammalian $tRNA^{Sec}$ population consists of two subpopulations containing either mcm^5U or the ribose-methylated derivative mcm^5Um in the wobble position. Interestingly, ribose methylation of mcm^5U in $tRNA^{Sec}$ appears to have a role in regulating selenoprotein synthesis, as the expression of some selenoproteins, such as glutathione peroxidase 1 (Gpx1), appears to be promoted by mcm^5Um-containing $tRNA^{Sec}$ (5, 7, 9, 32).

Some years ago, the *Escherichia coli* AlkB protein was found to be a 2-oxoglutarate- and iron-dependent dioxygenase capable of demethylating the lesions 1-methyladenosine and 3-methylcytosine in DNA (13, 42). Multicellular organisms generally possess several different AlkB homologues (ALKBH), and bioinformatics analysis has identified eight different mammalian ALKBH proteins, denoted ALKBH1 to ALKBH8 in humans and Alkbh1 to Alkbh8 in mice, as well as the somewhat-less-related, obesity-associated FTO protein (2, 16, 30). Among the ALKBH proteins of unknown function, ALKBH8 is the only one containing additional annotated protein domains. Here, the AlkB domain is localized between an N-terminal RNA recognition motif (RRM)

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and a C-terminal methyltransferase (MT) domain. Interestingly, the MT domain has sequence homology to the *S. cerevisiae* tRNA methyltransferase Trm9, which has been shown to catalyze the methyl esterification of modified wobble uridine (U34) residues of $tRNA^{Arg}$ and $tRNA^{Glu}$, resulting in the formation of mcm^5U and mcm^5s^2U, respectively (23, 43). Until recently, human ALKBH8 was incorrectly annotated in the protein sequence database, and another human protein, KIAA1456, has been designated the human Trm9 homologue (3, 23).

We have generated for this study *Alkbh8*-targeted mice that lack exons critical for both the MT and AlkB activities of Alkbh8. The mice did not display any overt phenotype, but tRNA from these mice was completely devoid of mcm^5U, mcm^5s^2U, and mcm^5Um, and the relevant tRNA isoacceptors instead contained the acid form 5-carboxymethyluridine (cm^5U) and/or the amide forms ncm^5U/ncm^5s^2U. Furthermore, we show that recombinant ALKBH8 and TRM112 form a heterodimeric complex capable of catalyzing the methyl esterification of cm^5U and cm^5s^2U to mcm^5U and mcm^5s^2U, respectively. In agreement with the involvement of mcm^5Um in selenoprotein synthesis, we observed a reduced level of Gpx1 in the *Alkbh8*$^{-/-}$ mice, and $tRNA^{Sec}$ from these mice showed a reduced ability to decode the UGA stop codon to Sec.

MATERIALS AND METHODS

Targeting of the mouse *Alkbh8* gene. Based on the analysis of the *Alkbh8* gene structure and the predicted Alkbh8 protein structure (Fig. 1A and B), a targeting strategy was designed to disrupt the murine *Alkbh8* gene by deletion of exons 9 and 10, encoding a part of the AlkB and the methyltransferase domains, respectively. Full details of the strategies for gene-targeting and screening methods (to verify homologous recombination events) are available on request. Three correctly targeted E14Tg2a embryonic stem (ES)-cell clones (ES cells are derived from 129P2/Ola mice) were expanded and selected for injection into C57BL6/J blastocysts. Among the first nine pups born, six male chimeras presented chimerism from 40 to 100%. Gene targeting, blastocyst injections, and breeding of mice to generate heterozygous animals were performed by GenOway, Lyon, France.

Construction of plasmids. Human ALKBH8-coding sequences were amplified by PCR on human cDNA templates. Primers ALKBH8-f1 and ALKBH8-r1 (primers are listed in Table 1) were used to obtain a PCR product encoding full-length ALKBH8 (amino acids [aa] 1 to 664), primers ALKBH8-f1 and ALKBH8-r2 for its AlkB domain (aa 1 to 354), and primers ALKBH8-f2 and ALKBH8-r1 for the MT domain (aa 352 to 664). The PCR products were cloned into the first multiple cloning site (MCS) in pET-Duet1 (Novagen), yielding pDuet-ALKBH8, pDuet-8AlkB and pDuet-8MT, respectively. Plasmid pDuet-ALKBH8 was used as a template for QuikChange II (Stratagene)-mediated mutagenesis using primers 414/416-f and 414/416-r and 431-f and 431-r to create pDuet-8MTmut, encoding a G414A/G416A/G431A (amino acid numbering corresponds to the full-length protein) triple amino acid substitution mutant. The TRM112 coding sequence was obtained by PCR on a cDNA clone (IMAGE: 3163327) using primers TRM112-f and TRM112-r, and the resulting PCR product was subsequently cloned into the second MCS of pET-Duet1 (pDuet-TRM112) to allow the coexpression of untagged recombinant TRM112 in *E. coli*. Bicistronic pET-Duet1 vectors (pDuet-ALKBH8/TRM112, pDuet-8AlkB/TRM112, pDuet-8MT/TRM112, and pDuet-8MTmut/TRM112) were generated by placing NotI-XhoI fragments from pDuet-TRM112 into the same sites of the plasmids containing ALKBH8-coding sequences in the first MCS. For the expression of 6×His-tagged TRM112, the NdeI-XhoI fragment from pDuet-TRM112 was transferred to pET28a (Novagen) to obtain pET28-TRM112. To generate pET28-SBP2 encoding the carboxy-terminal part (aa 409 to 854) of human SECIC binding protein 2 (SBP2), PCR was performed on pSport1-SECISBP2 (GenBank accession number AL136881) using primers m13F and SBP2, and the DNA fragment obtained was placed into the NdeI and NotI restriction sites of pET28a.

To create the dual luciferase reporter construct pDualLuc-Gpx1, the *Renilla reniformis* luciferase gene (Rluc) was obtained by PCR on pDualLuc-IRES3 (44) using primers RLuc-f and RLuc-r, and the resulting PCR fragment replaced the β-galactosidase gene located between the HindIII and BamHI sites of pBPLUGA-Gpx1 (21). The HindIII-KpnI fragment from the resulting plasmid was placed into the same sites of pCMV-Script (Stratagene) to obtain pDualLuc-Gpx1.

Protein purification. Proteins were expressed from pET-Duet1-derived and pET28a-derived constructs in the *E. coli* strain BL21-CodonPlus(DE3)-RIPL (Stratagene). Expression was performed overnight at 16°C in LB medium containing 0.075 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were pelleted and resuspended in equilibration buffer (50 mM sodium phosphate, pH 7.6, 150 mM NaCl, 10% glycerol, 0.5% NP-40, protease inhibitors [complete EDTA-free protease inhibitor tablets {Roche}], 5 mM imidazole, and 0.5 mg/ml lysozyme [Fluka]). Bacterial extracts were obtained by French press treatment, and proteins were subsequently purified from the extracts using TALON metal affinity resin (Clontech) according to the manufacturer's instructions. Protein purity and yield were assessed by 15% SDS–PAGE followed by Coomassie brilliant blue staining of the gel.

Isolation of total tRNA and tRNA isoacceptors from mice. Total tRNA was purified from mouse tissue using an RNA/DNA maxi kit (Qiagen) according to the manual provided. Individual $tRNA^{Sec}$, $tRNA^{Glu(UUC)}$, and $tRNA^{Arg(UCU)}$ isoacceptors were purified from total tRNA using 3′-biotinylated DNA oligonucleotides (Sec1, Glu1, and Arg1, respectively) (Table 1) immobilized on streptavidin-conjugated M-280 magnetic Dynabeads (Invitrogen). The beads were made RNase free according to the manufacturer's instructions, washed once with buffer A (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 M NaCl), and finally resuspended in buffer A. Subsequently, 2 μM 3′-biotinylated oligonucleotides in water were mixed with an equal volume of Dynabeads in buffer A and incubated at room temperature for 30 min with gentle mixing. The oligonucleotide-coated Dynabeads were then washed four times in buffer B (5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl) and then equilibrated in 6× SCC solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The oligonucleotide-coated Dynabeads and the tRNA in 6× SSC were incubated for 5 min at 75°C, pooled, and incubated for 5 more minutes at 75°C. Thereafter, the suspension was placed at room temperature for 40 min to allow tRNA binding to the beads. The supernatant that contained unbound tRNA was taken and kept for subsequent tRNA isoacceptor isolations. The oligonucleotide-coated Dynabeads were washed, in succession, three times with 3× SSC, twice with 1× SSC, and several times with 0.1× SCC until the absorbance of the wash solution at 260 nm was near zero. tRNA retained on the beads was eluted three times with 0.1× SCC at 65°C for 3 to 4 min. tRNA was concentrated by 1 M ammonium acetate (NH_4Ac)–isopropanol precipitation in the presence of 20 μg of glycogen (Roche).

LC–MS-MS analysis. The RNA samples were enzymatically hydrolyzed to nucleosides using nuclease P1, phosphodiesterase I from *Crotalus adamanteus* venom, and alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) as described previously (10), followed by the addition of 3 volumes of methanol and centrifugation (16,000 × *g* for 30 min). The supernatants were dried under vacuum, and the resulting residues dissolved in 50 μl 5% (vol/vol) methanol in water for nucleoside analysis by liquid chromatography coupled to two-dimensional mass spectrometry (LC–MS-MS). A portion of each sample was diluted for the quantitation of the unmodified nucleosides adenosine (A), cytidine (C), guanosine (G), and uridine (U).

Chromatographic separation of nucleosides was performed on a Shimadzu Prominence high-performance liquid chromatography system with a Zorbax SB C_{18} 2.1- by 150-mm inner diameter (3.5 μm) reverse-phase column equipped with an Eclipse XDB-C_8 2.1- by 12.5-mm inner diameter (5 μm) guard column (all from Agilent Technologies, CA) at ambient temperature and a flow rate of 0.2 ml/min. The mobile phase consisted of A (0.1% formic acid in water) and B (0.1% formic acid in methanol), for modified nucleosides starting with 95% A, 5% B for 0.5 min, followed by a 6.5-min linear gradient of 5 to 50% B, 2 min with 50% B, and 6 min of re-equilibration with the initial mobile phase conditions. Chromatography of unmodified nucleosides was performed under isocratic conditions, with a mobile phase of 90% A, 10% B at a flow rate of 0.2 ml/min and a total analysis time of 4 min per sample. Online mass spectrometry detection was performed using an Applied Biosystems/MDS Sciex 5000 triple-quadrupole mass spectrometer (Applied Biosystems Sciex, CA) with a TurboIonSpray probe operating in positive electrospray ionization mode. The nucleosides were monitored by multiple reaction monitoring using the mass (mass-to-charge ratio) transitions 317.2→185.1 (mcm^5U), 317.2→153.1 (mcm^5U), 333.2→201.1 (mcm^5s^2U), 333.2→169.1 (mcm^5s^2U), 331.2→185.1 (mcm^5Um), 331.2→153.1 (mcm^5Um), 303.2→171.1 (cm^5U), 319.2→187.1 (cm^5s^2U), 302.2→170.1 (ncm^5U), 318.2→186.1 (ncm^5s^2U), 268.2→136.1 (A), 244.2→112.1 (C), 284.2→152.2 (G), and 245.2→113.1 (U). Quantitation was accomplished by

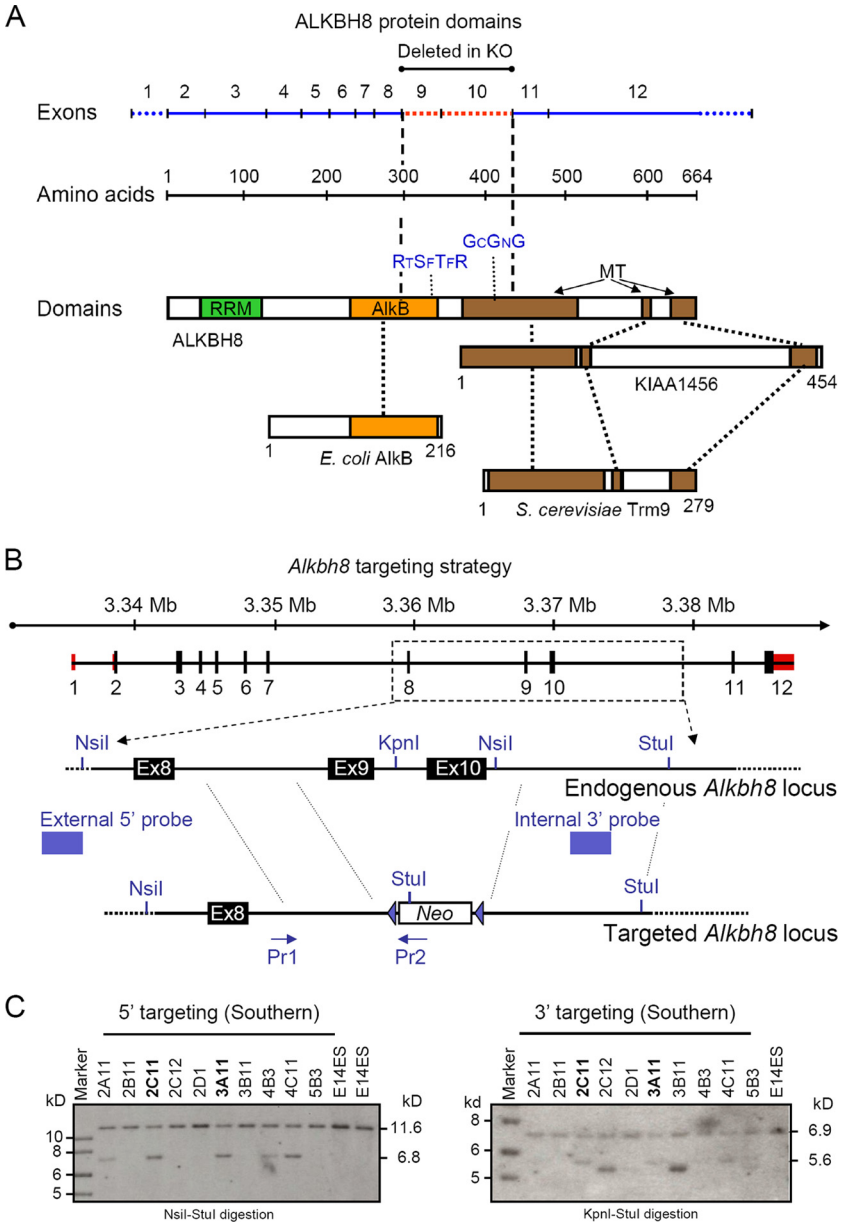


FIG. 1. ALKBH8 domain architecture and outline of the gene-targeting strategy. (A) Exons are numbered 1 to 12. The blue dashed line represents noncoding exonic regions, whereas the red dashed line represents exons deleted in the *Alkbh8*$^{-/-}$ mice. The predicted RNA recognition motif (RRM) is indicated in green, and the region of ALKBH8 that has sequence similarity to *E. coli* AlkB is shown in orange. The regions of ALKBH8 that display sequence similarity to the human protein KIAA1456 and to the *S. cerevisiae* tRNA methyltransferase Trm9 are indicated in brown. The localization of the RTSFTFR and GCGNG motifs predicted to be essential for oxygenase and MT activities, respectively, is indicated. KO, knockout. Nonconserved amino acids are indicated by small capitals. (B) Schematic organization of the genomic *Alkbh8* locus and the gene-targeting strategy. Black rectangles represent *Alkbh8* coding sequences, red rectangles represent noncoding exon portions, and the solid line represents the chromosome. Dashed lines point out the chromosomal region targeted by homologous recombination. The *Neo* cassette is indicated by an open rectangle, and the LoxP sites by blue triangles. Restriction sites, probes used for Southern blotting, and PCR primers required for verification of homologous recombination are indicated in blue. All other details of gene targeting are available upon request. Diagram is not depicted to scale. (C) Results of Southern blot analysis for verification of accurate 5′ (left panel) and 3′ (right panel) homologous recombination in ES cells (see panel B for details). The genomic DNA of 10 PCR-tested (not shown) ES cell clones (2A11, 2B11, 2C11, 2C12, 2D1, 3A11, 3B11, 4B3, 4C11, and 5B3) was extracted from expanded ES cells and compared with wild-type DNA (E14ES). Digested DNA was blotted on a nylon membrane and hybridized with either the external 5′ probe to screen for 5′ homologous recombination events or the internal 3′ probe to screen for 3′ homologous recombination events. As shown by the results in the left panel, the 5′ external probe detected the expected wild-type and recombined allele in 5 clones. Three of these clones (2C11, 3A11, and 4C11) revealed wild-type and targeted signals of equal intensity by the 3′ internal probe and were particularly well suited for blastocyst injections. Clones in bold (2C11 and 3A11) were injected into blastocysts for the generation of gene-targeted mice.

TABLE 1. Oligonucleotides used in this study[a]

Purpose	Name	Sequence (5′–3′)
Verification of homologous recombination	Pr1 5′ PCR	5′-GCCTTCTTGACGAGTTCTTCTGAGG-3′
	Pr2 5′ PCR	5′-AAACTACCAGTCTGGGAGCAGTCTCC-3′
	Pr 1 5′ probe	5′-TGAAAGTAGACTCATAACCTTTCTTGGTGATAACC-3′
	Pr 2 5′ probe	5′-TGTGAGAAAAAGAAAATACACAAAGTGTTCACAAC-3′
	Pr 1 3′ probe	5′-TATGAACTTAACCATTCTAGATCCAGAGGTAAAGC-3′
	Pr 2 3′ probe	5′-TCTAAGTTCCCTTAATTGCAGTGGTAGTATTTCC-3′
PCR genotyping	Alkbh8−/− f	5′-TATCGCCTTCTTGACGAGTTC-3′
	Alkbh8−/− r	5′-AGTTGTGTCCTGTGCAAGG-3′
	Alkbh8 wt f	5′-AGCCATGGCCACGCATAGTA-3′
	Alkbh8 wt r	5′-TGTATGAAGAATTGTGGGCAG-3′
$tRNA^{Sec}$ isolation	Sec1	CGCCCGAAAGGTGGAATTGAACCACTCTGTCGCTA-biotin
$tRNA^{Glu}$ isolation	Glu1	TTCCCACACCGGGAGTCGAACCCGGGCC-biotin
$tRNA^{Arg}$ isolation	Arg1	CGACTCCGCCGGGACTCGAACCCGGAAC-biotin
Cloning	RLuc-f	GC*AAGCTT*CGAGGGATCTGTGACATGATGACTTCGAAAGTTTATG
	RLuc-r	G*GGATCC*TCGGGATCATTGTTCATTTTTGAGAACTCG
	TRM112-f	AGCTTT*AGATCT*CAAACTGCTTACCCACAATCTG
	TRM112-r	AATCCG*CTCGAG*TCAACTCTCAGTTTCCTCTTCAC
	ALKBH8-f1	ACTACTGG*CATATG*GACAGCAACCATCAAAGTAATTAC
	ALKBH8-r1	ATGTAGAC*GTCGAC*TCAGGCCTTTTGAAGAATCACACACC
	ALKBH8-f2	ATAAC*GAGCTC*CCAGAGGAAAGAGACTCCCC
	ALKBH8-r2	ATGTAGAC*GTCGAC*TCATTTCCTCTGGCTATCACAGACCAAC
Mutagenesis	414/416-f	GTGGCTGATATTGCATGTGCTAATGGAAAGTATCTTG
	414/416-r	CAAGATACTTTCCATTAGCACATGCAATATCAGCCAC
	431-f	GTTATATATGATTGCTTGTGATCGTAG
	431-r	CTACGATCACAAGCAATCATATATAAC
Cloning	m13F	GTAAAACGACGGCCAG
	SBP2	GGCAGCCATATGTTTCCCAACCTGGCAGTTGC

[a] Restriction sites are italicized, and mutated nucleotides are underlined. Abbreviations: f is forward, r is reverse.

comparison with pure nucleoside standards run alongside the samples. Nucleoside standards for mcm^5U and mcm^5s^2U were obtained from Andrzej Malkiewicz (Lodz, Poland). The corresponding amides (ncm^5U and ncm^5s^2U) were generated from mcm^5U and mcm^5s^2U as previously described (14), while cm^5U and cm^5s^2U were generated by alkaline hydrolysis of mcm^5U and mcm^5s^2U, respectively, as follows: the nucleosides were incubated for 1 h at 25°C in a solution of 0.1 M NaOH in a 1:1 (vol/vol) mixture of H_2O and pyridine, followed by conversion of the resulting Na^+ salt to free acid by cation exchange with the H^+ form of Dowex beads. No nucleoside standard was available for mcm^5Um, but additional authentication of this nucleoside was provided by its strong enrichment in $tRNA^{Sec}$, as well as the presence of a characteristic, expected qualifier ion corresponding to the loss of a methanol (32 Da) neutral.

RNase T1 digestion and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry. The tRNA digestion mixture contained approximately 1 pmol/μl tRNA, 50 mM 3-hydroxypicolinic acid (Aldrich), and 20 U/μl RNase T1 (Ambion). Digestion was performed for 2 to 4 h at 37°C.

Samples for mass spectrometry were prepared by mixing 1 μl of tRNA digestion mixture with 0.7 μl of 0.5 M 3-hydroxypicolinic acid in 50% acetonitrile and approximately 0.1 μl of ammonium-loaded cation exchange beads (50W-X8; Bio-Rad), after which the sample was left to air dry at room temperature. MALDI mass spectrometry was performed on a PerSeptive Voyager STR instrument detecting positive ions in reflector time-of-flight geometry. Spectrum processing was done with *MoverZ* free software (Genomic Solutions).

Saponification. To remove the methyl moiety from the methoxycarbonylmethyl group, the methyl ester linkage was broken by saponification of the tRNA substrate as described previously (27). Briefly, 10 μl of a tRNA solution was mixed with 1.25 μl of 1 M NaOH and the mixture was incubated at room temperature for 8 min. The solution was neutralized with 1.25 μl of 1 M acetic acid.

Methyltransferase assay. Total tRNA isolated from mouse organs, *Bos taurus* liver (Novagene), *E. coli* (Roche), or *Saccharomyces cerevisiae* (Roche) was incubated with purified recombinant protein for 12 min at 37° in a 50-μl reaction mixture containing 1.33 μM *s*-adenosyl-*l*-[*methyl*-^{3}H]methionine (GE Healthcare), 25 μM *s*-adenosyl-*l*-methionine (NEB), 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 25 mM NH_4Ac, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 10 U RNasin Plus RNase inhibitor (Promega). The methylation reaction was stopped by adding 950 μl of 10% trichloroacetic acid (TCA), and the solution was spotted onto GF/C filters (Whatman) that were placed on a filter holder manifold (Millipore) under vacuum. Filters were washed with 10 ml of 10% TCA and 2 ml of 96% ethanol, and the amount of radioactivity retained on the filters was determined by scintillation counting. When enzyme-treated tRNA was analyzed by MALDI-TOF MS or LC-MS-MS, *s*-adenosyl-*l*-[*methyl*-^{3}H]methionine was omitted from the reaction mixture, and reaction mixtures were precipitated with 1 volume of isopropanol in the presence of 1 M NH_4Ac and 10 μg glycogen. Pellets were washed with 70% ethanol and dried.

***In vivo* ^{75}Se labeling.** ^{75}Se-labeled sodium selenite (1,006 Ci/mmol; University of Missouri Research Reactor Facility) was adjusted with NaOH to pH 7.4, and mice were injected intraperitoneally with 50 μCi of ^{75}Se/g and sacrificed 48 h after injection as described before (7). Protein extracts were separated by 8% SDS–PAGE, and the gel was stained with Coomassie blue before drying and visualization of ^{75}Se-containing proteins by phosphorimaging and quantitation as described previously (31).

Glutathione peroxidase activity assay. Measurement of liver glutathione peroxidase activity was carried out using a glutathione peroxidase cellular activity assay kit (Sigma-Aldrich) according to the manufacturer's instructions. H_2O_2 was used to start the reaction, and the pH of the glutathione peroxidase buffer was adjusted to 7.0 with HCl.

***In vitro* selenocysteine incorporation assay.** To obtain DualLuc-Gpx1 reporter RNA, runoff *in vitro* transcription was performed on KpnI-linearized pDualLuc-Gpx1 using a MEGAscript T3 kit (Ambion). Rabbit reticulocyte lysates (RRL; Promega) were depleted of tRNA according to previously described methods (20). One hundred nanograms of DualLuc-Gpx1 RNA, 1 μg of tRNA, 20 U of RNasin plus RNase inhibitor, and 1 pmol of recombinant hSBP2 were added to 25 μl of tRNA-dependent RRL. Firefly luciferase (Fluc) and *Renilla* luciferase (Rluc) activities were measured using a luminometer (TD-20/20; Turner

FIG. 2. Alignment of the ALKBH8-MT (methyltransferase) domain with TRM9 and KIAA1456. The amino acid sequence alignment shown was extracted from a slightly more extensive alignment that was generated with MAFFT (25) from 13 sequences, including, in addition to the sequences shown, ALKBH8 from *Caenorhabditis elegans* and *Drosophila melanogaster*, ALKBH8 and KIAA1456 from *Gallus gallus* and *Xenopus tropicalis*, and Trm9 from *Arabidopsis thaliana*. Arrows indicate a GXGXG motif expected to be critical for methyltransferase activity. Hs, *Homo sapiens*; Mm, *Mus musculus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*.

Designs), and a dual-luciferase reporter assay performed according to the manufacturer's protocol (Promega).

RESULTS

Alkbh8 gene structure and targeting strategy. ALKBH8 contains, in addition to the characteristic AlkB domain, an RNA recognition motif (RRM) and an *S*-adenosylmethionine (SAM)-dependent methyltransferase (MT) domain (Fig. 1). The ALKBH8 MT domain is the mammalian protein with the highest sequence similarity to *S. cerevisiae* Trm9, being slightly more similar to Trm9 than another previously identified but as-yet-uncharacterized homologue, KIAA1456 (Fig. 2) (23). The regions of the sequence with homology to Trm9 span the C-terminal half (aa 366 to 663) of ALKBH8 (664 aa). The entire mouse *Alkbh8* gene consists of 12 exons, of which exons 2 to 12 constitute the open reading frame, and spans more than 51 kb of genomic DNA. To fully eliminate the putative AlkB and MT activities of mouse Alkbh8, we deleted exons 9 and 10 in mouse embryonic stem (ES) cells (Fig. 1). Exon 9 encodes the RXSXTXR motif of the 2-oxoglutarate binding site (46), which is essential for AlkB activity, whereas the conserved SAM binding domain GXGXG (33), required for MT activity, is encoded by exon 10. The targeting strategy is shown in Fig. 1B. Genotyping of more than 160 live-born mice from intermating of heterozygous F_1 mice showed Mendelian segregation of the targeted *Alkbh8* locus. Despite the apparent effect of *Alkbh8* targeting on the modification of wobble uridine in tRNA (described below), no obvious phenotype was detected in homozygous *Alkbh8*$^{-/-}$ mice at 20 months of age.

Lack of mcm^{5}U, mcm^{5}Um, and mcm^{5}s^{2}U in total tRNA from *Alkbh8*$^{-/-}$ mice. *S. cerevisiae* Trm9 catalyzes a methyl esterification step during the generation of the wobble uridine nucleoside mcm^{5}U and its thio derivative, mcm^{5}s^{2}U (23). These modifications are also found in mammalian tRNA, which in addition contains the 2′-*O*-ribose-methylated derivative of mcm^{5}U, denoted mcm^{5}Um. To first address whether Alkbh8 deficiency had an effect on the modification status of wobble uridines, total tRNA was extracted from liver of wild-type and

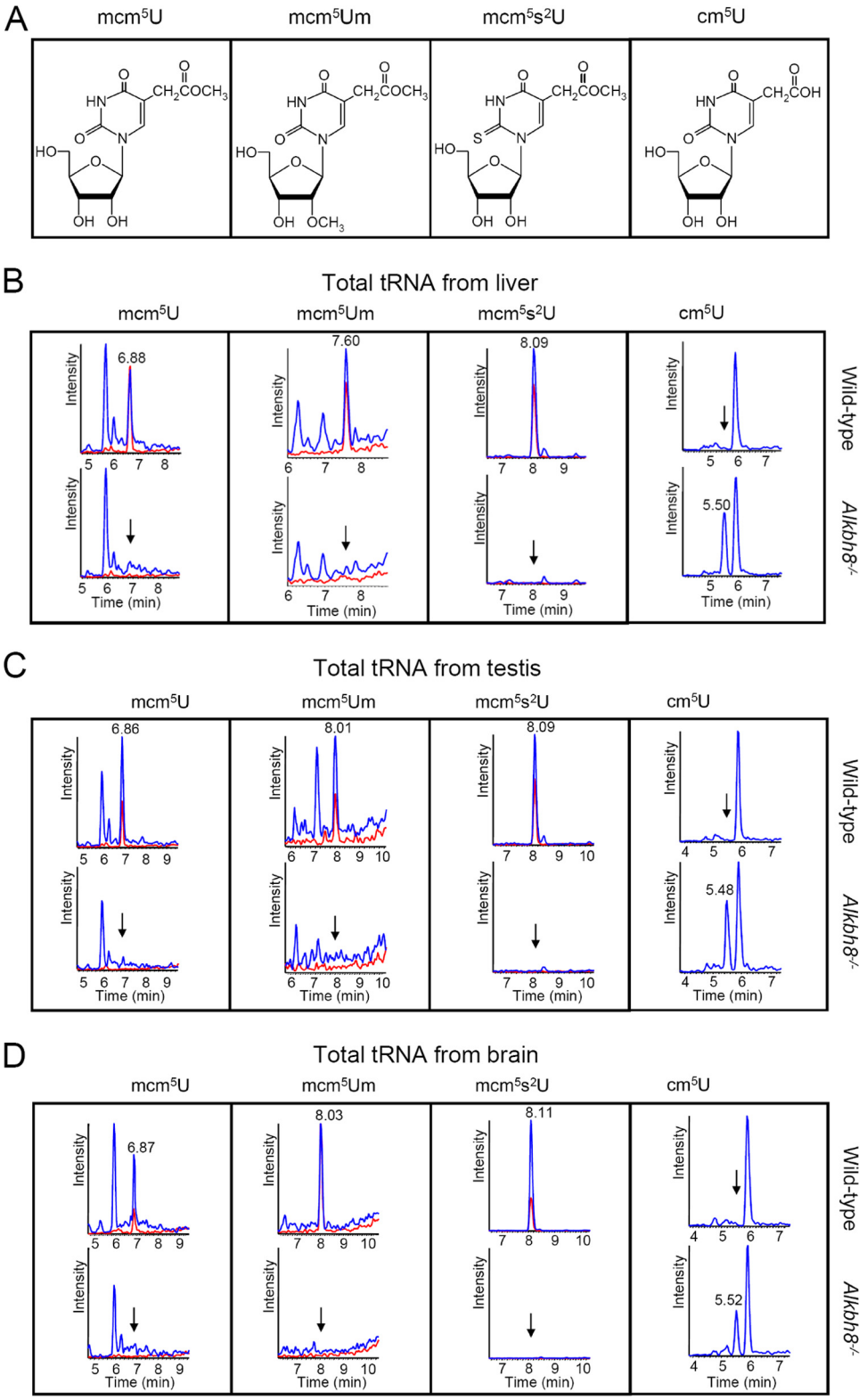
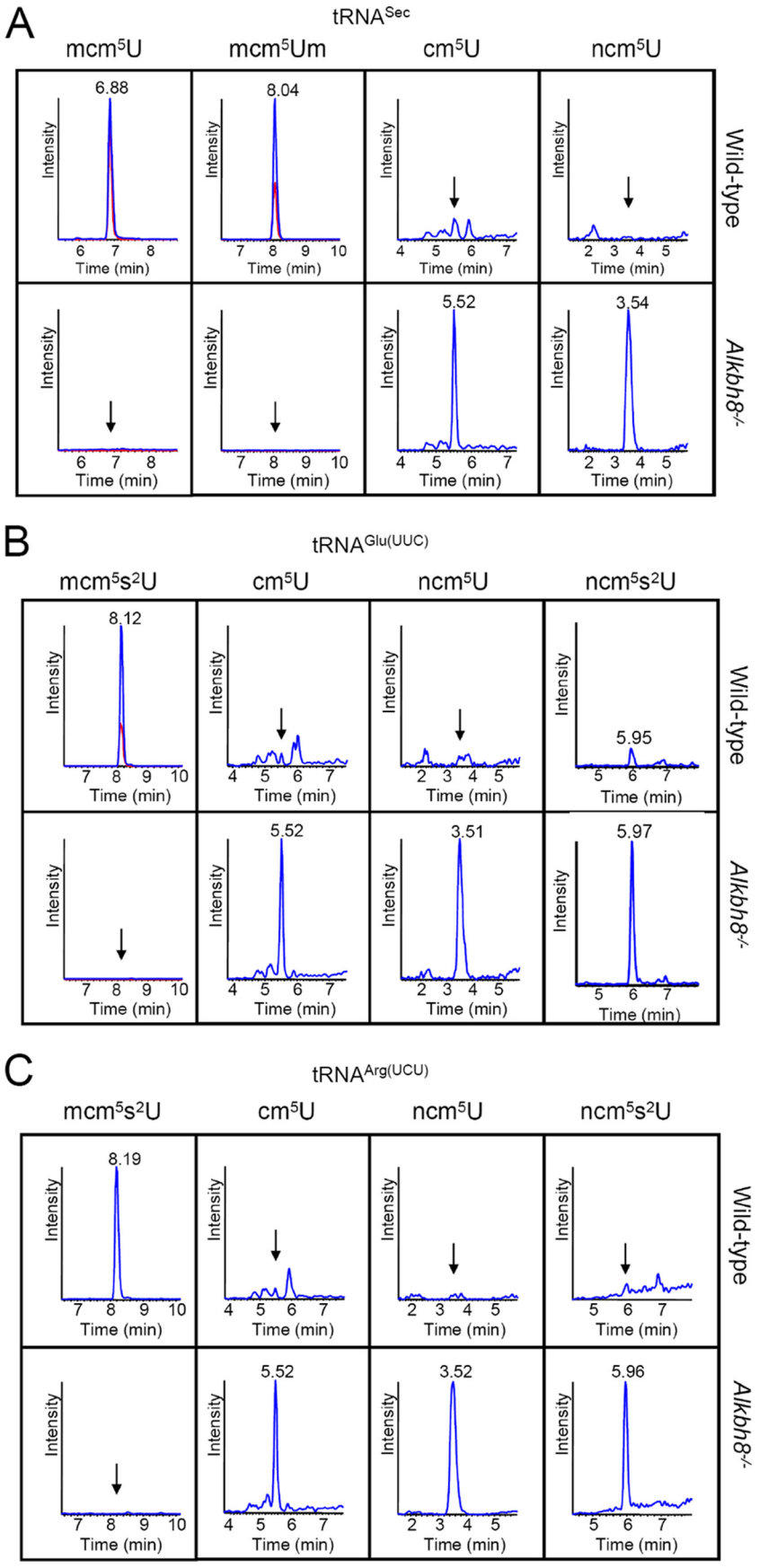


FIG. 3. Wobble uridine modifications in total tRNA from wild-type and *Alkbh8*$^{-/-}$ mice. (A) Chemical structures of the nucleosides investigated. (B to D) LC–MS-MS chromatograms for mcm^{5}U, mcm^{5}Um, mcm^{5}s^{2}U, and cm^{5}U of tRNAs purified from wild-type (upper inset chromatogram), and *Alkbh8*$^{-/-}$ (lower inset chromatogram) liver (B), testis (C), or brain (D). Relative quantity and retention time are indicated on the *y* and the *x* axis, respectively. Blue curves in the chromatograms correspond to mass transitions from loss of ribose neutrals (−132 Da), and red curves correspond to qualifier mass transitions from additional loss of the base methylester groups as methanol neutrals (−32 Da). The relevant peak is identified by its retention time (when detected). An arrow points to the expected position for the modified uridine nucleoside when absent or only just detectable.


A
tRNA^Sec
mcm5U
mcm5Um
cm5U
ncm5U
6.88
8.04
5.52
3.54
Wild-type
Alkbh8-/-
B
tRNA^Glu(UUC)
mcm5s2U
cm5U
ncm5U
ncm5s2U
8.12
5.95
5.52
3.51
5.97
C
tRNA^Arg(UCU)
mcm5s2U
cm5U
ncm5U
ncm5s2U
8.19
5.52
3.52
5.96
Intensity
Time (min)

Alkbh8$^{-/-}$ mice at 2 months of age. The isolated tRNA was then digested to nucleosides (Fig. 3A), which were analyzed by liquid chromatography coupled to LC–MS-MS. Each nucleoside was identified by its retention time, as well as its characteristic nucleoside-to-base ion transition. Both these properties were determined by using synthetic nucleoside standards. Notably, mcm^{5}U, mcm^{5}Um, and mcm^{5}s^{2}U were readily detected in total tRNA from wild-type livers but were completely absent from total tRNA from *Alkbh8*$^{-/-}$ mice (Fig. 3B). In contrast, we detected substantial amounts of cm^{5}U, the putative unmethylated precursor of mcm^{5}U, in total tRNA from *Alkbh8*$^{-/-}$ mice but not in total tRNA from wild-type mice. In order to substantiate these findings, total tRNA from testis and brain from the wild-type and *Alkbh8*$^{-/-}$ mice was also analyzed, with results similar to those obtained with liver tRNA (Fig. 3C and D). We could detect negligible amounts of the unmethylated forms of mcm^{5}s^{2}U and mcm^{5}Um, i.e., cm^{5}s^{2}U and cm^{5}Um, respectively, and there were no appreciable differences between wild-type and *Alkbh8*$^{-/-}$ mice (data not shown). The results indicated that the thiolation and ribose methylation enzymes required for the formation of mcm^{5}s^{2}U and mcm^{5}Um, respectively, use mcm^{5}U as substrate and, thus, rely on prior Alkbh8-mediated methylation.

Wobble uridine modification in tRNA^{Sec}, tRNA$^{Glu(UUC)}$, and tRNA$^{Arg(UCU)}$ isoacceptors from wild-type and *Alkbh8*$^{-/-}$ mice. The dramatic changes in the pattern of modified uridines in total tRNA from *Alkbh8*$^{-/-}$ mice (Fig. 3) clearly indicated that the wobble uridine modification status of several individual tRNA isoacceptors is affected. To verify this, tRNA^{Sec} (mcm^{5}U/mcm^{5}Um), tRNA$^{Glu(UUC)}$ (mcm^{5}s^{2}U), and tRNA$^{Arg(UCU)}$ (mcm^{5}s^{2}U) were purified from liver total tRNA by hybridization to immobilized complementary oligonucleotides (Table 1). tRNA^{Sec}, which recognizes the UGA stop codon as a selenocysteine codon, is particularly interesting, as the relative amounts of the two tRNA^{Sec} isoforms, containing either mcm^{5}U or mcm^{5}Um, appear to affect the efficiency of UGA recoding (1, 12). As expected, tRNA^{Sec} from wild-type mice contained high levels of mcm^{5}U and mcm^{5}Um, and these modifications were entirely missing in *Alkbh8*$^{-/-}$ tRNA^{Sec}, which instead contained the wobble cm^{5}U (Fig. 4A). In addition, the corresponding amide, ncm^{5}U, was observed in *Alkbh8*$^{-/-}$ tRNA^{Sec}. Similarly, mcm^{5}s^{2}U was found at high levels in tRNA$^{Glu(UUC)}$ and tRNA$^{Arg(UCU)}$ from wild-type mice but was absent in *Alkbh8*$^{-/-}$ tRNA$^{Glu(UUC)}$ and *Alkbh8*$^{-/-}$ tRNA$^{Arg(UCU)}$, which instead contained a mixture of cm^{5}U, ncm^{5}U, and ncm^{5}s^{2}U (Fig. 4B and C). These results indicate that in the absence of mouse Alkbh8, the cm^{5}U-containing tRNAs are partially channeled into the pathway involved in generating ncm^{5}U/ncm^{5}s^{2}U or, alternatively, that ncm^{5}U is a precursor of cm^{5}U and that both of these accumulate when the latter is not converted to mcm^{5}U. We have not performed an exact quantitation of modified nucleosides, but the purified isoacceptors typically contained one wobble modification per ~100 unmodified nucleosides. We observed a strong (~100-fold) enrichment of the expected wobble uridine modifications in purified isoacceptor tRNA preparations relative to the number in total tRNA. This indicates that the isolated isoacceptors are relatively pure, which is further supported by the results of MALDI-TOF mass spectrometry (see below).

MALDI-TOF mass spectrometry of RNase T1 fragments of tRNA^{Sec}, tRNA$^{Glu(UUC)}$, and tRNA$^{Arg(UCU)}$ from wild-type and *Alkbh8*$^{-/-}$ mice. Further confirmation of the modification status of tRNA isoacceptors, as well as of the localization of the modification to the anticodon loop, was provided by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry of fragments obtained by RNase T1 digestion. The RNase T1 fragments encompassing the anticodon loop (indicated in blue in Fig. 5A and B) were the only fragments of tRNA^{Sec} and tRNA$^{Glu(UUC)}$ where a mass difference between the wild-type and *Alkbh8*$^{-/-}$ samples was observed. Wild-type tRNA^{Sec} gave rise to two anticodon-containing RNase T1 fragments with a mass difference of 14 Da, corresponding to the mcm^{5}U- and mcm^{5}Um-containing isoforms (Fig. 5A). Due to the natural isotope distribution, each fragment gives rise to a cluster of peaks of approximately 1.0-Da spacing, where the leftmost peak corresponds to the monoisotopic mass. tRNA^{Sec} from *Alkbh8*$^{-/-}$ mice gave a single cluster of peaks, which displayed a mass reduction of 15.0 Da relative to the mass of the mcm^{5}U-containing fragment observed for wild-type tRNA^{Sec}. This is compatible with the observed (by LC–MS-MS) presence of cm^{5}U and ncm^{5}U, which have molecular masses that are 14.0 Da and 15.0 Da less, respectively, than that of mcm^{5}U. Wild-type tRNA$^{Glu(UUC)}$ gave rise to a fragment corresponding to the presence of wobble mcm^{5}s^{2}U, whereas *Alkbh8*$^{-/-}$ tRNA$^{Glu(UUC)}$ gave rise to a more complex peak pattern, compatible with the presence of cm^{5}U, ncm^{5}U, and ncm^{5}s^{2}U (Fig. 5B). tRNA$^{Arg(UCU)}$, which also contains wobble mcm^{5}s^{2}U, gave results similar to those obtained with tRNA$^{Glu(UUC)}$ (data not shown). In summary, these data strongly support the results obtained by LC–MS-MS analysis of nucleosides and, in addition, locate the modification to the anticodon loop.

ALKBH8 and TRM112 form a functional tRNA methyltransferase complex. A commissioned (Hybrigenics, Paris, France) yeast two-hybrid screen using human ALKBH8 as bait identified HSPC152/TRM112 as a likely partner (data not shown). A BLAST search revealed that TRM112 is the closest human homologue of yeast Trm112. Since yeast Trm112 is an accessory protein required for several methyltransferases, including Trm9 (15), we considered it likely that TRM112 may form a methyltransferase complex together with ALKBH8. To investigate the putative interaction between human TRM112 and ALKBH8, untagged TRM112 was coexpressed in *E. coli* with 6×His-tagged full-length ALKBH8 or with the individual AlkB (ALKBH8-AlkB; aa 1 to 354) or methyltransferase (ALKBH8-MT; aa 352 to 664) domains. 6×His-tagged protein was then affinity purified from the bacterial lysate by adsorp-

FIG. 4. Wobble uridine modifications in tRNA^{Sec}, tRNA$^{Glu(UUC)}$, and tRNA$^{Arg(UCU)}$ from wild-type and *Alkbh8*$^{-/-}$ mice. (A to C) LC–MS-MS analysis of modified uridine nucleosides in isoacceptors tRNA^{Sec} (A), tRNA$^{Glu(UUC)}$ (B), and tRNA$^{Arg(UCU)}$ (C) from wild-type and *Alkbh8*$^{-/-}$ mice. Blue and red curves are as described in the Fig. 3 legend. Chromatograms for nucleosides only present in trace amounts in a given isoacceptor (e.g., mcm^{5}s^{2}U in tRNA^{Sec}) are not shown. Retention times and arrows are as in Fig. 3.

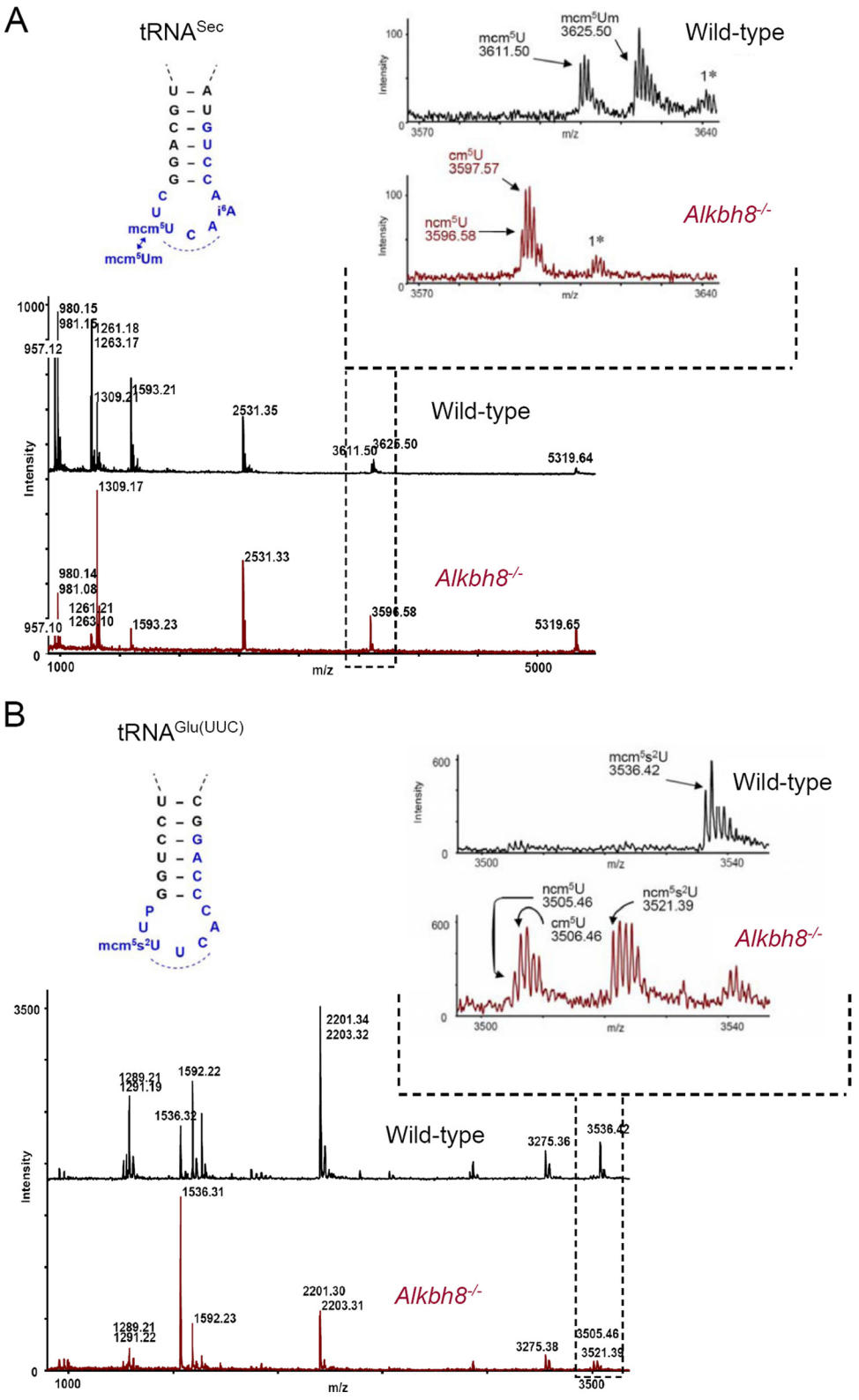


FIG. 5. MALDI-TOF mass spectrometry of RNase T1 fragments of $tRNA^{Sec}$ (A) and $tRNA^{Glu(UUC)}$ (B) purified from wild-type (black) and *Alkbh8*$^{-/-}$ (red) mice. Only one fragment (inset; representing the anticodon loop) displayed a difference in atomic mass when wild-type and *Alkbh8*$^{-/-}$ samples were compared, and the relevant *m/z* interval of this fragment is shown in greater detail. The 2′-3′-cyclic phosphate versions of the RNase T1 fragments represent the major peaks, and 1∗ indicates minor signals from the 3′-phosphate versions. The peaks corresponding to the modifications identified by LC–MS-MS analysis are indicated.

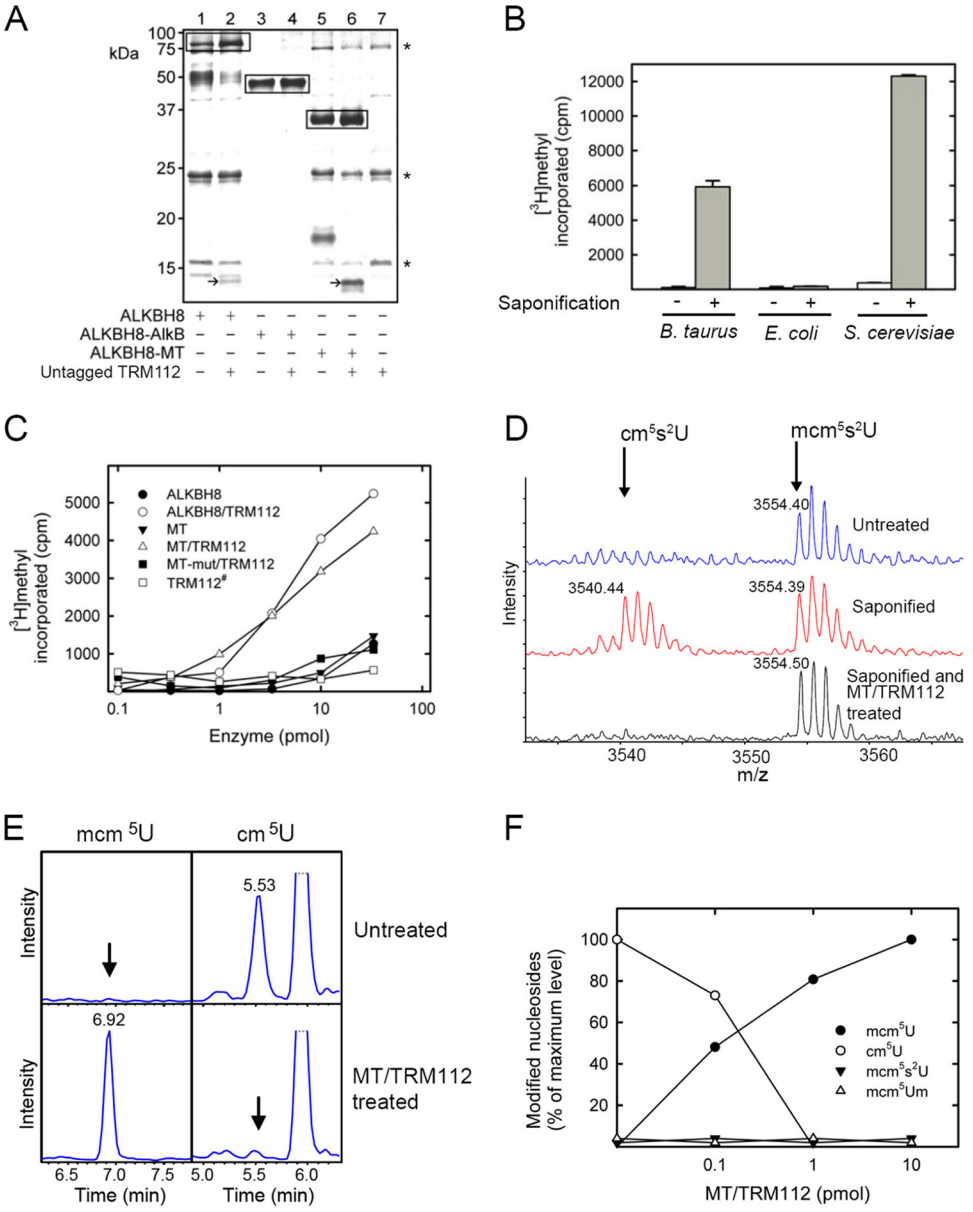


FIG. 6. Enzymatic activity of the human ALKBH8/TRM112 methyltransferase complex. (A) Copurification of human TRM112 (arrows) with the MT domain of human ALKBH8. 6×His-tagged ALKBH8 or its individual MT (amino acids 352 to 664) or AlkB (amino acids 1 to 354) domain (indicated by boxes) was coexpressed with untagged TRM112 in *E. coli* as indicated and affinity purified on Talon beads. Asterisks indicate background bands representing *E. coli* proteins (not visible for ALKBH8-AlkB, which gave higher expression levels, and hence, less eluate was loaded). (B) MT activity of 100 pmol ALKBH8-MT/TRM112 on 10 μg of total tRNA from different organisms. tRNA was saponified where indicated. (C) The MT activity of ALKBH8 requires TRM112. The methyltransferase activity on 10 μg of saponified calf liver tRNA using different amounts of purified recombinant protein was measured. MT-mut, mutant ALKBH8-MT with G416A, G418A, and G431A amino acid substitutions; TRM112#, 6×His-tagged TRM112. (D) ALKBH8-MT/TRM112-mediated remethylation of saponified $tRNA^{Glu(UUC)}$ from calf liver, as demonstrated by MALDI-TOF mass spectrometry of RNase T1 fragments. In this experiment, the RNase T1 digestion primarily yielded the 3′-phosphate version of the fragments (18.0 Da heavier than the 2′-3′-cyclic phosphate version). (E) LC–MS-MS analysis of nucleosides from total tRNA (3.2 μg) from livers of *Alkbh8*$^{-/-}$ mice, not treated or incubated with 100 pmol of ALKBH8-MT/TRM112. Retention times and arrows are as in Fig. 3. (F) Titration of ALKBH8-MT/TRM112 activity on *Alkbh8*$^{-/-}$ tRNA (3.2 μg). LC–MS-MS analysis was performed as described for panel E, and the peaks were quantified. The maximum levels of mcm^5U and cm^5U were estimated to be approximately 1 modification per 10,000 unmodified nucleosides. No peaks corresponding to mcm^5Um or mcm^5s^2U were detected.

tion to Talon beads, and the adsorbed material was analyzed by SDS-PAGE. As shown in Fig. 6A, untagged TRM112 copurified with immobilized ALKBH8 and ALKBH8-MT but not ALKBH8-AlkB, indicating that TRM112 indeed interacts with the MT domain of ALKBH8.

The methyl ester bond of mcm^5U^* is susceptible to mild alkaline hydrolysis, so-called "saponification," which yields cm^5U^* (27), the putative substrate for ALKBH8-MT (the sets consisting of the modifications $mcm^5U/mcm^5s^2U/mcm^5Um$, $ncm^5U/ncm^5s^2U/ncm^5Um$, and $cm^5U/cm^5s^2U/cm^5Um$ are re-

ferred to as mcm^{5}U*, ncm^{5}U*, and cm^{5}U*, respectively). To investigate the MT activity of recombinant ALKBH8, tRNA from calf (*B. taurus*), yeast (*S. cerevisiae*), and *E. coli* was subjected to saponification, followed by incubation with recombinant protein in the presence of the radiolabeled methyl donor *S*-adenosyl-*l*-[*methyl*-^{3}H]methionine. The results showed that the ALKBH8-MT/TRM112 complex can catalyze the incorporation of radiolabeled methyl groups into eukaryotic tRNA from yeast and calf, but only when the tRNA has been saponified (Fig. 6B). No such activity was observed toward bacterial tRNA from *E. coli*, which does not contain mcm^{5}U. We observed methyltransferase activity both for full-length ALKBH8 and for ALKBH8-MT but not for an ALKBH8-MT mutant (G414A/G416A/G431A) in which three putatively important and conserved glycine residues had been mutated to alanine (Fig. 6C). Only negligible MT activity was observed for ALKBH8 or ALKBH8-MT in the absence of TRM112 or for 6×His-tagged TRM112 alone. We also tested the other putative human Trm9 homologue, KIAA1456, in this assay but could not detect Trm9-like MT activity (data not shown).

In mammalian tRNA, mcm^{5}s^{2}U is a much more abundant modification than mcm^{5}U, which has been observed only in the low-abundance tRNA^{Sec}. Thus, the high level of MT activity observed using saponified calf tRNA as substrate may suggest that cm^{5}s^{2}U (resulting from saponification of mcm^{5}s^{2}U) also served as an MT substrate. This was indeed the case, as MALDI-TOF analysis of tRNA$^{Glu(UUC)}$ clearly showed that saponification partially hydrolyzed the methyl ester of mcm^{5}s^{2}U and incubation with the ALKBH8-MT/TRM112 complex entirely reversed this effect (Fig. 6D).

Enzymatic activity of ALKBH8-MT/TRM112 against *Alkbh8*$^{-/-}$ tRNA. As described above (Fig. 6B), tRNA from calf and yeast was not a substrate for the recombinant human ALKBH8-MT/TRM112 complex unless it was saponified. Our observation that cm^{5}U accumulated in tRNA from *Alkbh8*$^{-/-}$ mice suggested that their tRNA may function as a MT substrate even without saponification. Clearly, wobble cm^{5}U in purified *Alkbh8*$^{-/-}$ tRNA disappeared upon incubation with the human ALKBH8-MT/TRM112 complex, and a concomitant increase in mcm^{5}U was observed (Fig. 6E and F). We were unable to detect any formation of mcm^{5}s^{2}U or mcm^{5}Um, in accordance with the observed absence of cm^{5}s^{2}U and cm^{5}Um in the *Alkbh8*$^{-/-}$ tRNA.

Efficiency of UGA decoding by *Alkbh8*$^{-/-}$ tRNA. Selenocysteine (Sec) is the 21st amino acid and is encoded by the UGA stop codon. The correctly modified tRNA^{Sec} and the SECIS (*se*lenocysteine *i*nsertion *s*equence) secondary structural motif in the 3′ untranslated region of the selenoprotein mRNA are both required for recoding of the UGA stop codon to Sec (18). There are several indications that mcm^{5}Um-modified tRNA^{Sec} is important for efficient synthesis of several selenoproteins, like Gpx1 (5, 12, 18). Since tRNA^{Sec} from *Alkbh8*$^{-/-}$ mice lacked mcm^{5}Um (see above), an effect on selenoprotein synthesis in the *Alkbh8*$^{-/-}$ mice might be expected. We therefore studied the selenoproteome in liver, heart, kidney, lung, spleen, and brain by ^{75}Se labeling (injections of ^{75}Se-labeled sodium selenite as described in Materials and Methods) and only detected altered expression of a single liver selenoprotein, Gpx1 (Fig. 7A). The amount of another major liver selenoprotein, thioredoxin reductase 1 (Txnrd1), which is not reported to be regulated by mcm^{5}Um levels in liver (5), was the same in wild-type and *Alkbh8*$^{-/-}$ livers. These data agree with the observation that the Gpx1 level in liver is particularly affected when the selenocysteine tRNA gene, *Trsp*, is mutated (7). Since Gpx1 represents the majority of the glutathione peroxidase activity in liver (6), total glutathione peroxidase activity was determined as an indirect measure of Gpx1 activity. In agreement with the reduced Gpx1 expression levels, *Alkbh8* targeting also resulted in decreased glutathione peroxidase activity (Fig. 7B).

A dual luciferase (DualLuc) reporter assay was developed to examine the efficiency of selenocysteine incorporation *in vitro* (Fig. 7C). The *Renilla* luciferase (Rluc) and firefly luciferase (Fluc) genes in the DualLuc-Gpx1 RNA reporter are interrupted by a UGA codon, and the Gpx1 SECIS element is present in the 3′ untranslated region to promote selenocysteine incorporation (Fig. 7C). Thus, the Fluc but not the Rluc activity will depend on efficient recoding of UGA to Sec. The luciferase activities from this reporter were measured in rabbit reticulocyte lysates depleted of endogenous tRNA (20) and supplemented with tRNA from wild-type or *Alkbh8*$^{-/-}$ livers. The results were comparable with those of the ^{75}Se-labeling experiment; that is, a modest but significant reduction of UGA recoding was observed for *Alkbh8*$^{-/-}$ tRNA^{Sec} relative to the level seen with wild-type tRNA^{Sec} (Fig. 7C).

DISCUSSION

Through studies of *Alkbh8*$^{-/-}$ knockout mice and the recombinant ALKBH8/TRM112 methyltransferase complex, we demonstrate here that ALKBH8 performs the final methylation step during formation of the wobble uridine modification mcm^{5}U in mammals. Furthermore, ALKBH8-catalyzed methylation is required for the subsequent 2-thiolation and ribose methylation leading to mcm^{5}s^{2}U and mcm^{5}Um, respectively. This agrees well with the results of a previous study of *Leishmania tarentolae*, which also recognized mcm^{5}U as a common modification intermediate for thiolation and ribose methylation (24). In accordance with the results of previous studies of *S. cerevisiae* (22, 34), we observed that mcm^{5}U and its derivatives were replaced by ncm^{5}U/ncm^{5}s^{2}U in *Alkbh8*$^{-/-}$ tRNA, but we also observed accumulation of the unmethylated precursor cm^{5}U. The conserved CTU1/CTU2 complex is responsible for 2-thiolation of mcm^{5}U, and the observation that mcm^{5}s^{2}U was replaced by mcm^{5}U in yeast strains with Ctu1 deleted (11) also supports the notion that ALKBH8-mediated methylation of cm^{5}U occurs prior to thiolation. In summary, our data give novel insights into the sequential order of the modification reactions occurring on wobble uridines (summarized in Fig. 8).

Sec is often referred to as the 21st amino acid, and tRNA^{Sec} mediates the cotranslational insertion of Sec into the 25 selenoproteins in mammals (29). Two populations of tRNA^{Sec} are found in mammalian cells; one contains mcm^{5}U in the wobble position, whereas the other contains mcm^{5}Um (12, 28). It has been proposed that the relative levels of the two forms are actively regulated and that the mcm^{5}Um-containing form is required for efficient expression of certain stress-related selenoproteins. Although several lines of indirect evidence have clearly supported this notion (5, 7, 9, 32), we have abrogated

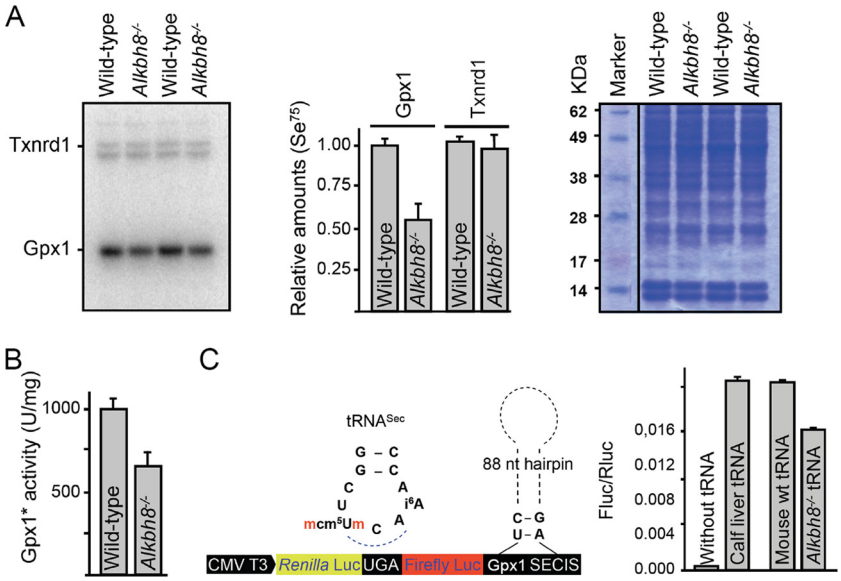


FIG. 7. Selenoprotein expression in wild-type versus *Alkbh8*$^{-/-}$ mice. (A) ^{75}Se labeling and selenoprotein analysis. Mice were labeled with ^{75}Se for 48 h, and liver protein was extracted from wild-type and *Alkbh8*$^{-/-}$ mice prior to electrophoresis to detect ^{75}Se-labeled proteins. Gpx1 and Txnrd1 were detected by gel electrophoresis and phosphorimaging, and the corresponding band intensities are shown in the bar graphs. Identification of Gpx1 and Txnrd1 was based on the method of a previous study (6). A Coomassie blue-stained gel (right) was used to correct for loading differences. (B) Measurement of glutathione peroxidase activity in liver extracts from *Alkbh8*$^{-/-}$ and wild-type mice. Error bars represent standard deviations in the results of triplicate experiments. The *y* axis represents nanomoles of NADPH/min/mg protein. *, Gpx1 is responsible for the majority of the glutathione peroxidase activity in liver and thus is indicated on the *y* axis. (C) Reduced efficiency of stop codon recoding by *Alkbh8*$^{-/-}$ relative to that of wild-type tRNA. DualLuc-Gpx1 reporter RNA (schematically represented in the left panel) was translated in tRNA-depleted rabbit reticulocyte lysates in the presence of calf liver tRNA or tRNA from wild-type or *Alkbh8*$^{-/-}$ mice, luciferase activities were measured, and the Fluc/Rluc ratios were calculated. Error bars represent ranges between results for duplicate samples in a typical experiment. nt, nucleotide.

for this study the ribose methylation of tRNA^{Sec} wobble uridine and see rather subtle effects on selenoprotein expression and activity. Thus, our data indicate that the U34 modification status has a smaller effect on the synthesis of stress-related selenoproteins than previously thought.

In yeast, most U34-containing tRNAs have the wobble uridine modifications mcm^{5}U or ncm^{5}U or their derivatives, and these modifications are also present in mammals (22, 26, 40). The knowledge of the enzymes introducing such modifications in mammalian tRNA is very limited. However, in yeast and in *Caenorhabditis elegans*, the Elongator complex, which is also involved in transcriptional regulation, has been shown to be required for the early steps of generating these modifications (19, 41). Our observations clearly indicate that the pathways leading to the formation of mcm^{5}U and ncm^{5}U are also interconnected in mammals, supporting the notion that the Elongator complex here is also involved in tRNA modification. Mutations in the human genes encoding the Elongator components Elp1 and Elp3 are associated with the neurological diseases familial dysautonomia and amyotrophic lateral sclerosis, respectively (36, 39), and Elp1 and Elp3 mutants of *C. elegans* also displayed neurological symptoms (8). The *Alkbh8*$^{-/-}$ mice appeared normal, but it is tempting to speculate that the extensive mismodification of wobble uridines reduces the fidelity and efficiency of protein translation, possibly leading to the accumulation of insoluble protein aggregates, a hallmark of many neurological diseases. Thus, it will be of great interest to subject the *Alkbh8*$^{-/-}$ mice to analysis aimed at revealing minor changes in the appearance and performance of the neural system.

The presence of the Trm9 function in all eukaryotes (23) clearly indicates that the mcm^{5}U modifications have an important role in protein translation. Nonetheless, like *Alkbh8*$^{-/-}$ mice, the *trm9* mutant of *S. cerevisiae* exhibits no phenotype under normal conditions, despite the complete absence of mcm^{5}U and mcm^{5}s^{2}U. However, the *trm9* mutant is hypersensitive toward paromomycin, which introduces translational stress, and toward the genotoxic agent methyl methanesulfonate (3, 23). Besides, a recent study found that small interfering RNA-mediated knockdown of ALKBH8 induced apoptosis in a bladder cancer-derived cell line and also inhibited tumor growth in a corresponding mouse model (38). Correspondingly, the mouse Alkbh8 function may not be crucial for development and survival in a protected, noncompetitive environment but may still provide improved fitness to animals living in a more competitive natural habitat.

We have here presented strong evidence that the MT domain of ALKBH8 is responsible for converting cm^{5}U to mcm^{5}U in the wobble position of several tRNA species. The present study has not shed further light on the role of the RRM and AlkB portion of the protein, but the results implicate this region in tRNA modification also. Indeed, bifunctional tRNA modification enzymes have already been described (4, 35). Initially, we were intrigued by the possibility that the putative demethylase activity of the AlkB domain could remove the esterified methyl group in mcm^{5}U or the ribose methylation of mcm^{5}Um, but we have not been able to detect any such activity associated with the recombinant protein. Thus, we are currently developing and characterizing gene-targeted mouse

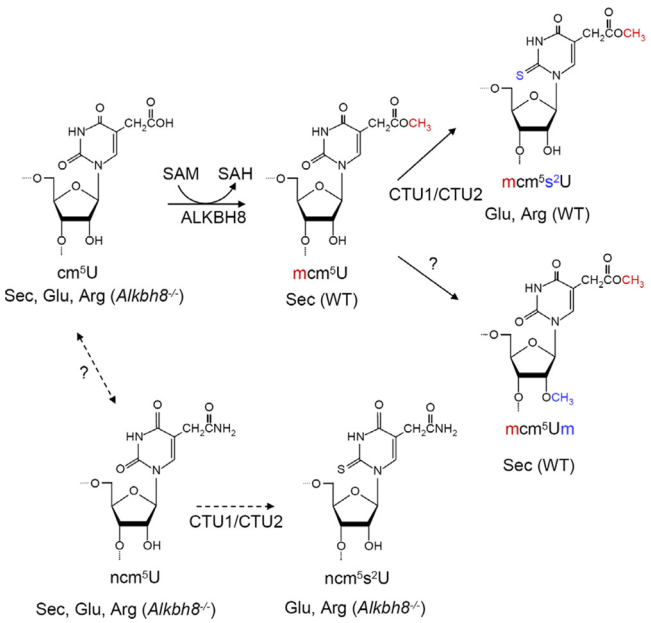


FIG. 8. The role of ALKBH8 in the biogenesis of wobble uridine modifications in higher eukaryotes. The indicated scheme integrates the results of the present study with current knowledge of wobble uridine modifications. Red print indicates modifications directly formed by ALKBH8, and blue print indicates modifications that rely on ALKBH8-generated substrates for their formation. The isoacceptors addressed in the present study and their modification status in the wild-type (WT) and *Alkbh8*$^{-/-}$ mice are indicated.

models which express Alkbh8 with one inactivated enzymatic domain (MT or AlkB). Hopefully, tRNA from these mice will give new insights into the enigmatic function of the RRM and AlkB domains.

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