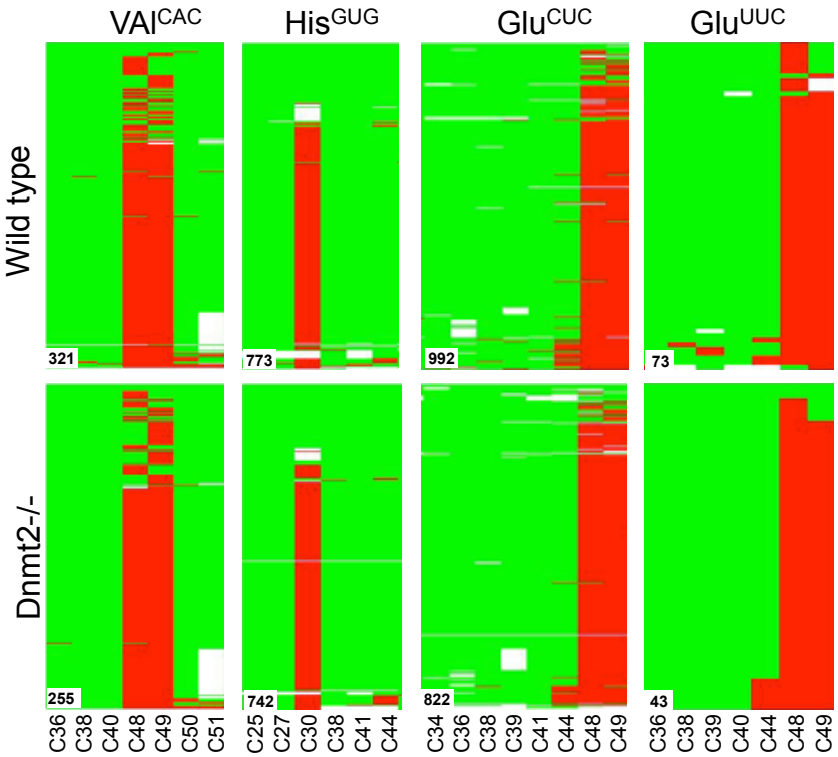
Appendix

Tuorto et al.: The tRNA methyltransferase Dnmt2 is required for accurate polypeptide synthesis during haematopoiesis

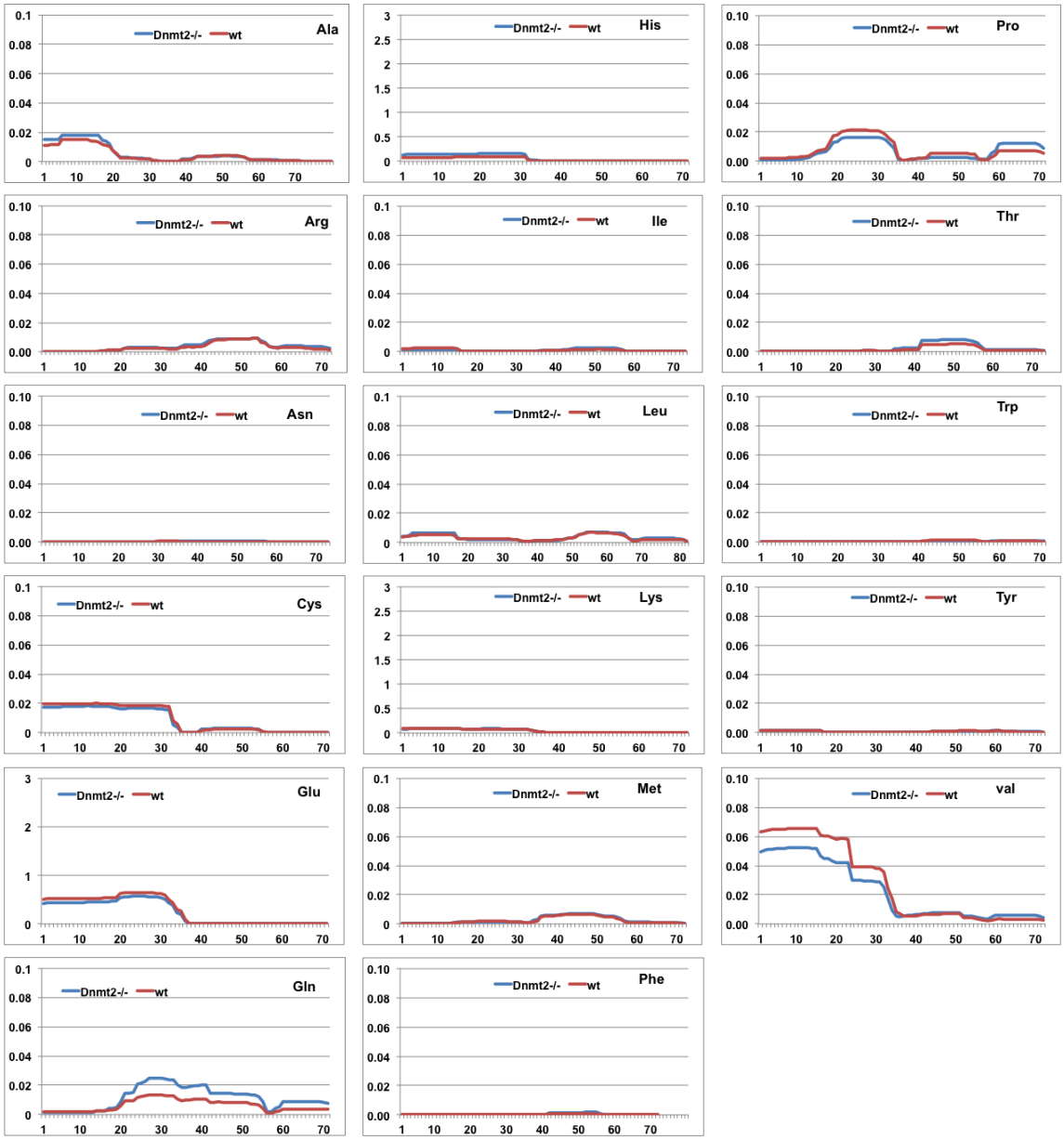
Contents:

Appendix Figure S1



Bisulfite sequencing maps for four tRNAs in wild type and *Dnmt2*$^{-/-}$ bone marrow. Each row represents one sequence read, each column a cytosine residue. Green boxes represent unmethylated cytosine residues, red boxes indicate methylated cytosine residues, sequencing gaps are shown in white. Numbers in maps indicate the number of reads, the positional number of each specific cytosine residue is shown at the bottom.

Appendix Figure S2



tRNA fragments coverage distribution obtained by small RNA sequencing of 8-day old bone marrow RNA. Wild type coverage is represented in red and *Dnmt2*$^{-/-}$ in blue. The plots complete the data of Figure 4C.

Appendix Table S1 Sequences of PCR primers and oligonucleotides

454 bisulfite sequencing	
mAsp-for	TGTTAGTATAGTGGTGAGTAT
mAsp-rev	CTCCCCATCAAAAAATTA
mVal-for	GTTTTTGTAGTGTAGTGGTTAT
mVal-rev	TATTTCCACCCAATTTCAAACC
mGly-for	GGTGGTTTAGTGGTAGAATT
mGly-rev	TACATTAACCAAAAATC
mGluCUC-for	TTTAGTGGTTAGGATTTGGT
mGluUUC-for	TTTAGTGGTTAGGATTTTTG
mGlu-rev	TTCCCTAACCAAAAATCAAACCC
mHis-for	GTTGTGATTGTATAG
mHis-rev	AACTCAAATTCAAACCAAGGTT
Northern probes	
tRNAAsp	ACCACTATACTAACGAGGA
tRNAGly	TCTACCACTGAACCACCGAT
tRNASer	CACTCGGCCCACCTCGTC
5SRNA	GGGTGGTATGGCCGTAGAC
qPCR primers	
OsterixF	AGCGACCACTTGAGCAAACAT
OsterixR	TTGGCTTCTTCTTCCCCGAC
OsteocalcinF	GCAATAAGGTAGTGAACAGACTCC
OsteocalcinR	GTTTGTAAGGCGGTCTTCAAGC
C/EBPalphaF	CAGCTCAGCTAGTGCGTCAG
C/EBPalphaR	TCACTGGTCAACTCCAGCAC
Glut4F	GATTCTGCTGCCCTTCTGTC
Glut4R	CAGCTCAGCTAGTGCGTCAG
Dnmt2-for	AGCCTGTGGCTTTCAGTATCA
Dnmt2-rev	TTGGCTGACTTTCTTCAACTACTGC
Gapdh-for	CATGGCCTTCCGTGTTCCTA
Gapdh-rev	TGCTTCACCACCTTCTTGATGT

Appendix Supplementary Methods

Mice and ethics Statement

Dnmt2$^{-/-}$ (B6;129-Trdmt1$^{tm1Bes/J}$) mice in the original mixed background (Goll et al, 2006) were obtained from The Jackson Laboratory (http://jaxmice.jax.org/strain/006240.html). The mice were backcrossed and maintained in a BL6J background and the phenotype analysis was carried out with the 10th generation backcross.

All mouse husbandry and experiments were carried out at the German Cancer Research Center pathogen-free animal facility according to applicable laws and regulations.

Histology and immunohistochemistry

Tibias and femurs from 8 day old pups were fixed in formalin, decalcified, and embedded in paraffin. Sections (4 μm) were either stained according to standard histology protocols or processed using a standard protocol for fluorescence immunostaining, incorporating an antigen retrieval step (citrate buffer microwave method). For primary antibodies, anti-GLS (*Griffonia simplicilolia* Lectin from Sigma, 1:50), Nestin (Acris, 1:50), and Periostin (Abcam, 1:50) were used. Pictures were taken using a Confocal microscope (Leica TCS SL).

Peripheral blood cell counts

Using EDTA treated collection tubes (Microvette CB300), peripheral blood samples were collected from *Dnmt2*$^{-/-}$ and age-matched wild type littermates, mixed (1:3) with NaCl 0.9% and analyzed using the Advia Analyzer System.

Colony formation assay

Bone marrow cells were harvested, washed with HBSS (Sigma) supplemented with 2% FBS (PAN-Biotech, Germany) and counted using Tuerck Solution (Sigma). For myeloid CFU-GM assays, 1.25x10^4 total bone marrow cells were plated using Methocult M3434 (StemCell Technologies) in two replicates, incubated for 10-14 days at 37°C and 5%CO_2. On day 15, cells were harvested, washed and 0.1% of single cell suspensions were re-plated for secondary colony formation. For CFU-preB assays, 5x10^4 cells were plated using Methocult M3630 (StemCell Technologies), incubated for 10-14 days at 37°C and 5% CO_2. On day 15, cells were

harvested, washed and 10% of single cell suspensions were re-plated for secondary colony formation under 37°C and 5%CO_2.

Osteogenic and adipogenic differentiation

Osteogenic differentiation of the MSCs was induced by 100 nM dexamethasone, 50 µg/ml ascorbic acid, and 10 mM β-glycerolphosphate (all Sigma-Aldrich). For the adipogenic differentiation, cells were plated in DMEM high glucose (Sigma-Aldrich), and the medium was supplemented with 0.2 mM indomethacin, 1 mM IBMX, 0.1 mg/mL insulin, and 1 µM dexamethasone (all Sigma-Aldrich). Osteogenic differentiation was visualized by Alizarin Red and adipogenic differentiation by Oil Red O after 14 days. For quantitative analysis, stained cells were counted in 5 random independent high-power fields using ImageJ. MSCs and differentiated cells were harvested at different time points and RNA was isolated using TRIZOL and used for qPCR or bisulfite methylation analysis. qPCR expressions values were normalized to GAPDH. Primers are listed in probes are listed in Appendix Table S1.

Small RNA sequencing

Based on Qubit quantification and sizing analysis multiplexed sequencing libraries were normalized, pooled and clustered on the cBot (Illumina, Inc.) with a final concentration of 10 pM (spiked with 1% PhiX control v3, Cat No. FC-110-3001) using the TruSeq SR Cluster Kit v3 (Cat no. GD-401-3001). Sequencing on HiSeq 2000 (Illumina) was performed using the 50 cycles TruSeq SBS Kit v3 according to the manufacturer's protocol.

Ribosome footprinting

Cells were lysed with 15 mM Tris-HCl (pH 7.4), 15 mM $MgCl_2$, 300 mM NaCl, 1% Triton X-100, 0.1% 2-Mercaptoethanol, 100 µg/ml cycloheximide, 500 µg/ml Heparin, and 1x Complete Protease Inhibitors (Roche). 400 µl aliquots of lysate were treated with 4 U DNase I (ThermoScientific) and 800 U of RNase I (Ambion), for 45 minutes at room temperature with gentle shaking. 800 U of RNasin Ribonuclease Inhibitor (Promega) was added to quench the reaction. The samples were run on a 17.5-50% sucrose gradient to isolate monosomes. Footprint monosome RNA was end-repaired with T4 polynucleotide kinase (TaKaRa), and size selected for 28-31 nucleotide sizes on a 15% polyacrylamide TBE-urea gel. The sequencing libraries

were prepared according the protocol of the NEB NEXT Small RNA library Prep Set for Illumina (Multiplex Compatible) E7330. After quality control and adapter trimming (AGATCGGAAGAGCACACGTCT), 20 nt reads were used as seed to align to an rRNA fasta index file built from RefSeq, followed by the discarding of any mapped reads. Using Bowtie, the remaining reads were mapped against the mouse mm9 Canonical Known Genes reference transcript database downloaded from the UCSC Genome Browser (http://genome.ucsc.edu). Here, 20 nucleotides of each read were used as the seed region, allowing alignments with up to 1 mismatch. The total reads for each replicate were between 14 and 17 million reads, of which 3 to 7 million reads mapped uniquely to CDS regions. Data analysis was performed using custom Python and R scripts. Footprint alignments were assigned to a specific A site according to the offset from the initial site of translation when mapping 5' ends of reads of various lengths to the initiation site (Ingolia, 2010). The offsets from the 5' end of the footprints were +15 for 26-30 nt read lengths and +16 for 31-32 nt read lengths. Reads were assigned to a codon when mapped to -1, 0, +1 relative to the first nucleotide of the codon. Bulk codon occupancy at each ribosomal site for each codon was calculated by normalizing the sum of all codons to the average of reads at +2 and -2 outer sites (Zinshteyn & Gilbert, 2013). The fold changes were then calculated by computing the log2 ratio of these relative ribosome dwell times for each codon in *Dnmt2*$^{-/-}$ versus wild type cells. To compute the single codon occupancy metric for each codon, the sum of the codons mapped to the A site for each gene was divided by the average read density for that gene (Zinshteyn & Gilbert, 2013).

In-solution protein digest

Peptides were generated according to (de Godoy et al, 2008). Briefly, samples were reduced with 1 mM tris(2-carboxyethyl) phosphine (TCEP) and free sulfhydryl groups carbamidomethylated using 5.5 mM choloroacetamide. Proteins were pre-digested with 0.5 µg sequencing grade endopeptidase LysC (Wako) for 3 h at room temperature and subsequently diluted with four volumes of 50 mM ammonium-bicarbonate (ABC). Tryptic digestion occurred for 10 h at room temperature using 1 µg sequencing grade trypsin (Promega). The reaction was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 1% resulting in a final pH of 2. The peptides were purified by using C18 stage-tips (3M) (Rappsilber et al, 2007).

Mass spectrometry

Peptides eluted from C18 stage-tips were run on an LC-MS setup. The fractionated and unfractionated samples were measured by LC-MS/MS on a Q Exactive orbitrap mass spectrometer (Thermo) connected to a Proxeon nano-LC system (Thermo) in data-dependent acquisition mode using the top 10 peaks for HCD fragmentation. Four-hour gradients for the unfractionated dimethyl labeled samples or three-hour gradients for all the other samples (solvent A: 5% acetonitrile, 0.1% formic acid; solvent B: 80% acetonitrile, 0.1% formic acid) was applied using an in-house prepared nano-LC column (0.074 mm x 250 mm, 3 µm Reprosil C18, Dr Maisch GmbH). A sample volume of 5 µl was injected and the peptides were eluted with 3 or 4 h gradients of 4% to 76% ACN and 0.1% FA in water at flow rates of 0.25 µl/min. MS acquisition was performed at a resolution of 70'000 in the scan range from 300 to 1700 m/z. Dynamic exclusion was set to 30 s and the normalized collision energy was specified to 26. The eluent was directly sprayed into a Q Exactive mass spectrometer (Thermo Fisher Scientific,) equipped with a nano electrospray ion source. The recorded spectra were analyzed using MaxQuant software package version 1.2.2.5 (Cox & Mann, 2008) with Andromeda search against the UniProt *Mus musculus* database (version 2012) with a false discovery rate of 1% (peptides and proteins). The fixed and variable modifications were set to carbamidomethylation of cysteines and methionine oxidation, respectively. For further data analysis, the R statistical software package was conducted. For amino-acid transition analysis we used MaxQuant version 1.4.1.4. Transitions were set as variable modifications allowing only two transitions in separate MaxQuant runs. We searched the following transitions as variable modifications: Glu-Asp (-14 Da), Asp-Glu (+14 Da), Cys-Trp (+83 Da), Ser-Arg (+70 Da), Asn-Lys (+14 Da), His-Gln (-9 Da) and Phe-Leu (-34 Da). In each run we included carbamidomethylation on Cys as fixed modification and oxidized Met as an additional variable modification. The Amino acid change frequency was calculated dividing the number of peptides showing an amino acid substitution by the total number of peptide identified in the MaxQuant run.

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