The METTL20 Homologue from Agrobacterium tumefaciens Is a Dual Specificity Protein-lysine Methyltransferase That Targets Ribosomal Protein L7/L12 and the β Subunit of Electron Transfer Flavoprotein (ETFβ)*

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Jędrzej Małecki, Helge-André Dahl, Anders Moen, Erika Davydova, and Pål Ø. Falnes

From the Department of Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo 0316, Norway

Human METTL20 is a mitochondrial, lysine-specific methyltransferase that methylates the β-subunit of electron transfer flavoprotein (ETFβ). Interestingly, putative METTL20 orthologues are found in a subset of α-proteobacteria, including Agrobacterium tumefaciens. Using an activity-based approach, we identified in bacterial extracts two substrates of recombiant METTL20 from A. tumefaciens (AtMETTL20), namely ETFβ and the ribosomal protein RpL7/L12. We show that AtMETTL20, analogous to the human enzyme, methylates ETFβ on Lys-193 and Lys-196 both in vitro and in vivo. ETF plays a key role in mediating electron transfer from various dehydrogenases, and we found that its electron transferring ability was diminished by AtMETTL20-mediated methylation of ETFβ. Somewhat surprisingly, AtMETTL20 also catalyzed monomethylation of RpL7/L12 on Lys-86, a common modification also found in many bacteria that lack METTL20. Thus, we here identify AtMETTL20 as the first enzyme catalyzing RpL7/L12 methylation. In summary, here we have identified and characterized a novel bacterial lysine-specific methyltransferase with unprecedented dual substrate specificity within the seven β-strand class of lysine-specific methyltransferases, as it targets two apparently unrelated substrates, ETFβ and RpL7/L12. Moreover, the present work establishes METTL20-mediated methylation of ETFβ as the first lysine methylation event occurring in both bacteria and humans.

Methylation is a common biochemical reaction catalyzed by various methyltransferases (MTases) and involving transfer of a methyl group from a methyl donor, usually S-adenosyl-L-methionine (AdoMet), to a wide range of acceptor molecules, including small metabolites and macromolecules such as RNA, DNA, and proteins (1). Proteins can be methylated on several amino acid residues but most commonly on lysines and arginines (2–4). Lysine residues can be mono-, di-, and trimethylated on the ε-amino group in reactions catalyzed by lysine (K)-specific protein MTases (KMTs). Lysine methylation leaves the overall charge unaffected but decreases the potential for hydrogen bond formation and increases the bulkiness. Lysine methylation can modulate many different aspects of protein function, such as stability, intracellular localization, enzymatic activity, and the ability to interact with other molecules (5).

The human genome has been predicted to encode ~200 MTases, and the two main groups are the seven-β-strand (7BS) MTases, which have a characteristic core fold of seven β-strands, and the SET proteins, which contain a defining SET domain (6). Whereas SET proteins mainly encompass KMTs, many of which target histones (7), the 7BS MTases have been shown to methylate a wide range of small molecules and macromolecular substrates, including lysine and arginine residues in proteins. In recent years, several of the human members of a group of related 7BS MTases, denoted “methyltransferase family 16” (MTF16), were established as KMTs, and these include CaM-KMT that methylates calmodulin (8), VCP-KMT that targets the ATP-dependent chaperone valosin-containing protein (9), HSPA-KMT (METTL21A) that methylates several HSPA (Hsp70) proteins (10, 11), METTL22 that targets Kin17 (10), and eEF2-KMT (FAM86A) that methylates eukaryotic elongation factor 2 (12). Another human MTF16 member is METTL20 (ETFβ-KMT), a mitochondrial enzyme that methylates the β-subunit of electron transfer flavoprotein (ETFβ) (13, 14). Interestingly, putative orthologues of human METTL20 are found in some bacteria, contrasting with other human MTF16 members, which are restricted to eukaryotes.

Eukaryotic ETF is a mitochondrial protein composed of two subunits, α and β (ETFα and ETFβ), and it binds two cofactors, FAD and AMP (15). ETF mediates transfer of electrons from several mitochondrial FAD-containing dehydrogenases (DHs) to the ETF:quinone oxidoreductase (ETF-QO), leading to reduction of the quinone pool of the mitochondrial respiratory chain (15). In humans, there are ~13 ETF-dependent DHs, including sarcosine dehydrogenase and dimethylglycine dehydrogenase (DMGDH), which are involved in choline metabolism, as well as several acyl-CoA dehydrogenases (ACADs) involved in degradation of amino acids or in β-oxidation of fatty acids (e.g. medium-chain acyl-CoA dehydrogenase (MCAD))
Dual Specificity METTL20 Homologue from *A. tumefaciens*

(16–18). Structural studies have shown that the so-called “recognition loop” in ETFβ, encompassing residues 191–200, is important for its interaction with MCAD, and it has been suggested that this loop is also involved in the interaction with the remaining DHs (19). We and others recently showed that human METTL20 methylates ETFβ on Lys-200 and Lys-203, which partly overlap with the recognition loop, and that methylation affects electron transfer from DHs to ETF (13, 14). Interestingly, bacterial METTL20 homologues are mostly limited to α-proteobacteria (13), and ETFs from α-proteobacteria are, in contrast to most other bacterial ETFs, highly similar to their human counterparts (20). As α-proteobacteria are considered the evolutionary precursors of mitochondria (21), this suggested that ETFβ methylation may be conserved from bacteria to eukaryotes.

Protein lysine methylation appears much less common in bacteria than in eukaryotes, and many of the reported methylation events involve components of the protein synthesis machinery, e.g. ribosomal proteins and translation factors (22). For example, the 50S ribosomal protein Rpl11 is methylated at multiple sites by the MTase PrmA (23). Another example is Rpl7/L12, which is part of the multimeric ribosomal stalk shown to be important for recruiting translational factors to the ribosome (24, 25). Rpl7/L12 is methylated at a single lysine residue (Lys-82 in *Escherichia coli* Rpl7/L12) in a wide range of prokaryotic species (26–29). However, the identity of the responsible enzyme(s) as well as the functional significance of the methylation remains elusive.

In the present study, we have taken an unbiased approach to identify the substrates of the putative METTL20 orthologue from *Agrobacterium tumefaciens* (AtMETTL20). Through extensive in vitro and in vivo studies, we demonstrate that AtMETTL20 is a dual specificity KMT that targets both ETFβ and Rpl7/L12. By analyzing several ETF-dependent dehydrogenases from *A. tumefaciens*, we show that methylation of ETFβ impairs the ability of ETF to extract electrons from these dehydrogenases.

**Experimental Procedures**

**Cloning and Mutagenesis**—Genes encoding bacterial proteins were amplified from genomic DNA isolated from *A. tumefaciens* strain C58 (ATCC 33970) or *Rhizobium etli* strain CFN 42 (ATCC 51251) using Phusion DNA Polymerase HF (Thermo Fisher Scientific). cDNA generated from HeLa cells was used to amplify ORFs for human proteins. Mutagenesis was performed using mutagenic primers designed with the PrimerX program. All constructs were sequence-verified.

**Bioinformatics Analysis**—The NCBI Basic Local Alignment Tool (BLAST) was used to identify protein sequences homologous to *A. tumefaciens* METTL20, ETFβ, and Rpl7/L12 (30). Multiple protein sequence alignments were performed using algorithms embedded in the Jalview (v2.8) interface (31).

**Cell Cultures**—*A. tumefaciens* strains (C58 and GV3101 pM90) were grown at 28 °C in LB medium supplemented with 10–100 μg/ml rifampicin (Rif) and 10–50 μg/ml gentamycin (Gent). The growth rate of different strains of *A. tumefaciens* at 28 °C in LB or minimal medium with 0.2% glucose was followed by monitoring absorbance at 600 nm, starting from cultures with identical *A*₅₆₀ (~0.2).

**Generation of *A. tumefaciens* Strain with AtMETTL20 Gene Knock-out—** *A. tumefaciens* strain GV3101 pM90 (tetracycline-sensitive) was used to generate bacteria with AtMETTL20 gene knock-out (KO) using the TargeTron Gene Knock-out System (Sigma-Aldrich) according to the manufacturer’s protocol with some modifications. In short, the pBL1 plasmid (32) was mutated to contain an intron that would self-insert into *A. tumefaciens* METTL20 gene between nucleotides 261 and 262, thus introducing a premature stop codon, located downstream of the SGSG sequence within Motif 1 (Fig. 1A). The resulting plasmid (pBL1-MT20(261)KO) was transformed into bacteria that were selected for Rif (100 μg/ml), Gent (50 μg/ml), and tetracycline (12.5 μg/ml) resistance. Positive colonies were grown at 28 °C in MGL medium (5 g/liter tryptone, 2.5 g/liter yeast extract, 5 g/liter mannitol, 1 g/liter monopotassium l-glutamate, 1 μg/liter biotin, 250 mg/liter KH₂PO₄, 100 mg/liter NaCl, 100 mg/liter MgSO₄·7H₂O) supplemented with Rif, Gent, and tetracycline until *A*₅₆₀ reached 0.3, and then intron insertion was induced with 5 mM m-toluic acid for 3 h. Cells were harvested, plated, and screened for the presence of the AtMETTL20 gene with a 900-bp intron insertion. The pBL1-MT20(261)KO plasmid was removed (cured) from the bacteria by overnight growth at 28 °C in LB medium (Rif + Gent), plating on LB-agarose (Rif + Gent), and screening for tetracycline-sensitive colonies. Finally, *A. tumefaciens* colonies with AtMETTL20 gene knock-out were tested for absence of pBL1 plasmid (by colony PCR) and sequence-verified to contain mutated AtMETTL20 gene with properly inserted intron.

**Expression and Purification of Recombinant Proteins**—Human A38-METTL20 (with the N-terminal 38 amino acids deleted) and human ETFα/β heterodimer were cloned, expressed, and purified as described previously (13). The gene encoding *A. tumefaciens* ETFα was cloned into pETDuet-1, whereas other relevant bacterial genes were cloned into pET28a. All proteins were expressed as N-terminally His₅-tagged proteins in the *E. coli* strain BL21-CodonPlus(DE3)-RIPL (Agilent Technologies). Protein expression was routinely carried out at 16 °C (for 18 h) by induction with 0.1 mM isopropyl-1-thio-β-d-galactopyranoside except in the case of Rpl7/L12 from *A. tumefaciens*, which as indicated was expressed at either 16 °C or 37 °C (for 4 h). Cells were harvested by centrifugation and frozen at −20 °C.

Unless indicated otherwise, all proteins were purified according to the same purification protocol as described below. Bacteria were lysed in Lysis Buffer 1 (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5% glycerol, 30 mM imidazole) supplemented with 2 mM β-mercaptoethanol, 1× Complete (EDTA-free) protease inhibitor mixture (Roche Applied Science), and 10 units/ml Benzonase nuclease (Sigma-Aldrich). His-tagged proteins were loaded onto Ni-NTA-agarose column (Qiagen) equilibrated in Lysis Buffer 1, washed extensively with Lysis Buffer 1, and finally eluted with Lysis Buffer 1 supplemented with 200 mM imidazole. During purification of FAD-containing proteins, Lysis Buffer 1 was additionally supplemented with 2 μM FAD, and during purification of ETF-QO, Lysis Buffer 1 was...
supplemented with 2 μM FAD and 1% Triton X-100. Proteins eluted from Ni-NTA-agarose were buffer-exchanged to Storage Buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol) using centrifugal concentrators with a molecular mass cutoff of 10–50 kDa (Sartorius, Goettingen, Germany) and stored at −20 °C, except recombinant A. tumefaciens METTL20 and RpL7/L12, which were stored at −20 °C in Storage Buffer containing 25% glycerol. All proteins were 90–95% pure as assessed by SDS-PAGE and Coomassie Blue staining except for human METTL20, which was ~50% pure. Protein concentration was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Theoretical molecular mass of recombinant proteins was used to calculate molar concentrations.

Purification of Recombinant A. tumefaciens ETFα/β Heterodimer—E. coli cells expressing His6-tagged ETFα and His6-tagged ETFβ were lysed separately (in Lysis Buffer 1 supplemented with 2 μM FAD) and then mixed together, and the mixed lysate was used to purify ETFα/β heterodimer on Ni-NTA-agarose similarly as described in the previous section. The eluate from Ni-NTA-agarose was buffer-exchanged to Storage Buffer using centrifugal concentrators with a molecular mass cutoff of 50 kDa and frozen at −20 °C. After thawing on ice, the precipitated protein, containing mostly free ETFβ, was removed by centrifugation, whereas the supernatant, containing mostly ETFα/β heterodimer, was again buffer-exchanged to Storage Buffer. Finally, the amount of glycerol in the sample was increased to 25%, and the protein was stored at −20 °C. For ETFα/β and other FAD-containing enzymes, the A275/A436 ratio was ~7, indicating that FAD remained strongly associated with these proteins throughout the purification procedure.

Preparation and Fractionation of Bacterial Cell Extracts—A. tumefaciens cells were lysed at 4 °C for 10 min in Lysis Buffer 2 (50 mM Tris-HCl, pH 6.5, 100 mM NaCl, 1% Triton X-100, 5% glycerol, 0.5 mM DTT, 1× Complete (EDTA-free) protease inhibitor mixture), and the lysate was sonicated and cleared by centrifugation. Cell extracts were fractionated at 4 °C by ion exchange chromatography using either Pierce Strong Cation Exchange (S) or Pierce Strong Anion Exchange (Q) Spin Columns (Thermo Fisher Scientific). First, the NaCl concentration was reduced to 50 mM by diluting cell lysates with Dilution Buffer (50 mM Tris-HCl, pH 6.5, 1% Triton X-100, 5% glycerol), and then extracts were applied onto the S-column equilibrated with Dilution Buffer. Material bound to the S-column was eluted by a step gradient of increasing NaCl concentrations prepared in Dilution Buffer. Typically, four fractions were collected: 0.2S (eluted between 0.05 and 0.15 M NaCl), 0.3S (eluted between 0.15 and 0.3 M NaCl), 0.5S (eluted between 0.3 and 0.5 M NaCl), and 0.75S (eluted between 0.5 and 0.75 M NaCl). Unbound material, which was released in the S-column flow-through, was reapplied onto the Q-column equilibrated with Dilution Buffer. The unbound material, present in the Q-column flow-through, was designated FTQ, whereas material bound to the Q-column was eluted using Dilution Buffer with a step gradient of increasing NaCl concentrations, similarly as for the S-column, in four fractions, 0.15Q, 0.3Q, 0.5Q, and 0.75Q.

Isolation of Ribosomes from A. tumefaciens Cells—Ribosomal particles were isolated from A. tumefaciens cells at 4 °C following a previously published procedure (27) with some modifications. Cells were lysed in Ribosome Isolation Buffer (20 mM Tris-HCl, pH 7.5, 50 mM magnesium acetate, 100 mM NH4Cl, 1 mM EDTA) supplemented with fresh 2 mM DTT, 1 unit/ml DNase I (Qiagen), and 1× Complete (EDTA-free) protease inhibitor mixture and sonicated. Cellular debris was removed by centrifugation, and the supernatant was filtered (0.2 μm). Three milliliters of clear lysate was carefully layered on top of 1.5 ml of 1.1 m sucrose in Ribosome Isolation Buffer and centrifuged at 100,000 × g for 2 h using an Optima Max ultracentrifuge with MLS-50 rotor (Beckman-Coulter). The supernatant and the sucrose cushion were carefully removed, and the ribosomal pellet was washed, finally resuspended in Ribosome Isolation Buffer, aliquoted, and stored at −80 °C. Typically, ribosome solutions had an A280/A550 ratio of ~1.9. Ribosomes were quantified by RNA content, assuming a molecular extinction coefficient at 260 nm of ε260 = 39.1 × 106 M−1 cm−1 (33).

In Vitro Methyltransferase Assays Using 1HAdoMet—MTase activity of METTL20 was tested by setting up on ice 10-μl reactions containing 1× MTase Assay Buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM KCl, 1 mM MgCl2, 10% glycerol), 3–5 μl recombinant substrate or 20–60 μg of proteins from cell extracts (and/or equivalent fractions obtained from ion exchange chromatography), 50–80 pmol of METTL20, and 0.5 μCi of [3H]AdoMet (PerkinElmer Life Sciences) ([AdoMet]total = 0.64 μM; specific activity = 78.2 Ci/mmol). Reaction mixtures were then incubated at 28 °C for 2 h. Proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and stained with Ponceau S. The membrane was dried, sprayed with EN3HANCE spray (PerkinElmer Life Sciences), and exposed to Kodak BioMax MS film (Sigma-Aldrich). When recombinant protein substrates were used, [3H]AdoMet was diluted with non-radioactive AdoMet (New England Bio-labs) ([AdoMet]total = 32.6 μM; specific activity = 1.53 Ci/mmol). All fluorography experiments were performed three times with similar results, and results of representative experiments are shown.

For scintillation counting and titration experiments, reaction mixtures (10 μl) contained 1× MTase Assay Buffer, 0.1 mg/ml BSA (as carrier), 0.5 μCi of [3H]AdoMet ([AdoMet]total = 32.6 μM), increasing concentrations of METTL20 (0–20 μM), and fixed and equal concentrations of ETFα/β or RpL7/L12 (~2 μM). Reactions were stopped by precipitation with 10% trichloroacetic acid, and trichloroacetic acid-insoluble material was subjected to scintillation counting.

Preparation of Samples for MS Analysis—In vitro methyla- tion of recombinant or cellular (in extract) proteins for the purpose of mass spectrometry (MS) analysis was performed as in the above section except that [3H]AdoMet was replaced with non-radioactive AdoMet (1 mM). Proteins were resolved by SDS-PAGE and stained with Coomassie Blue; the portion of the
gel containing the protein of interest was cut out and subjected to \textit{in}-g\textit{el} trypsin (Sigma-Aldrich), Asp-N (Roche Applied Science), or Glu-C (Promega) digestion; and the resulting proteolytic fragments were analyzed as described previously (9, 11). MS data were analyzed with an in-house maintained A. \textit{tumefaciens} protein sequence database using SEQUEST\textsuperscript{TM} and Proteome Discoverer\textsuperscript{TM} (Thermo Fisher Scientific). The mass tolerances of a fragment ion and a parent ion were set as 0.5 Da and 10 ppm, respectively. Methionine oxidation and cysteine carbamidomethylation were selected as variable modifications. MS/MS spectra of peptides corresponding to methylated ETF\textbeta using a molecular extinction coefficient of CoQ1 (oxidized at 275 nm for 2 min, and the rate of CoQ1 reduction was allowed to equilibrate at room temperature for 5 min, and then

Results

\textit{Bacterial METTL20 Homologues Have Protein Methyltransferase Activity}—During our efforts to characterize the human MTase METTL20 (13), we noticed the presence of proteins displaying high sequence homology to METTL20 in a few bacteria. The bacterial METTL20 proteins are primarily found in \textalpha-proteobacteria, especially in the Rhizobiales order, where they seem to be ubiquitous. In addition, some scattered members of \textbeta-proteobacteria, \textepsilon-proteobacteria, and actinobacteria also have a METTL20-like protein. Interestingly, the majority of these proteins have already been annotated as “50S ribosomal protein L11 methyltransferase” because they were found to display some sequence homology to the lysine-specific MTase PrmA from \textit{E. coli} that targets ribosomal protein L11 (36). However, as illustrated by the sequence alignment shown in Fig. 1A, they are much less similar to PrmA than to human METTL20, suggesting that their function is different from that of PrmA. Besides containing the 7BS MTase hallmark motifs (I, Post I, and II), the bacterial METTL20 proteins have the (D/E)XX(Y/F) motif characteristic of MTF16 members (Fig. 1A) (9).

To investigate the function of bacterial METTL20 proteins, we expressed and purified such enzymes from \textit{A. tumefaciens} and \textit{R. etli} as recombinant proteins in \textit{E. coli}. Reasoning that an \textit{A. tumefaciens} cell extract may contain substrates for bacterial METTL20 proteins, we incubated such extracts with recombinant enzymes in the presence of \textsuperscript{3}H-labeled AdoMet and then analyzed the samples by SDS-PAGE and fluorography. Interestingly, extracts incubated with AtMETTL20 showed radiolabeling of two proteins with apparent molecular masses of \textasciitilde28 and \textasciitilde15 kDa (Fig. 1B, lane 5). Two putatively inactive mutant AtMETTL20 enzymes, D81A and D105A, where key catalytic residues important for AdoMet binding had been mutated (indicated in Fig. 1A) were included as negative controls. Extracts incubated with these AtMETTL20 mutants showed no labeling of the \textasciitilde28- and \textasciitilde15-kDa substrates, thus excluding the possibility that the labeling was due to an \textit{E. coli}-derived contaminant rather than the activity of the recombinant enzyme (Fig. 1B). Also, both the \textasciitilde28- and \textasciitilde15-kDa substrates were methylated by the METTL20 homologue from \textit{R. etli}, whereas only the \textasciitilde28-kDa protein was labeled by human METTL20 (Fig. 1C). These results indicate that bacterial homologues of METTL20 are protein MTases with a substrate specificity partially overlapping with that of human METTL20.

Identification of ETF\textbeta and Rpl7/L12 as Likely Substrates of \textit{Bacterial METTL20}—To reveal the identity of the \textasciitilde28- and the \textasciitilde15-kDa substrates of bacterial METTL20 proteins, \textit{A. tumefaciens} extracts were fractionated using ion exchange chromatography, and the fractions were subjected to methyla-
tion by AtMETTL20 and analyzed by fluorography (Fig. 2A).

The ~28-kDa substrate bound poorly to an S-column and was found mostly in the S-column flow-through, whereas the ~15-kDa substrate bound partially to the S-column and could be eluted predominantly between 0.15 and 0.3 M NaCl (0.3S) (Fig. 2A, lane 5). When the S-column flow-through fraction was reapplied onto a Q-column, both the ~28- and the ~15-kDa proteins were found to partially bind and were predominantly eluted between 0.05 and 0.15 M NaCl (0.15Q) (Fig. 2A, lane 9).

For the 0.15Q fraction, regions of interest were excised from a Coomassie-stained protein gel (Fig. 2B) and subjected to trypsin digestion followed by protein identification through MS. Because human METTL20 was previously shown to methylate ETFβ (13, 14) and because the ~28-kDa substrate from A. tumefaciens was methylated also by human METTL20, we anticipated this protein to be A. tumefaciens ETFβ (AtETFβ), which has a molecular mass of ~28 kDa. Indeed, AtETFβ was identified as the predominant protein in the ~28-kDa region of the 0.15Q fraction (Table 1).

Ribosomal protein RpL7/L12 from A. tumefaciens (AtRpL7/L12) was identified as the most abundant protein in the ~15-kDa region of the 0.15Q fraction (Table 2), and also for this
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A

Cell lysate

S column

Eluted S fractions

Flow-through (FTS)

Q column

Eluted Q fractions

Flow-through (FTQ)

AtMETTL20

~28 kDa substrate

auto-
methylated AtMETTL20

~15 kDa substrate

B

C

D

E

[Image of gel electrophoresis and mass spectrometry results]
protein, a methylated lysine residue was identified, namely Lys-86, which was found primarily in the monomethylated state (>97% monomethylation) (Fig. 2D). E. coli RpL7/L12 has been reported to be associated with ribosomes (37), and to further investigate whether AtRpL7/L12 is likely to be the ~15-kDa substrate, ribosomes isolated from A. tumefaciens were subjected to in vitro methylation. Reassuringly, labeling of the ~15-kDa substrate, but not of the ~28-kDa substrate, was observed (Fig. 2E, lane 5). We used MS to establish the identity of the ~15-kDa substrate present in ribosomes, and again AtRpL7/L12 was found to be the most abundant protein in the ~15-kDa region (Table 3). In summary, the above results suggested two proteins, namely AtETFβ and AtRpL7/L12, as likely substrates of AtMETTL20.

Lys-193 and Lys-196 in A. tumefaciens ETFβ Are Methylated by METTL20 in Vitro—Based on sequence similarity, ETFs have been categorized into three main groups: I, II, and III (20). Interestingly, group I includes ETFs from humans and other eukaryotes but also ETF from a few bacteria, including those possessing METTL20 proteins. In contrast, other bacteria have been categorized into three main groups: I, II, and III (20). Our previous studies of human METTL20 had demonstrated that human ETFβ is methylated on two neighboring residues, namely Lys-200 and Lys-203, which correspond to Lys-193 and Lys-196 in AtETFβ. To verify that ETFβ is a substrate of AtMETTL20, we generated recombinant ETFα/β dimers to be used in enzymatic assays. To also investigate the cross-species compatibility of these enzymes and substrates, we investigated the activity of METTL20 from both human and A. tumefaciens on ETFα/β from both these organisms. The results showed that human and bacterial METTL20 were both active on ETFβ from these two organisms, although the enzymes were slightly more active on their cognate substrates (Fig. 3B). In accordance with the absence of a METTL20 homologue in E. coli, AtETFβ was unmethylated at Lys-193 when expressed in this host (Fig. 3C). Most importantly, incubation of E. coli-expressed AtETFβ with AtMETTL20 shifted the state of Lys-193 into predominantly mono- and dimethylation, showing that AtMETTL20 can indeed methylate this residue (Fig. 3C).

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FIGURE 2. Identification of ETFβ and RpL7/L12 as likely substrates of recombinant AtMETTL20. A. partial purification of AtMETTL20 substrates from A. tumefaciens extracts or ribosomes incubated with [3H]AdoMet and AtMETTL20 was assessed by fluorography (right; 1-week exposure). B, indication of protein bands used for AtMETTL20 substrate identification. Indicated fractions were resolved by SDS-PAGE and stained with Coomassie Blue. Indicated bands (arrows), corresponding to protein substrates visualized by fluorography, were identified by MS to contain predominantly AtETFβ, ribosomes incubated with [3H]AdoMet and AtMETTL20 was assessed by fluorography (upper panel; 3 weeks exposure). The band corresponding to the ~15-kDa protein substrate visualized by fluorography (lower panel, arrow in Ponceau S-stained membrane) was identified by MS to contain predominantly A. tumefaciens RpL7/L12 (Table 3). FT, flow-through.
these residues together or individually into arginine and investigated the effect on METTL20-mediated methylation. Indeed, whereas methylation was substantially reduced (to ~60%) when these residues were mutated individually, methylation was completely abolished in the K193R/K196R double mutant (Fig. 3, D and E). To substantiate these findings, we analyzed by MS an Asp-N-generated peptide encompassing Lys-193 and Lys-196 in ETF before and after treatment with AtMETTL20. In the absence of AtMETTL20 treatment, this peptide displayed in the case of all the ETF variants a mass corresponding to a complete lack of methylation (Fig. 3F). After AtMETTL20 treatment, the peptide from wild-type ETF displayed primarily tri- and tetramethylation, whereas the K193R and K196R mutants were found predominantly in the dimethylated state (Fig. 3F). (Note that in principle independent confirmation of these findings could have been provided by MS/MS sequencing of the Asp-N peptide, but we were unable to obtain such data). Taken together, the above results demonstrate that AtMETTL20 specifically and independently methylates Lys-193 and Lys-196 in AtETF and indicate that AtMETTL20 is a non-processive enzyme that typically introduces two methyl groups at each of these sites.

FIGURE 3. Recombinant AtMETTL20 methylates recombinant AtETF on Lys-193 and Lys-196. A, sequence alignment of ETF proteins from A. tumefaciens (At; NP_357017.1) and H. sapiens (Hs; NP_001976.1) and FixA from E. coli (Ec; NP_752004.2). Arrows indicate the position of Lys residues methylated by METTL20. B, METTL20-mediated methylation of ETF. ETF, either A. tumefaciens or human (Hs), was incubated with [3H]AdoMet and METTL20, either A. tumefaciens or human. [3H]Methyl incorporation into proteins was assessed by fluorography (upper panel; 1-day exposure) of Ponceau S-stained membrane (lower panel). C, Lys-193 in AtETF is methylated by AtMETTL20. AtETF was incubated with non-radioactive AdoMet and AtMETTL20 as indicated. Methylation of Lys-193 in AtETF-derived trypsin-digested peptides (as in Fig. 2C) was analyzed by MS. Shown are the mean relative intensities of signals gated for the different methylation states of Lys-193 in AtETF with error bars indicating the range of values from three independent experiments. D, mutational analysis indicates that both Lys-193 and Lys-196 in AtETF are methylated by AtMETTL20. AtETF with WT or mutated ETF as indicated was incubated with [3H]AdoMet and AtMETTL20, and ETF methylation was assessed by fluorography (1-day exposure). E, quantitation of AtMETTL20-mediated methylation of Lys-193 and Lys-196 in WT and mutant AtETF. AtETF with WT or mutant ETF was incubated with [3H]AdoMet and AtMETTL20, and ETF methylation was assayed by scintillation counting of trichloroacetic acid-precipitated material. Shown are averaged data from two experiments run in triplicate. Sample variation is expressed as S.D. (error bars) (n = 6). F, AtMETTL20-catalyzed methylation introduces multiple methyl groups at Lys-193 and Lys-196 in AtETF. AtETF with WT or mutated ETF was incubated with non-radioactive AdoMet and AtMETTL20, and methylation of the indicated Asp-N-generated AtETF-derived peptide, encompassing residues 177–199, was analyzed by MS. Shown are the mean relative intensities of signals gated for the different methylation states of the peptide with error bars indicating the range of values from three independent experiments. Red color indicates the location of the methylated residues Lys-193 and Lys-196 within the peptide sequence.
METTL20 Modulates the Ability of ETF to Receive Electrons from FAD-Containing Dehydrogenases—In humans, ETF acts as a mobile electron carrier that shuttles electrons from several mitochondrial DHs to ETF-QO. Interestingly, the residues targeted by METTL20 (Lys-200 and Lys-203) were shown to be a part of the recognition loop that interacts with one of these DHs, namely MCAD (19). Sequence homology with characterized ETF-dependent DHs from other organisms indicates the presence of more than 10 such enzymes in A. tumefaciens, but these remain uncharacterized experimentally. To study the possible effects of methylation on the ability of AtETF to receive electrons from DHs, we cloned the genes encoding various putative ETF-dependent DHs from A. tumefaciens, and we were able to express and purify several of the corresponding recombinant proteins from E. coli. To assess the ETF dependence and substrate specificity of these DHs, we used a colorimetry-based assay where DCIP served as the final electron acceptor. ETF-dependent activity of the DHs was monitored as reduction-induced bleaching of DCIP (Fig. 4A). For the enzymes belonging to the ACAD family, the activity of the recombinant protein was tested against a panel of relevant CoA-containing substrates. We found one of the DHs (NP_356237.1), which already is annotated as an A. tumefaciens glutaryl-CoA dehydrogenase, to display the expected specificity toward glutaryl-CoA (Fig. 4B). Similarly, we identified another enzyme (NP_357132.2) that preferred isovaleryl-CoA, thereby representing an A. tumefaciens isovaleryl-CoA dehydrogenase (Fig. 4B). A third enzyme (NP_357135.2) denoted A. tumefaciens short-chain acyl-CoA dehydrogenase oxidized both butyryl- and isobutyryl-CoA, thereby displaying activities characteristic of both short-chain acyl-CoA dehydrogenase and isobutyryl-CoA dehydrogenase. Finally, a fourth enzyme (NP_353529.2) denoted A. tumefaciens long-chain acyl-CoA dehydrogenase had octanoyl- and palmitoyl-CoA as preferred substrates, thereby displaying both long-chain acyl-CoA dehydrogenase- and MCAD-like activities. In addition to these putative ACAD family members, we characterized another enzyme (NP_354018.1) denoted AtDMGDH as it showed high sequence similarity to human DMGDH and tested its ability to mediate transfer of electrons from AtETF to the electron acceptor CoQ1, as monitored through a change in absorption (275 nm) of CoQ1 (Fig. 4A). Somewhat surprisingly, although AtETF-mediated electron transfer to DCIP was observed with a number of different DHs (see above), the reduction of CoQ1 through the DH/ETF-QO system was only observed with AtDMGDH but not with any of the tested AtACADs. Notably, we found that wild-type AtMETTL20, but not the inactive D81A mutant, reduced the ability of AtETF to extract electrons from AtDMGDH and shuttled them to AtETF-QO (Fig. 4G, right panel). In summary, the above results indicate that methylation of AtETFβ by AtMETTL20 impairs the ability of AtETF to mediate electron transfer between ETF-dependent dehydrogenases and ETF-QO.

METTL20 Methylates A. tumefaciens RpL7/L12 on Lys-86 in Vitro—The results shown in Fig. 2 suggested AtRpL7/L12 to be a substrate for AtMETTL20. To further investigate this, we expressed and purified recombinant AtRpL7/L12 from E. coli, assessed AtMETTL20-mediated methylation of AtRpL7/L12 in an MTase assay using [3H]AdoMet, and detected protein methylation by fluorography. Reassuringly, we observed AtMETTL20-mediated methylation of wild-type AtRpL7/L12 but not of the K86A mutant where the putative methylation site had been mutated (Fig. 5A). RpL7/L12 from E. coli shows high sequence similarity to AtRpL7/L12 (Fig. 5B) and has been shown to be monomethylated on the corresponding site, Lys-82, by a yet unidentified MTase (29). Accordingly, we observed by MS analysis that AtRpL7/L12 expressed in E. coli was already partially methylated on Lys-86 (Fig. 5, C and D). Interestingly and in agreement with previous studies of endogenous E. coli RpL7/L12 (38), we found methylation at Lys-86 of recombinant E. coli-expressed AtRpL7/L12 to decrease dramatically with increasing growth temperature; the protein was mostly monomethylated when expressed at 16 °C (~85% monomethylated), whereas it existed predominantly in the unmethylated state when expressed at 37 °C (~95% unmethylated) (Fig. 5C). Importantly, in either case, the level of Lys-86 methylation could be further increased by treatment of recombinant AtRpL7/L12 with AtMETTL20 (Fig. 5C). In summary, the above results clearly demonstrate that AtMETTL20 can methylate Lys-86 in AtRpL7/L12 in vitro and show that E. coli expresses an MTase capable of targeting the same site and that likely is also responsible for methylation of Lys-82 in endogenous RpL7/L12 from E. coli.

Because E. coli possesses an enzyme capable of methylating AtRpL7/L12, the possibility existed that the apparent activity of recombinant AtMETTL20 on AtRpL7/L12 originated from an E. coli-derived contamination rather than from the recombinant enzyme itself. However, no methylation of AtRpL7/L12 was observed either with Homo sapiens (Hs) METTL20 or with the inactive AtMETTL20 D81A mutant, thus excluding this possibility (Fig. 5E).
To further investigate the observed dual specificity of AtMETTL20 toward AtRpL7/L12 and AtETFβ, we assessed the relative activity of the enzyme on these two substrates. Thus, in vitro methylation reactions were performed using a constant concentration of recombinant AtETFα/β or AtRpL7/L12 (expressed at 37 °C) and varying concentrations of AtMETTL20. Although equal amounts of substrates were used, a substantially higher methylation level (~2.5-fold higher) was observed for AtETFβ compared with AtRpL7/12 (Fig. 5F), which may be explained by the higher number of methyl groups received by
AtETFβ (compare Figs. 3F and 5C). Otherwise, the titration curves for the two substrates were similar, supporting the notion that methylation of both substrates is biologically relevant.

AtMETTL20 Is Responsible for in Vivo Methylation of AtETFβ and AtRpL7/L12 in A. tumefaciens—Next, we set out to investigate whether AtMETTL20 is also responsible for in vivo methylation of AtETFβ and AtRpL7/L12 in A. tumefaciens as well as to further investigate functional consequences of AtMETTL20-mediated methylation. For these purposes, we generated an A. tumefaciens strain with AtMETTL20 gene KO. AtMETTL20 KO cells were viable, and their growth in LB or glucose-supplemented minimal medium was indistinguishable from that of wild-type cells (Fig. 6A). To indirectly assess AtRpL7/L12 and AtETFβ methylation status in KO versus wild-type bacteria, extracts were subjected to AtMETTL20-mediated methylation. Substantially higher levels of AtMETTL20-induced methylation of both AtRpL7/L12 and AtETFβ were observed with the KO bacteria, indicating that these proteins are hypo- or unmethylated in the KO bacteria (Fig. 6B). A similar pattern was observed when human METTL20 was used except that this enzyme methylated AtETFβ but not AtRpL7/L12 as observed previously.

The methylation status of AtETFβ from wild-type and AtMETTL20 KO bacteria was also directly assessed by MS analysis of the Asp-N-generated peptide encompassing both Lys-193 and Lys-196. Whereas these two residues together carried up to four methyl groups in the wild-type bacteria, they were exclusively found in the unmethylated state in the KO bacteria (Fig. 6C). The methylation status of AtRpL7/L12 present in the ribosomal fraction was directly assessed by MS analysis of the Glu-C-generated peptide containing Lys-86. AtRpL7/L12 was found predominantly in the monomethylated state in the wild-type bacteria but was found to be completely unmethylated in the KO bacteria (Fig. 6D). Taken together, the above experiments firmly establish AtMETTL20 as the enzyme responsible for lysine methylation of AtETFβ and AtRpL7/L12 in vivo.

Several translation factors with GTPase activity bind to the ribosome through the so-called RpL7/L12 stalk, and the GTPase activity has been shown to be stimulated by RpL7/L12 and ribosome binding (39, 40). Thus, we tested ribosomes from wild-type and METTL20 KO bacteria for their ability to stimulate such GTPase activity and found that they were similarly active in stimulating the GTPase activity of EF-G (Fig. 6E) as well as of IF2 and RF3 (data not shown). In summary, these results demonstrate that AtMETTL20 is a dual specificity KMT and the sole enzyme responsible for lysine methylation of ETFβ and RpL7/L12 in A. tumefaciens.

Discussion

We have demonstrated here, using a wide range of in vitro and in vivo approaches, that METTL20 from A. tumefaciens is a novel bacterial KMT with unprecedented dual specificity, targeting both ETFβ and RpL7/L12. Taken together with the corresponding results obtained with METTL20 from R. etli, this indicates that other bacterial METTL20-like proteins are also likely to display similar activities. Moreover, we have here established lysine methylation of ETFβ as the first example of a lysine-specific modification that occurs both in bacteria and humans.

Protein lysine methylation is much more prevalent in eukaryotes than in prokaryotes. For example, the SET domain KMTs, which constitute close to a third of the MTases in humans, are rare in bacteria where they do not target endogenous bacterial proteins but rather act as effectors that modify histone proteins in the infected host (41). Accordingly, almost all the bacterial KMTs acting on endogenous proteins belong to the 7BS MTase family. Of these, PrmA, which targets ribosomal protein RpL11, has been studied in the most detail and appears to be present in all bacteria (36). Pseudomonas and certain other species of γ-proteobacteria and firmicutes possess a 7BS MTase, EftM (EF-Tu-modifying enzyme), which methylates Lys-5 in translation elongation factor EF-Tu, thus promoting bacterial infectivity (42). Similarly, two related 7BS MTases, PKM1 and PKM2, that methylate the outer membrane protein OmpB on several Lys residues in Rickettsia subspecies, are also important for bacterial virulence (43, 44). Thus, bacterial KMTs seem to primarily target components of the translational machinery as well as determinants of bacterial virulence. The AtMETTL20 enzyme reported here is apparently unrelated (beyond the shared 7BS fold) to the above enzymes, thus representing a new type of bacterial KMT.

We found that AtETFβ is methylated by AtMETTL20 on both Lys-193 and Lys-196, corresponding to the previously reported target sites of the human enzyme on HsETFβ, namely Lys-200 and Lys-203 (13, 14). These residues are part of the recognition loop, which has been shown to interact with human
MCAD (19), and we have previously shown that methylation of HsETF by human METTL20 impairs the ability of HsETF to extract electrons from human MCAD and glutaryl-CoA dehydrogenase (13). In the present work, we report similar findings, i.e., that enzymatically active AtMETTL20 inhibits electron flow from various A. tumefaciens ETF-dependent dehydrogenases to ETF.

Three different classes of ETFs are found in bacteria (20), and interestingly, α-proteobacteria, which represent the evolutionary precursors of mitochondria, have an ETF that shows high similarity to eukaryotic ETFs (AtETF and HsETF show ~60% sequence identity). In contrast, the E. coli equivalent of ETFβ, FixA, is more distantly related to HsETFβ, and we did not observe AtMETTL20-mediated methylation of FixA in vitro (data not shown). Although a eukaryotic-like ETF appears to be present in all α-proteobacteria, a METTL20-encoding gene is found only in a subset of these, thus resembling the scattered distribution of METTL20 orthologues in Eukaryota. Taken together, this indicates that Lys methylation is not required for basic ETF function but rather has a biologically relevant modulatory role that gives a selective advantage in certain organisms.

We here report AtMETTL20-catalyzed lysine methylation of AtRpL7/L12 at Lys-86, and the corresponding residue appears.

FIGURE 5. Recombinant AtMETTL20 methylates recombinant AtRpL7/L12 on Lys-86. A, AtMETTL20 mediated methylation of WT but not K86A mutated recombinant AtRpL7/L12. AtRpL7/L12 (WT or K86A mutant) expressed and purified from E. coli was incubated with [3H]AdoMet and AtMETTL20, and its methylation was assessed by fluorography (1-day exposure). B, sequence alignment of RpL7/L12 homologues from A. tumefaciens (At; NP_354932.1) and E. coli (Ec; NP_290617.1) and mRpL12 from H. sapiens (Hs; NP_002940.2). The arrow indicates the position corresponding to Lys-86 in AtRpL7/L12. C, MS analysis of in vitro methylation and temperature-dependent in vivo methylation of E. coli-expressed recombinant AtRpL7/L12. Recombinant AtRpL7/L12 was expressed in E. coli at the indicated temperature, purified, and then incubated with AtMETTL20 as indicated in the presence of non-radioactive AdoMet. Methylation of Lys-86 in the indicated Glu-C-generated AtRpL7/L12-derived peptide, encompassing residues 77–87, was analyzed by MS. Shown are the mean relative intensities of signals gated for the different methylation states of Lys-86 in AtRpL7/L12 incubated with or without AtMETTL20 with error bars indicating the range of values from three independent experiments. Red color indicates the location of the methylated residue Lys-86 within the peptide sequence. D, MS/MS fragmentation of Glu-C-digested peptides obtained as in C supporting mono- and dimethylation of Lys-86 in AtRpL7/L12. E, inability of D81A mutated AtMETTL20 and HsMETTL20 to methylate AtRpL7/L12. AtRpL7/L12 incubated with the indicated METTL20 enzymes in the presence of [3H]AdoMet, and methylation was assessed by fluorography (1-day exposure). F, AtMETTL20 shows similar activity on AtETFβ and AtRpL7/L12. Equal concentrations of AtETF or AtRpL7/L12 (expressed at 37 °C) were incubated with [3H]AdoMet and increasing concentrations of AtMETTL20. Incorporation of [3H]methyl into proteins was assayed by scintillation counting of trichloroacetic acid-precipitated material. Shown are data from a representative experiment run in triplicate. Sample variation is expressed as S.D. (error bars).
Dual Specificity METTL20 Homologue from A. tumefaciens

The majority of the 7BS KMTs characterized so far methylate a single residue in a unique substrate (or in a group of highly related substrates), but some enzymes, e.g. the bacterial PrmA and PKMT1/PKMT2 as well as human METTL20, have been shown to methylate several Lys residues within the same substrate (13, 14, 23, 43). However, AtMETTL20 is the first 7BS KMT shown to methylate two different substrates, and thus it possesses unprecedented dual substrate specificity. The two substrates methylated by AtMETTL20, namely AtETF and AtRpL7/L12, appear functionally and structurally unrelated. Thus, it is pertinent to ask whether both these methylation events are biologically relevant, and as elaborated above, we believe this is likely the case. This is further supported by our observations that both substrates were methylated in vivo and were similarly amenable to AtMETTL20-mediated methylation in vitro.

As METTL20-mediated RpL7/L12 methylation occurs in α-proteobacteria, the evolutionary precursors of mitochondria, the possibility existed that HsMETTL20 methylates mRpL12, the mitochondrial counterpart of bacterial RpL7/L12. However, human mRpL12 does not have a lysine residue in the position corresponding to Lys-86 in AtRpL7/L12 (Fig. 5A), and we were unable to detect any in vitro MTase activity of HsMETTL20 on either AtRpL7/L12 (Fig. 5E) or mRpL12 (data not shown). Taken together, this suggests that during evolution to be commonly methylated in prokaryotes. Proteomic studies of bacterial ribosomal proteins have reported di- or monomethylation at this residue in E. coli (29), Caulobacter crescentus (28), and Rhodopseudomonas palustris (27), and the reported molecular mass of Thermus thermophilus RpL7/L12 also suggests methylation (26). However, this modification appears not to be present in all bacteria as Bacillus subtilis RpL7/L12 was found to be unmodified (45). Interestingly, except for R. palustris, none of the bacteria with reported RpL7/L12 methylation possess a putative METTL20 orthologue. This indicates that bacteria independently have evolved at least two types of KMTs for methylation of RpL7/L12, i.e. METTL20 and the yet unidentified KMT(s) predicted to exist in the METTL20-less bacteria with methylated RpL7/L12, such as E. coli. This likely example of convergent evolution strongly suggests that RpL7/L12 methylation is functionally important, although we were not able to detect any functional consequences of AtMETTL20 gene knock-out. Notably, the extent of RpL7/L12 methylation in E. coli has been reported to be enhanced at low temperature (38), and in agreement with this, we found that E. coli-expressed recombinant AtRpL7/L12 showed a dramatically higher methylation level when the bacteria were grown at 16 °C relative to 37 °C. This suggests that RpL7/L12 methylation may play an adaptive role in regulating protein translation in response to external cues.
Dual Specificity METTL20 Homologue from A. tumefaciens

eukaryotic METTL20 has lost the ability to methylate Rpl7/L12 or alternatively that bacterial METTL20 proteins acquired the ability to methylate Rpl7/L12 after the endosymbiotic event that led to the generation of mitochondria.

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