METTL21B Is a Novel Human Lysine Methyltransferase of Translation Elongation Factor 1A: Discovery by CRISPR/Cas9 Knockout*

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Lysine methylation is widespread on human proteins, however the enzymes that catalyze its addition remain largely unknown. This limits our capacity to study the function and regulation of this modification. Here we used the CRISPR/Cas9 system to knockout putative protein methyltransferases METTL21B and METTL23 in K562 cells, to determine if they methylate elongation factor eEF1A. The known eEF1A methyltransferase EEF1AKMT1 was also knocked out as a control. Targeted mass spectrometry revealed the loss of lysine 165 methylation upon knockout of METTL21B, and the expected loss of lysine 79 methylation on knockout of EEF1AKMT1. No loss of eEF1A methylation was seen in the METTL23 knockout. Recombinant METTL21B was shown in vitro to catalyze methylation on lysine 165 in eEF1A1 and eEF1A2, confirming it as the methyltransferase responsible for this methylation site. Proteomic analysis by SILAC revealed specific upregulation of large ribosomal subunit proteins in the METTL21B knockout, and changes to further processes related to eEF1A function in knockouts of both METTL21B and EEF1AKMT1. This indicates that the methylation of lysine 165 in human eEF1A has a very specific role. METTL21B exists only in vertebrates, with its target lysine showing similar evolutionary conservation. We suggest METTL21B be renamed eEF1A-KMT3. This is the first study to specifically generate CRISPR/Cas9 knockouts of putative protein methyltransferase genes, for substrate discovery and site mapping. Our approach should prove useful for the discovery of further novel methyltransferases, and more generally for the discovery of sites for other protein-modifying enzymes.

Protein methylation is emerging as an important regulator of diverse cellular processes, in all kingdoms of life (1–4). Recent studies have demonstrated the widespread occurrence of protein methylation in human (5–12), however the biological function of most of these modifications remains unknown. To investigate the role of protein methylation, it is necessary to identify the enzymes that catalyze it.

Protein methyltransferases catalyze the methylation of lysine or arginine residues, and some other amino acids to a lesser degree. Protein lysine methyltransferases can be classified as either SET domain or seven-beta-strand methyltransferases, based upon their catalytic domain (13, 14). Although all SET domain methyltransferases specifically methylate lysine residues, seven-beta-strand methyltransferases methylate a wide range of molecules, including proteins, nucleic acids and metabolites (15). For this reason, discovery of new seven-beta-strand lysine methyltransferases is relatively difficult. Despite this, there have been many seven-beta-strand lysine methyltransferases discovered in both Saccharomyces cerevisiae and human in recent years (14).

One group of seven-beta-strand methyltransferases, the Family 16 methyltransferases (Pfam: PF10294), have all so far proven to be protein-specific. Most of them specifically target lysines. In yeast, elongation factor methyltransferases Efm2 and Efm3 methylate lysines in translation elongation factor 2, whereas Efm6 and Efm7 methylate lysines in elongation factor 1A, with Efm7 also methylating its N terminus (16–21). Yeast Rkm5 and Hpm1 methylate ribosomal proteins RPL1A/B and RPL3, however only Rkm5 is a lysine methyltransferase, as Hpm1 methylates a histidine residue (22, 23). In human, CaMK-methylates calmodulin, VCP-KMT methylates valosin-containing protein (VCP), HSPA-KMT methylates 70 kDa heat shock proteins, ETFB-KMT methylates electron transfer flavoprotein beta (ETF), EEF2-KMT methylates translation elongation factor 2 and METTL22 methylates KIN17 (19, 24–29). These are lysine methyltransferases. METTL18, METTL21B, METTL21C and METTL23 are the four remaining human Family 16 methyltransferases without described substrates. These are also likely to be protein methyltransferases, with METTL18 being the likely orthologue of yeast Hpm1 (14).
Knockout Discovery of a New Human Protein Methyltransferase

Eukaryotic elongation factor 1A (eEF1A)\(^1\) is a translation factor which delivers amino-acyl tRNA to the ribosome during translational elongation. It is also involved in many other cellular functions, including actin cytoskeleton dynamics, proteasomal degradation and nuclear export (30). It shows high levels of methylation, with S. cerevisiae eEF1A being targeted by five different methyltransferases (20, 21, 31, 32). In human, eEF1A has at least six known methylation sites, namely methylation at its N terminus and at lysines 36, 55, 79, 165, and 318 (21, 33). Of these, methyltransferases are known only for lysines 79 and 318, which are eEF1A-KMT1 and eEF1A-KMT2 respectively (21, 34). Human eEF1A has two different isoforms: eEF1A1, which is highly expressed in almost every tissue type, and eEF1A2, which is predominantly expressed in the brain, heart and skeletal muscle (35, 36). Although methylation has been predominantly studied on eEF1A1, eEF1A2 is also known to be methylated at a number of the same residues as eEF1A1 (37).

In this study we knocked out the genes encoding two putative methyltransferases of eEF1A, METTL21B, and METTL23, in human K562 cells. This was done using the CRISPR/Cas9 system, along with knockouts of methyltransferase eEF1AKMT1 as a control, using duplicate knockouts per gene. Changes in eEF1A methylation were then studied by mass spectrometry. Knockouts of EEF1AKMT1 showed a complete loss of lysine 79 methylation, which is its described target site (21). Knockouts of METTL21B showed a complete loss of lysine 165 methylation, a site for which a methyltransferase had not yet been described. Purified METTL21B could methylate purified eEF1A1 and eEF1A2 in vitro, confirming that METTL21B is the methyltransferase responsible for this site of methylation in both eEF1A isoforms. Proteomic analysis of METTL21B knockout by stable isotope labeling by amino acids in cell culture (SILAC) revealed changes in biological processes and complexes related to eEF1A function, including an upregulation of large ribosomal subunit proteins. In accordance with the recent naming of eEF1A-KMT1 and eEF1A-KMT2, we suggest that METTL21B be renamed as eEF1A-KMT3.

**EXPERIMENTAL PROCEDURES**

**Experimental Design and Rationale—**Three methyltransferases, EEF1AKMT1, METTL21B and METTL23, were targeted for knockout in K562 cells by CRISPR/Cas9 genome editing, to observe any changes in eEF1A methylation. To minimize the chance that any observed changes were because of off-target effects, two separate small guide RNAs (gRNAs) were used to knockout each methyltransferase. The knockout of EEF1AKMT1, a known eEF1A methyltransferase, served as a proof of concept. The methylation of eEF1A in each knockout was analyzed by targeted mass spectrometry followed by database searches and manual data analysis.

For SILAC proteomic analysis of the METTL21B and EEF1AKMT1 knockout cell lines, both gRNA knockouts for each methyltransferase were analyzed with forward (light wild-type and heavy knockout) and reverse (heavy wild-type and light knockout) labeling. This gave four different quantified ratios of each methyltransferase knockout compared with wild-type, accounting for any label or gRNA-specific effects.

**Cloning of gRNA Plasmids for CRISPR/Cas9 Genome Editing—**For CRISPR/Cas9 genome editing a plasmid encoding both the Cas9 protein and the gRNA was used, pSpCas9(BB)-2A-GFP (pX458) was a gift from Feng Zhang (Addgene plasmid #48138) (38). The Cas9 sequence is coupled to a T2A site and EGFP. Expression of the Cas9 protein results in simultaneous expression of EGFP allowing for selection of positively transfected cells. gRNA sequences were designed using the optimized CRISPR design online tool (http://crispr.mit.edu/) provided by the Zhang lab from Massachusetts Institute of Technology, Boston. For each target gene, two different gRNAs were designed. For primers see supplemental Table S1.

**Culturing K562 Cells—**K562 cells were maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS) and 1 × penicillin, streptomycin and L-glutamine. Cells were transfected by nucleofection using a Neon Transfection System (Invitrogen, Carlsbad, CA). Cells (10\(^7\)) were resuspended in nucleofection buffer T and given three pulses of 1450 V for 10 ms. Cells were then cultured for 48–72 h in RPMI1640 supplemented with 10% FCS before fluorescent activated cell sorting.

**Screening of Knockout Clones—**Forty-eight to 72 h after transfection, EGFP positive single cells were sorted into 96-well plates and the plates were left in the incubator for 7–14 days or until small colonies could be seen by the naked eye. Clonal populations were then transferred into one master 96-well plate. Fifty microliters of each well of 70% confluent cultures were transferred into a PCR plate and spun down. Supernatant was discarded by flicking the plate. Each cell pellet was resuspended in 50 μl of QuickExtract\(^\text{TM}\) DNA Extraction Solution (Epicenter, Madison, WI), the plate was heated to 98 °C for 2 min followed by incubation at 65 °C for 6 min. This extract was then used in a PCR of the genomic region that had been targeted for knockout. PCR products were then Sanger-sequenced to identify clones that would result in frameshifts and truncated protein products. Sequence alignment and genomic PCR primer design was carried out using Snapgene software (GSL Biotech LLC, Chicago, IL). For primers see supplemental Table S2.

For METTL23 clones, which showed allelic variation of mutations, the allelic genotype of these clones was determined by TA cloning using the pGEM-T Easy cloning system (Promega, Madison, WI). To add adenine overhangs, blunt-ended PCR products from knockout screening were treated with Taq Polymerase. A-tailed PCR products were purified, ligated into the pGEM-T Easy vector and transformed into Escherichia coli. For each clone, 12 successful transformants were picked, plasmids extracted and Sanger-sequenced.

**Sample Preparation and Mass Spectrometry for Methylation Analysis—**K562 cells (wild-type and knockouts) were pelleted, washed once with phosphate-buffered saline, before being resuspended in lysis buffer (50 mM HEPES, 100 mM NaCl, 0.5% v/v Triton X-100, 2 mM DTT, 2 mM EDTA, pH 7.5) and incubated on ice for 30 min. Lysates were clarified by centrifugation (40 min at 14,000 rpm and 4 °C) and separated by SDS-PAGE, after which gel bands were excised, digested and prepared for mass spectrometry, as described previously (21). Samples were analyzed by LC-MS/MS on a Q Exactive Plus (Thermo Fisher Scientific, Waltham, MA), as described previously (21).

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\(^1\) The abbreviations used are: eEF1A, eukaryotic elongation factor 1A; KMT, lysine methyltransferase; AdoMet, S-adenosyl L-methionine; METTL, methyltransferase like; gRNA, small guide RNA; CRISPR, Clustered regularly interspaced short palindromic repeats; SILAC, stable isotope labelling by amino acids in cell culture.
Knockout Discovery of a New Human Protein Methyltransferase

Raw data were converted to Mascot Generic Format (.mgf) using RawConverter (39) (v. 1.0.0.0), with “Experiment Type” set to “Data Dependent” and “Select monoisotopic m/z in DDA” selected. Converted data were then searched against the SwissProt database (2016_03, 550,740 sequences through to 2016_09, 552,259 sequences) using Mascot (v. 2.4, Matrix Sciences) hosted by the Walter and Eliza Hall Institute for Medical Research (Melbourne, Australia) with the following settings: Enzyme: Trypsin (trypsin-digested samples), Asp-N (AspN-digested samples), or LysArginase (Lysarginase-digested samples); Max missed cleavages: 2; Precursor ion tolerance: 4 ppm; Fragment ion tolerance: 20 ppm; Peptide charge: 2+, 3+ and 4+; Instrument: ESI_HCD; Variable modifications: Oxidation (M), Methyl (K), Dimethyl (K), Trimethyl (K) and Methyl (DE); Methyl (NTerm), Dimethyl (N-term) and Propyl (N-term) were additionally selected as variable modifications for samples pertaining to N-terminal methylation. For samples from lysates (K562 wild-type and mutants), Taxonomy was set as Homo sapiens (human) and the contaminants database (139 sequences) was additionally selected. For METTL23 knockout samples, the Enzyme was set as Asp-N_ambic, and Max missed cleavages was set as 3. All peptides were identified with expect values <0.05, and spectra were manually annotated to only include fragment ions with 20 ppm error or less.

Peptides and their methylation states were analyzed by taking extracted ion chromatograms of the monoisotopic peak (±10 ppm) in Thermo Xcalibur Qual Browser 2.2 SP1.48, as described previously (21). A tolerance of ±5 ppm was used instead for the eEF1A2 AspN peptide DSTEPAYSEKRY1-2 and its methylated states, because of the presence of near-isobaric, co-eluting, non-monoisotopic peaks. Mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE (40) partner repository with the dataset identifier PXD005497.

Cloning, Expression and Purification of METTL21B, eEF1A1, and eEF1A2—METTL21B was cloned into pET15b for bacterial expression with the Gibson Assembly® Cloning Kit (New England Biolabs, Ipswich, MA), with a C-terminal 6× His tag. The resultant plasmid was confirmed by Sanger sequencing, transformed into E. coli (Rosetta DE3) and METTL21B expression induced with IPTG (1 mM) overnight at 18 °C. eEF1A1 and eEF1A2 were cloned into pD1204-GAL1, with confirmed by Sanger sequencing, transformed into K562 wild-type and mutants. Cell
culture (SILAC), K562 wild-type and knockouts of METTL21B, METTL22B, and METTL23 were grown in RPMI1640 medium without arginine, leucine, lysine, and phenol red (Sigma-Aldrich, St. Louis, MO), supplemented with 50 mg/L L-leucine and 300 mg/L L-proline. Cells were then lysed by immobilized metal affinity chromatography, as described previously (17).

In Vitro Methylation Assay—Purified eEF1A proteins (10 μM) were incubated with or without purified METTL21B (10 μM) in the presence of S-adenosyl l-methionine (AdoMet, 500 μM) in in vitro methylation buffer (50 mM HEPES-KOH, 20 mM NaCl, 1 mM EDTA, pH 7.4) at 37 °C overnight. Reactions were then resolved by SDS-PAGE and eEF1A proteins prepared for mass spectrometry as above.

SILAC ProteomeXchange Analysis—For stable isotope labeling by amino acids in cell culture (SILAC), K562 wild-type and knockouts of METTL21B and EEF1AKMT1 were grown in RPMI1640 medium without arginine, leucine, lysine, and phenol red (Sigma-Aldrich, St. Louis, MO), supplemented with 50 mg/L L-leucine and 300 mg/L L-proline. Cells were either supplemented with 220 μM L-lysine HCl and 240 μM L-arginine (Sigma-Aldrich), for light labeling, or 220 μM 13C6-L-lysine HCl and 240 μM 13C6-L-arginine HCl (Silantes, Munich, Germany), for heavy labeling. Cells were grown for five doublings in order to ensure >95% labeling, which was confirmed before mixing.

Cells were then lysed as above, and lysates from heavy and light conditions were mixed 1:1 before separation by SDS-PAGE as above. Each gel lane was cut into 25 bands, which were prepared for mass spectrometry as above, with the exception that reduction (10 mM DTT at 37 °C for 1 h) and alkylation (55 mM chloroacetamide at room temperature for 1 h) were included before digestion by trypsin.

SILAC data were analyzed with MaxQuant (v.1.5.8.0) (42). Data were searched against human SwissProt proteins (03-2017, 20170 sequences) and contaminants (245 sequences) with the following settings: Multiplicity: 2 (heavy labels designated as Lys8 and Arg10); Enzyme: Trypsin/P; Max missed cleavages: 2; Fixed modification: Carbamidomethyl (C); Variable modifications: Oxidation (M), Acetyl (Protein N-term); Precursor ion tolerance: 4.5 ppm; Fragment ion tolerance: 20 ppm. Peptides with the Carbamidomethyl (C) modification were used in protein quantification and “Match between runs” was selected. Peptide-spectrum matches were filtered at a false discovery rate of 1%, estimated using the target-decoy approach. Peptides for which SILAC ratios were obtained across all eight comparisons (3307 in total) were then further analyzed using Perseus (43). Each methyltransferase knockout was analyzed for significantly changing proteins using a one-sample t test and applying a 10% Benjamini-Hochberg false discovery rate. Significantly up- and down-regulated proteins for METTL21B and EEF1AKMT1 knockouts were analyzed for gene ontology (GO) term enrichment (biological process and cellular compartment) using the PANTHER (44) (v. 11.1, released 2016-10-24) overrepresentation test, with all 3307 quantified proteins set as background. All quantified proteins from enriched GO terms were compared for differences in average SILAC ratios between METTL21B and EEF1AKMT1 knockouts using a Mann-Whitney test. p values were then adjusted for multiple testing by multiplying by the number of GO terms tested (n = 37). All raw mass spectrometry data, as well as MaxQuant outputs, have been deposited to the ProteomeXchange Consortium via the PRIDE (40) partner repository with the data set identifier PXD005497.

RESULTS

Generation of EEF1AKMT1, METTL21B, and METTL23 Knockout Cell Lines—Given that METTL21B and METTL23 are likely to be protein methyltransferases and are also ubiquitously expressed (36), we investigated them as potential methyltransferases of eEF1A. To do this, we sought to knock out both in the human erythroid progenitor K562 cell line using the CRISPR/Cas9 system. Additionally, as a proof of principle, we sought to knockout the recently described eEF1A methyltransferase, EEF1AKMT1, which methylates lysine 79 (21). To minimize the chance that any effects are because of off-target cleavage by the Cas9 nuclease, we used a CRISPR knockout design that utilized two different small guide RNAs (gRNAs) for EEF1AKMT1 (exon 2), for METTL21B (exon 1) and for METTL23 (exon 2) (Fig. 1A, 1B and supplemental Fig. S1). The gRNAs were designed to target the first protein-coding exon of all known isoforms for each gene, ensuring an absence of functional protein if successful. We transected wild-type K562 cells with plasmids containing the Cas9 protein, as well as gRNA sequences targeting the gene of interest, and then established clonal populations that were screened for knockouts. Successful knockout of the gene of interest was determined by genomic PCR across the targeted locus followed by Sanger sequencing of the PCR product and infer-
ence of truncated protein products (Fig. 1
C, D, supplemental Fig. S1). Any allelic variation was verified by allele-specific sequencing. We generated two replicate K562 knockout cell populations for each of the three methyltransferases, providing us with cellular models for all subsequent analyses of protein methylation.

**Knockouts of EEF1AKMT1 and METTL21B in K562 Cells Result in Loss of Methylation at Lysines 79 and 165 in eEF1A**—Mammalian elongation factor 1A has many sites of methylation reported in high-throughput studies (5, 6, 8–10). To validate these, we used targeted mass spectrometry of eEF1A1 from wild-type K562 cells with three different proteases: trypsin, LysargiNase (45) and AspN. Our analysis confirmed N-terminal methylation and methylation at lysines 36, 55, 79, 165 and 318, which is consistent with results from small-scale studies (21, 33) (see Table I for summary, see supplemental Table S3 for peptides and see supplemental Fig. S2 for representative MS/MS spectra). Although other methylation sites may occur in various cell types or conditions, these sites may be considered as the canonical sites of methylation.

To confirm that our cellular knockout system was suitable for methyltransferase discovery we checked the methylation status of lysine 79 in the two knockout cell populations of EEF1AKMT1 (gRNAs #1 and #2), Gel bands corresponding to EEF1A1 in K562 cells were transfected with CRISPR/Cas9 plasmids containing one of the two gRNAs targeting EEF1AKMT1 or METTL21B, respectively. Clonal populations were established and successful knockout was determined by genomic PCR followed by sequencing with primers spanning the targeted region. The 20-nucleotide gRNA sequence is shown with a red box and the PAM sequence with a blue box. To ensure knockout on the protein level, clonal populations were chosen that resulted in premature stop codons (*) or frameshift mutations resulting in an altered protein product (indicated by red letters in the protein sequence, with the residues that match the WT protein product shown in black letters).
cells. In association with the previously described in vitro methylation assays (21), this confirms that EEF1AKMT1 is the sole methyltransferase responsible for this site.

In human eEF1A, the methyltransferases responsible for methylation at its N terminus and lysines 36, 55 and 165 are unknown. We therefore sought to observe any loss of these methylation sites in the METTL21B and METTL23 knockout cells. To do this, digests of eEF1A1 from METTL21B and METTL23 knockouts were analyzed by LC-MS/MS. Two knockouts per methyltransferase were analyzed independently. Interestingly, both knockouts of METTL21B (gRNAs #1 and #2) resulted in a complete loss of the mono-, di- and tri-methylated forms of the eEF1A1 tryptic peptide containing lysine 165 (MDSTEPPYSQKR, lysine 165 is underlined), when compared with wild-type K562 cells (Fig. 2B). There was also a corresponding increase in the unmethylated form of this peptide in both knockouts of METTL21B (Fig. 2B). Additionally, we were able to identify the mono- and di-methylated forms of the eEF1A2 AspN peptide containing lysine 165 (DSTEPAYSEKR, lysine 165 is underlined) in wild-type K562 cells (see supplemental Fig. S3 and supplemental Table S3).

Both methylated states of this peptide were lost in knockouts of METTL21B, with a corresponding increase in the unmethylated form of this peptide (see supplemental Fig. S4). Both knockouts of METTL21B showed no loss of methylation on any of the other sites in eEF1A (Table I). These data indicate that knockouts of METTL21B result in a complete loss of lysine 165 methylation in both eEF1A1 and eEF1A2 and, therefore, that METTL21B is the sole methyltransferase responsible for lysine 165 methylation in eEF1A1 and eEF1A2. Despite this, and the fact that eEF1A1 is essential for translation and thus growth, there was no obvious growth defect observed in either of the METTL21B knockout cell populations (see supplemental Fig. S5). Regarding METTL23, neither of the two knockouts (gRNAs #1 and #2) showed an effect on methylation of any of the canonical sites on eEF1A (Table I), indicating that it is unlikely to be responsible for any methylation of eEF1A.

**METTL21B Methylates eEF1A1 and eEF1A2 In Vitro**—Given that knockout of METTL21B resulted in loss of lysine 165 methylation in both eEF1A1 and eEF1A2, we sought to determine whether METTL21B could methylate eEF1A1 or eEF1A2 in vitro. To test this, human eEF1A1 and eEF1A2, expressed and purified from wild-type yeast, were incubated with or without purified METTL21B (see supplemental Fig. S6 for purification), in the presence of the methyl-donor AdoMet. eEF1A1 and eEF1A2 were then digested with trypsin and analyzed by LC-MS/MS. In the absence of METTL21B, the eEF1A1 tryptic peptide containing lysine 165 (MDSTEPPYSQKR, lysine 165 is underlined) was only found in the unmethylated state (Fig. 3A). However, when incubated with METTL21B, the monomethylated form of this peptide was observed (Fig. 3A). Similarly, the eEF1A2 tryptic peptide containing lysine 165 (MDSTEPPYSQKR, lysine 165 is underlined) was found to be monomethylated only when incubated with METTL21B (Fig. 3B). This demonstrates that METTL21B has direct methyltransferase activity on lysine 165 of both eEF1A1 and eEF1A2, confirming that it is the methyltransferase responsible for this methylation site. In accordance with the recent naming of the eEF1A methyltransferases eEF1A-KMT1 and eEF1A-KMT2 (14, 21, 34), we suggest METTL21B be renamed eEF1A-KMT3.

**Proteomic Analysis of METTL21B and EEF1AKMT1 Knockouts**—To gain insight into the specific function of METTL21B-mediated methylation of lysine 165 in eEF1A, we used SILAC followed by LC-MS/MS to analyze the proteome of METTL21B knockouts and compare them to wild-type K562 cells. Knockouts of EEF1AKMT1 were also analyzed for proteomic changes and were compared with wild-type K562 to determine the effects of loss of lysine 79 methylation. The two knockouts of each methyltransferase, arising from the use of two different guide RNAs in each case, were analyzed separately. They were analyzed with forward (light-labeled wild-type and heavy-labeled knockout) and reverse (heavy-labeled wild-type and light-labeled knockout) SILAC labeling. In total, 3307 proteins were consistently quantified across these eight comparisons (see supplemental Table S4 for all protein identifications and their quantification). Hierarchical clustering revealed that forward and reverse labels clustered together, indicating minimal label effect (see supplemental Fig. S7). It also revealed there were apparent off-target effects associated with each gRNA.

**Comparison of the METTL21B knockout proteome and that from K562 wild-type revealed 600 proteins to be significantly**

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**Table I**

| eEF1A residue | Highest methylation degree | Previously described methyltransferase | References |
|---------------|----------------------------|----------------------------------------|------------|
| N-terminus    | Tri                        | (21) and this study                    |            |
| K36           | Tri                        | (8, 9) and this study                  |            |
| K55           | Di                         | (5-8, 10) and this study               |            |
| K79           | Tri                        | EEF1AKMT1 (5, 6, 8, 10, 21) and this study |        |
| K165          | Tri                        | (5-10) and this study                  |            |
| K318          | Tri                        | EEF1AKMT2 (METTL10)                    |            |

**a** A dash indicates no change to methylation. A cross indicates loss of methylation.

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Knockout Discovery of a New Human Protein Methyltransferase
differentially expressed. This included 291 downregulated and 309 upregulated proteins. A total of 838 proteins were found to be differentially expressed upon knockout of \textit{EEF1AKMT1}, with 353 downregulated and 485 upregulated proteins. Gene ontology (GO) enrichment analysis revealed the overrepresentation of proteins involved in a number of biological processes and cellular components (Table II). Notably, many of these processes are related to known functions of eEF1A (30). In the \textit{METTL21B} knockout, proteins involved in actin cytoskeleton organization were found to be downregulated, whereas proteins involved in ribosomal large subunit biogenesis, rRNA processing, mRNA metabolism, as well proteins of the preribosome and cytosolic large ribosomal subunit, were found to be upregulated. In the \textit{EEF1AKMT1} knockout, proteins involved in tRNA aminoacylation and proteins from the nuclear exosome, a complex involved in RNA processing, were

\[\text{Fig. 2. Knockouts of EEF1AKMT1 and METTL21B result in complete loss of methylation at lysines 79 and 165, respectively, in eEF1A.} \]

\[\text{A, Knockouts of EEF1AKMT1 generated with gRNAs \#1 and \#2 show a complete loss of mono-, di-, and tri-methylation of lysine 79 in eEF1A, confirming that it is the sole methyltransferase responsible for this site of methylation. The methylation status of lysine 79 was analyzed by taking mass windows (±10 ppm) corresponding to all relevant methylation states of the eEF1A AspN peptide DISLWKETFSTYFYVTIL. B, Knockouts of the putative methyltransferase METTL21B generated with gRNAs \#1 and \#2 show a complete loss of mono-, di-, and tri-methylation of lysine 165 in eEF1A, indicating that it is the methyltransferase responsible for this site of methylation. The methylation status of lysine 165 was analyzed by taking mass windows (±10 ppm) corresponding to all relevant methylation states of the eEF1A tryptic peptide MDSTEPPYSQKR. Peaks were normalized to the most abundant ion for each methylation state. Elution times of peptides are shaded; peaks outside shading are unrelated, near-isobaric ions. me0: unmethylated peptide; me1: monomethylated peptide; me2: dimethylated peptide; me3: trimethylated peptide.}\]
found to be downregulated whereas proteins involved in the regulation of ubiquitination and proteins from the small-subunit processome, a ribosome assembly intermediate, were found to be upregulated. Overall, this strongly suggests that both METTL21B- and eEF1AKMT1-mediated methylation modulate many well-characterized eEF1A functions in protein translation, ubiquitin-mediated protein degradation and the cytoskeleton (30, 46).

We next investigated whether there were any changes in the proteome that were specific to either methyltransferase knockout. This was done by statistically analyzing the average SILAC ratios (log₂ fold-change in knockout relative to wild-type) of proteins that led to enriched GO terms. This revealed that 32 of the 37 GO terms in Table II had the same distribution of average SILAC ratios between knockout of METTL21B and EEF1AKMT1, indicating that they are common to knockouts of both methyltransferases and similar in magnitude. Notably, actin cytoskeleton organization, mRNA metabolism, tRNA aminoacylation, ribosomal large subunit biogenesis, regulation of ubiquitination, the preribosome and the small-subunit processome were all found to have SILAC ratios that were not significantly different between knockouts of METTL21B and EEF1AKMT1. This indicates that these processes are similarly affected upon loss of lysine 79 and lysine 165 methylation of eEF1A. However, the constituent proteins of five GO terms showed a significant difference in SILAC ratios between the two methyltransferase knockouts. These were the cytosolic large ribosomal subunit, showing the most dramatic difference, and rRNA processing, the mitochondrial matrix, the nuclear exosome complex and the mitotic cell cycle process (Table II).

The METTL21B knockout showed a highly significant increase in SILAC ratios for the large cytosolic ribosomal subunit proteins compared with the ratios in the EEF1AKMT1 knockout (Fig. 4A, Table II). This suggests that the loss of lysine 79 methylation could be affecting the function of the large ribosomal subunit, which the cell is seeking to rectify through upregulation of its constituent proteins. The small cytoplasmic ribosomal subunit, in contrast, did not show any variation (Fig. 4B). Interestingly, although the SILAC ratios of proteins involved in rRNA processing were significantly different between METTL21B and EEF1AKMT1 knockout, this difference was found to disappear when large ribosomal subunit proteins were removed from the comparison (Table II). This indicates that the SILAC ratios of the large ribosomal subunit proteins entirely accounted for this apparent difference.

The EEF1AKMT1 knockout showed a significant decrease in SILAC ratios for mitochondrial matrix proteins, compared with the ratios in the METTL21B knockout (Table II). Given the differences observed for cytosolic ribosomal proteins above, and the fact that cytosolic and mitochondrial ribosomes show aspects of synchronization (47), we investigated the SILAC ratios of mitochondrial matrix proteins with and without the mitochondrial ribosome (mitoribosome). We also investigated whether the mitochondrial ribosome proteins themselves showed different SILAC ratios between the two knockouts.

Interestingly, this revealed that nonribosomal mitochondrial matrix proteins showed no difference in SILAC ratios between the two methyltransferase knockouts (Fig. 4C), with both knockouts showing overall downregulation compared with wild-type. However there were significant differences in the SILAC ratios of the mitoribosome proteins, with the EEF1AKMT1 knockout showing lower ratios than the METTL21B knockout. These significant differences were present in both the large (Fig. 4D) and small (Fig. 4E) mitoribosome subunits. This implies that mitochondrial matrix proteins are similarly downregulated in the knockouts of EEF1AKMT1 and METTL21B, compared with wild-type, but in the METTL21B knockout the mitoribosomal proteins are upregulated relative to the rest of the mitochondria. Overall, these data show that although many processes related to eEF1A function are affected upon knockout of METTL21B or EEF1AKMT1, the large cytosolic ribosomal subunit and the mitoribosome are specifically upregulated upon knockout of METTL21B.

**Fig. 3.** eEF1A1 and eEF1A2 are methylated by METTL21B in vitro at lysine 165. Purified human eEF1A1 (A) and eEF1A2 (B) were incubated with or without purified METTL21B in the presence of AdoMet. Both eEF1A1 and eEF1A2 were found to be monomethylated at lysine 165 only when incubated with METTL21B. The methylation status of lysine 165 was analyzed by taking mass windows (±10 ppm) corresponding to all relevant methylation states of the tryptic peptides MDSTEPPYSQKR (eEF1A1) and MDSTEPAYSEKR (eEF1A2). Peaks were normalized to the most abundant ion for each methylation state. Elution times of peptides are shaded; peaks outside shading are unrelated, near-isobaric ions. me0: unmethylated peptide; me1: monomethylated peptide.

**Fig. 4.** A, C, and D show the differences in SILAC ratios for mitochondrial matrix proteins, compared with the ratios in the METTL21B knockout (Table II). Given the differences observed for cytosolic ribosomal proteins above, and the fact that cytosolic and mitochondrial ribosomes show aspects of synchronization (47), we investigated the SILAC ratios of mitochondrial matrix proteins with and without the mitochondrial ribosome (mitoribosome). We also investigated whether the mitochondrial ribosome proteins themselves showed different SILAC ratios between the two knockouts.

**Fig. 5.** Knockout Discovery of a New Human Protein Methyltransferase METTL21B and Its Target Lysine Show Similar Evolutionary Conservation. To understand the evolutionary conservation of METTL21B, we sought to identify orthologues in other species. We performed reciprocal BLAST searches between...
Knockout Discovery of a New Human Protein Methyltransferase

human METTL21B and a range of species, and proteins that returned METTL21B as the best human hit were true orthologs. Given its similarity with the other METTL21 proteins, the matches between the putative orthologues and METTL21A (HSPA-KMT), METTL21C, and METTL21D (VCP-KMT) were also examined, in the same manner as was done for METTL21A (26). This analysis revealed that METTL21B is present only in vertebrates, with no orthologues in any invertebrates or other eukaryotes (Table III). Within the vertebrates, however, METTL21B is not entirely conserved, as it is notably absent in some model organisms like zebrafish and chicken (Table III). The most distantly related organism to human for which a METTL21B orthologue could be found was *C. milii*, a cartilaginous fish. This contrasts with METTL21A and METTL21D, which are conserved across most eukaryotes (24, 26). Alignment of METTL21B with its orthologues in mouse, rabbit and frog revealed high conservation of characteristic seven-beta-strand motifs I, II, and the DXXY motif, which is characteristic of Family 16 methyltransferases (14, 24) (Fig. 5A).

TABLE II
Proteomic changes in knock outs of METTL21B and EEF1AKMT1

| Condition                          | Biological process/Cellular component                          | Fold enrichment | Corrected p value | Comparison between METTL21B and EEF1AKMT1 |
|------------------------------------|---------------------------------------------------------------|-----------------|-------------------|------------------------------------------|
| **METTL21B** knock out down-regulated | Alcohol metabolic process                                     | 3.60            | 2.13E-03          | n.s.                                     |
|                                    | Actin cytoskeleton organisation                                | 2.64            | 2.02E-02          | n.s.                                     |
|                                    | Endoplasmic reticulum-Golgi intermediate compartment          | 3.88            | 3.48E-02          | n.s.                                     |
|                                    | Extracellular space                                           | 2.30            | 2.74E-02          | n.s.                                     |
|                                    | Extracellular exosome                                         | 1.56            | 6.38E-07          | n.s.                                     |
|                                    | Cytosol                                                       | 1.22            | 4.96E-02          | n.s.                                     |
| **METTL21B** knock out up-regulated | Ribosomal large subunit biogenesis                            | 5.25            | 4.18E-06          | n.s.                                     |
|                                    | rRNA processing                                               | 2.98            | 1.76E-07          | **                                       |
|                                    | Nucleobase-containing compound catabolic process               | 2.13            | 3.89E-02          | n.s.                                     |
|                                    | mRNA metabolic process                                         | 2.06            | 1.24E-05          | n.s.                                     |
|                                    | RNA biosynthetic process                                       | 1.76            | 2.06E-03          | n.s.                                     |
|                                    | Regulation of gene expression                                  | 1.44            | 1.04E-02          | n.s.                                     |
|                                    | Preribosome                                                    | 4.81            | 3.72E-03          | n.s.                                     |
|                                    | Cytosolic large ribosomal subunit                              | 4.51            | 1.32E-05          | ****                                     |
|                                    | Nucleolus                                                     | 1.88            | 1.83E-03          | n.s.                                     |
|                                    | Nucleolar                                                     | 3.40            | 3.25E-05          | n.s.                                     |
|                                    | Coenzyme metabolic process                                    | 2.85            | 6.56E-05          | n.s.                                     |
|                                    | Alpha-amino acid metabolic process                            | 2.80            | 1.80E-02          | n.s.                                     |
|                                    | Carboxylic acid biosynthetic process                           | 2.73            | 2.68E-02          | n.s.                                     |
|                                    | Monocarboxylic acid metabolic process                          | 2.58            | 2.17E-04          | n.s.                                     |
|                                    | Carbohydrate metabolic process                                | 2.45            | 2.71E-02          | n.s.                                     |
|                                    | Nuclear exosome (RNase complex)                                | 6.84            | 2.37E-02          | *                                        |
|                                    | Mitochondrial matrix                                          | 2.21            | 3.00E-06          | *                                        |
| **EEF1AKMT1** knock out down-regulated | Tricarboxylic acid cycle                                      | 5.09            | 8.75E-03          | n.s.                                     |
|                                    | Dicarboxylic acid metabolic process                            | 4.25            | 6.88E-04          | n.s.                                     |
|                                    | tRNA aminocaylation for protein translation                   | 4.00            | 6.93E-03          | n.s.                                     |
|                                    | Nucleotide catabolic process                                  | 3.40            | 3.25E-05          | n.s.                                     |
|                                    | DNA biosynthetic process                                       | 3.13            | 2.15E-02          | n.s.                                     |
|                                    | Positive regulation of ubiquitin-protein transferase activity | 2.74            | 4.22E-02          | n.s.                                     |
|                                    | Mitotic cell cycle process                                     | 1.71            | 2.65E-02          | *                                        |
|                                    | Regulation of cellular macromolecule biosynthetic process     | 1.47            | 5.81E-04          | n.s.                                     |
|                                    | Cellular protein modification process                          | 1.46            | 6.40E-03          | n.s.                                     |
|                                    | Regulation of gene expression                                  | 1.33            | 3.09E-02          | n.s.                                     |
|                                    | Small-subunit processome                                       | 6.86            | 2.36E-02          | n.s.                                     |
|                                    | Replication fork                                              | 3.43            | 2.22E-02          | n.s.                                     |
|                                    | Nucleoplasm                                                   | 1.41            | 1.64E-06          | n.s.                                     |

*aA Mann-Whitney test was performed on the SILAC ratios for constituent proteins of each GO term. p values were then corrected for multiple testing by multiplying by the number of GO terms tests (n = 37). n.s. indicates p > 0.05, * indicates p = 0.05, ** indicates p < 0.01, **** indicates p < 0.0001.

bWith large ribosomal subunit proteins removed from the comparison.

With mitochondrial ribosome proteins removed from the comparison.
onto the crystal structure of METTL21B shows them in their three-dimensional contexts: Motifs I, Post I and II are key to

![Box plots showing SILAC ratios (Log$_2$ fold-change of knockout compared with wild-type) for subsets of proteins in METTL21B or EEF1AKMT1 knockouts compared with wild-type. Intervals show the range of 10–90% of values. P values were determined using a Mann-Whitney test. N.S. indicates p > 0.05, **** indicates p ≈ 0.0001. A, Proteins of the cytosolic large ribosomal subunit (n = 42) have significantly higher SILAC ratios in METTL21B knockout compared with EEF1AKMT1 knockout. B, Proteins of the cytosolic small ribosomal subunit (n = 29) exhibit no change in SILAC ratios between METTL21B knockout and EEF1AKMT1 knockout. C, The mitoribosome (n = 62) is upregulated relative to the other mitochondrial matrix proteins (n = 202) in knockout of METTL21B but not EEF1AKMT1. Additionally, mitochondrial matrix proteins, with mitoribosomal proteins excluded, do not change in SILAC ratios between METTL21B and EEF1AKMT1 knockout. D, Proteins of the mitochondrial large ribosomal subunit (n = 39) have significantly higher SILAC ratios in METTL21B knockout compared with EEF1AKMT1 knockout. E, Proteins of the mitochondrial small ribosomal subunit (n = 23) have significantly higher SILAC ratios in METTL21B knockout compared with EEF1AKMT1 knockout.]

Ribosomal proteins of the cytosolic large subunit and mitochondrion are differentially expressed upon knockout of METTL21B but not EEF1AKMT1. Box plots showing SILAC ratios (Log$_2$ fold-change of knockout compared with wild-type) for subsets of proteins in METTL21B or EEF1AKMT1 knockouts compared with wild-type. Intervals show the range of 10–90% of values. P values were determined using a Mann-Whitney test. N.S. indicates p > 0.05, **** indicates p ≈ 0.0001. A, Proteins of the cytosolic large ribosomal subunit (n = 42) have significantly higher SILAC ratios in METTL21B knockout compared with EEF1AKMT1 knockout. B, Proteins of the cytosolic small ribosomal subunit (n = 29) exhibit no change in SILAC ratios between METTL21B knockout and EEF1AKMT1 knockout. C, The mitoribosome (n = 62) is upregulated relative to the other mitochondrial matrix proteins (n = 202) in knockout of METTL21B but not EEF1AKMT1. Additionally, mitochondrial matrix proteins, with mitoribosomal proteins excluded, do not change in SILAC ratios between METTL21B and EEF1AKMT1 knockout. D, Proteins of the mitochondrial large ribosomal subunit (n = 39) have significantly higher SILAC ratios in METTL21B knockout compared with EEF1AKMT1 knockout. E, Proteins of the mitochondrial small ribosomal subunit (n = 23) have significantly higher SILAC ratios in METTL21B knockout compared with EEF1AKMT1 knockout.

three-dimensional contexts: Motifs I, Post I and II are key to
forming the core methyltransferase fold, whereas the DXXY motif is positioned near the active site (Fig. 5B).

We finally investigated the conservation of the lysine residue that is methylated by METTL21B. Interestingly, eEF1A lysine 165 showed a similar pattern of evolutionary conservation to the METTL21B enzyme. It is present in mouse, rabbit, chicken and frog, but not zebrafish, *D. melanogaster*, *C. elegans* or *S. cerevisiae*, where it is replaced by an alanine or serine (Fig. 5C). The lack of conservation of this residue is notable given the high conservation of eEF1A in general; more than 50% of its sequence is identical across all nine species. This co-occurrence of methyltransferase and lysine substrate is similar to what was observed for METTL22 and its substrate lysine in KIN17 (25). Overall, this suggests that METTL21B has an evolutionarily important role for the methylation of lysine 165 in eEF1A.

**DISCUSSION**

Here we have shown that METTL21B is a protein methyltransferase that methylates both isoforms of translation elongation factor eEF1A at lysine 165. During the revision of this manuscript, this was also reported by Malecki et al. using an orthogonal method to the one described here (48). Through ribosomal profiling, they found that knockout of *METTL21B* resulted in many similar changes to those we observed, namely the upregulation of ribosome biogenesis and mRNA metabolism and the downregulation of proteins of the endoplasmic reticulum. Interestingly, they observed that deletion of *METTL21B* does not affect global protein translation rate (48). This suggests that the upregulation of large ribosomal subunit proteins we have observed is compensatory. The relative upregulation of the mitochondrial ribosome observed upon knockout of *METTL21B* may be a secondary effect because of the changes to cytosolic translation, as mitochondrial translation is known to be highly regulated in response to the cellular environment (49, 50), and can even synchronize with cytosolic translation (47, 51). Overall, this indicates that METTL21B-mediated methylation of eEF1A at lysine 165 is important for the optimization of translation.

We have shown that eEF1A-KMT1 is the sole methyltransferase responsible for lysine 79 methylation in eEF1A. Proteomic analysis revealed that the effect on the translational apparatus seen upon knockout of *METTL21B* did not occur in the knockout of *EEF1AKMT1*. This suggests that lysine 165

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**TABLE III**

| Organism | Best match to METTL21B (Accession) | METTL21A (HSPA-KMT) | METTL21B (NP_056248.2) | METTL21C (NP_001010977.1) | METTL21D (VCP-KMT) |
|----------|-----------------------------------|---------------------|------------------------|-------------------------|-------------------|
| Vertebrates |                                   |                     |                        |                         |                   |
| Mammals |                                   |                     |                        |                         |                   |
| H. sapiens (human) | NP_056248.2 | 1e-49 | 7e-170 | 3e-20 | 2e-20 |
| M. musculus (mouse) | NP_001191965.1 | 3e-49 | 8e-141 | 5e-19 | 3e-18 |
| O. cuniculus (rabbit) | XP_002720992.1 | 6e-52 | 5e-151 | 3e-19 | 6e-22 |
| Ornithorhynchus anatinus (platypus) | XP_016081736.1 | 8e-46 | 5e-74 | 8e-17 | 7e-15 |
| Birds |                                   |                     |                        |                         |                   |
| G. gullus (chicken) | NP_001264773.1 | 9e-108 | 4e-44 | 5e-29 | 1e-26 |
| Coturnix japonica (Japanese quail) | XP_015742469.1 | 2e-37 | 9e-71 | 6e-22 | 5e-21 |
| Reptiles |                                   |                     |                        |                         |                   |
| Anolis carolinensis (Carolina anole) | XP_003216738.1 | 7e-39 | 3e-92 | 1e-22 | 9e-21 |
| Gekko japonicus (gecko) | XP_015277709.1 | 1e-41 | 3e-97 | 4e-23 | 4e-18 |
| Amphibians |                                   |                     |                        |                         |                   |
| X. laevis (African clawed frog) | XP_018102975.1 | 5e-38 | 2e-91 | 9e-29 | – |
| X. tropicalis (Westem clawed frog) | NP_001016600.1 | 3e-38 | 4e-92 | 3e-30 | – |
| Bony fishes |                                   |                     |                        |                         |                   |
| D. rerio (zebrafish) | NP_001013584.1 | 8e-94 | 3e-50 | 1e-26 | 9e-25 |
| Ostertogynaus mykiss (rainbow trout) | CDQ98192.1 | 8e-45 | 5e-82 | 2e-23 | 2e-16 |
| Cartilaginous fishes |                                   |                     |                        |                         |                   |
| Callorhinchus milii (Australian ghostshark) | XP_007884254.1 | 1e-39 | 2e-79 | 8e-26 | 3e-20 |
| Others |                                   |                     |                        |                         |                   |
| Crassostrea gigas (Pacific oyster) | XP_011442808.1d | 5e-72 | 3e-45 | 2e-22 | 1e-24 |
| D. melanogaster (fruit fly) | NP_573368.2 | 1e-08 | 7e-11 | 2e-05 | – |
| C. elegans (nematode) | NP_001122759.1 | 1e-11 | 3e-21 | 1e-12 | 9e-30 |
| S. cerevisiae (yeast) | NP_014374.1* | 9e-20 | 9e-13 | – | 2e-16 |

*Putative orthologues were matched back to human METTL21 proteins.
Bold indicates the best human match overall.
Best matched protein in the group.
This protein is the best matched to human METTL21B out of all organisms that are not vertebrates.
This protein is Efm6, and is therefore not the orthologue of METTL21A (20).
methylation affects the role of eEF1A in protein translation differently to lysine 79 methylation, and is therefore the first evidence that different methylation sites on eEF1A can have different effects on its function. More broadly, loss of methylation on lysine 165 or 79 was seen to have mostly similar effects on many processes related to eEF1A function, including actin cytoskeleton organization, tRNA aminoacylation and ribosome biogenesis. This could indicate that effects on one specific function of eEF1A have downstream consequences for other, connected processes that eEF1A is involved in. For example, translation is known to be highly regulated through the actin cytoskeleton (52–54), and in fact eEF1A has been suggested as a mediator between these processes (55). It will therefore be important to understand the mechanisms underlying the role of eEF1A methylation in order to tease apart the function of each methylation site. A critical part of this will be the discovery of the human methyltransferases responsible for methylation of the N terminus and lysines 36 and 55 of eEF1A (Fig. 6). In fact, the methyltransferase responsible for lysine 36 methylation was very recently described (56).

**METTL21B joins three other elongation factor methyltransferases known in human, namely eEF2-KMT, eEF1A-KMT1 and eEF1A-KMT2, yet it is the only one not conserved in yeast (19, 21, 34). METTL21B is expressed at medium to high levels in every tissue type (36), and has been reported to localize to the nucleus, the cytoplasm and centrosomes (25, 36, 48). Formerly known as FAM119B, it is noteworthy that METTL21B has been implicated in multiple sclerosis because...**

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**Fig. 5. **METTL21B and eEF1A lysine 165 show similar evolutionary conservation. A, Multiple sequence alignment of human METTL21B and its orthologues in *Mus musculus* (mouse), *Oryctolagus cuniculus* (rabbit) and *Xenopus laevis* (frog). Conserved motifs are colored as red (Motif I), blue (Motif Post I), yellow (Motif II) and orange (DXXY motif). B, The crystal structure of METTL21B (residues 24-226) bound to S-adenosyl L-homocysteine (PDB: 4QPN). Motifs are colored as in (A) and S-adenosyl l-homocysteine is colored in cyan. The active site is indicated with an arrow. Visualized in PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC.). C, Multiple sequence alignment of eEF1A from *H. sapiens*, *M. musculus*, *O. cuniculus*, *Gallus gallus* (chicken), *X. laevis*, *Danio rerio* (zebrafish), *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode) and *S. cerevisiae* (yeast), showing that lysine 165 is only conserved from frog to human. In species with two eEF1A isoforms, eEF1A1 was used. The equivalent residue to human eEF1A lysine 165 is colored as magenta (lysine), green (alanine) or cyan (serine). Multiple sequence alignments were generated using Clustal Omega (69) with default settings. Asterisks (*) indicate full conservation of a residue, colons (:) indicate highly similar residues and periods (.) indicate moderately similar residues.
of changes in its expression in a susceptibility genotype (57, 58). It may not be a causal gene, however, as there is evidence to suggest that changes to the expression of the nearby gene CYP27B1 is the causative factor (58).

METTL21B is a member of the Family 16 group of methyltransferases. As with all members of this family, it appears to be highly specific, as Malecki et al. demonstrated that in vitro methylation of cell extracts with $[^3H]$AdoMet resulted in the six canonical methylation sites (blue) and any known responsible human methyltransferases. Because the N-terminal glycine was not resolved in this structure, the most N-terminal residue (glutamate at position four) is shown instead. Visualized in PyMOL (The PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC.).

Acknowledgments—We thank Dr. Ignatius Pang for assistance with statistical analysis.

DATA AVAILABILITY

Data are available at https://www.ebi.ac.uk/pride/archive/ with project ID PXD005497.

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* JIH acknowledges support from an Australian Postgraduate Award and additional support from the University of New South Wales. MRW acknowledges support from the Australian Research Council and from the University of New South Wales. Mass spectrometric results were obtained at the Bioanalytical Mass Spectrometry Facility within the Mark Wainwright Analytical Centre of the University of New South Wales.

This article contains supplemental material.

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