Supplementary Information for

Three distinct 3-methylcytidine (m^3C) methyltransferases modify tRNA and mRNA in mice and humans

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Supplementary Materials and Methods

Generation of *Mettl2*, and *Mettl8* mutant cell lines. gRNAs were designed and cells were transfected in 24-well plates using 0.5 μg of the Cas9 expression plasmid and 0.3 μg of the RNA expression plasmid by calcium phosphate precipitation or lipofectamine® 2000 following manufacturer's instructions. Two days after transfection, cells were trypsinized and replated in 10 cm^2 dishes with a highly-diluted passage ratio for single clone selection. After about two weeks, single clones were picked and cultured in individual wells. The selection of positive clones was done first by Western blot analysis of METTL2 or METTL8. Genomic DNA were extracted subjected to PCR, TA cloning and sequencing to confirm the mutation of the targeted *loci*.

Calcium phosphate precipitation transfection. Calcium phosphate precipitation was done only in 293T when confluency reaches 40-60%. 20 μg of plasmid DNA in 450 μl autoclaved Milli-Q water was mixed with 50 μl of 2.5 M freshly-made calcium chloride. Then 500 μl of 2×BES buffer (50 mM BES, 15 mM Na_2HPO_4, 280 mM NaCl. Adjust pH to 6.95 with 1 M NaOH) was added and the whole solution was vortexed immediately for 1 min followed by 20 min incubation at ambient temperature to allow DNA precipitation. After incubation, the DNA solution was briefly spin down, pipetted up and down several times, and added evenly to dishes in a drop-wise manner. The transfected cells were incubated at 37 °C with 2.5% CO_2. After about 16 h, media was discarded and replaced with 10 ml fresh media. Then cells were moved back to 37 °C incubator with 5% CO_2 until cells reached desired confluency. All other transfections were performed using lipofectamine® 2000.

Western Blotting. Appropriate amount of lysates of samples were electrophoresed by SDS-PAGE, transferred onto PVDF membranes (0.45 μm, Millipore) in cold room. After blocking with 5% skim milk in PBST containing 0.1% Tween-20 at 37°C for 1 h, the membranes were probed with primary antibodies of interest at 4 °C overnight and then with HRP conjugated secondary antibody (Biorad). X-ray film development (Fig. 3) or enhanced chemiluminescence visualization method (by GE ImageQuant LAS 500 or Amersham Imager 600, Figs S2E, S2G and S3B) were used. The source of antibodies is indicated in figure legends.

MTS assay. MTS was performed according to manufacturer's instructions (Promega)

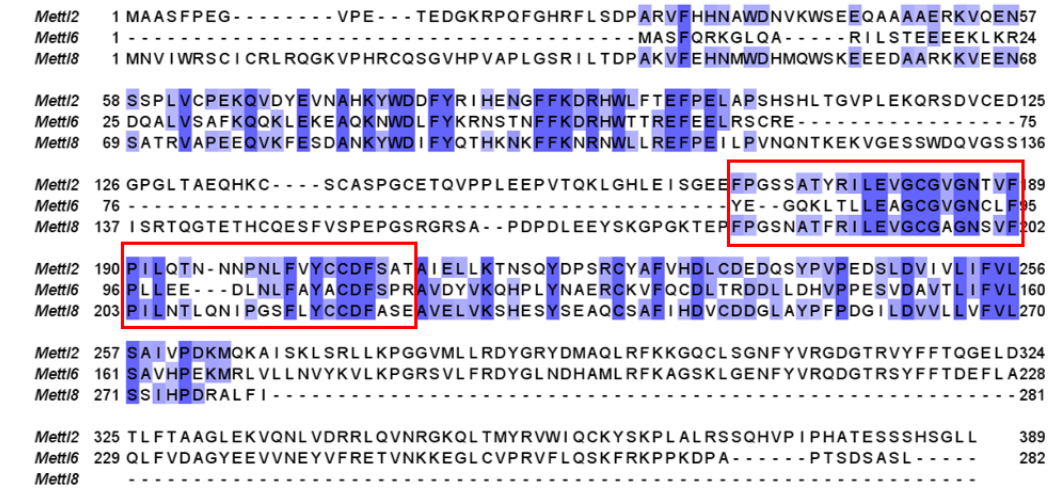
Polysome profiling. Polysome profiling was adapted from (1). Briefly, cells are treated with 100 μg/ml cycloheximide (Sigma) at 37 ℃ incubator for 10 minutes before harvesting. Trypsin was used and cell pellet was resuspended in 300 μl fresh 1× RSB buffer with cycloheximide. Take out 320 μl and put into a fresh cold 1.5ml tube. Add in the same volume of 320 μl fresh 1× lysis buffer. Mix gently and leave it on ice for 10 mins. Spin full speed for 3 mins to remove nuclei. Transfer about 600 μl extract into a new cold tube and spin full speed for 10 mins. Transfer into another new tube. Take out 10 μl extract to measure the OD units. Measure and load the same OD Units onto sucrose gradient (10%-50%), balanced the tubes with mixture of 1× Lysis Buffer and 1× RSB buffer with cycloheximide. Spin at 8 ℃, 36000rpm, 1.5hrs - 2hrs in Beckman centrifuge. Collect fractions and monitor 254nm UV reading by Biocomp gradient machine. 2× Resuspension Buffer (RSB): 20mM Tris-Hcl (pH7.4), 300mM NaCl. 30mM MgCl, 1× RSB with cycloheximide: 1x RSB with 500 unit SUPERase In RNase inhibitor (Thermo) per ml buffer, 100 μg/ml cycloheximide. 1× Lysis Buffer: 1× RSB add 1% Triton X, 2% Tween, 1% deoxycholate. All buffer use RNase free water and made fresh each day.

Supplementary Figures

Figure S1. Sequence alignment of METTL2, 6, 8 and their homologs. (A) Sequence alignments of the yeast Trm140 with human homologs METTL2A, 2B, 6 and 8 (accession numbers Q96IZ6, Q6P1Q9, Q8TCB7, Q9H825, respectively). **(B)** Sequence alignments of full length mouse METTL2, 6 and 8 proteins (accession numbers Q8BMK1, Q8BVH9 and A2AUU0, respectively). **(C)** Sequence alignments of Trm140 from baker's yeast with homologs from other organisms. Accession numbers as follows: Fruit fly (*Drosophila melanogaster*): NP 647636.3; Fission yeast (*Schizosaccharomyces pombe*): CAB76043.1; Baker's yeast (*Saccharomyces cerevisiae*): YOR239W; Common chimpanzee (*Pan troglodytes*): XP 001144324.1; Red junglefowl (*Gallus gallus*): NP 001006329.1; Gray wolf (*Canis lupus*): XP 537604.3; Cattle (*Bos taurus*): NP 001068714.1; Rat (*Rattus norvegicus*): NP 001102309.1; Worm (*Caenorhabditis elegans*): NP 001040827.1; Zebra fish (*Danio rerio*): NP 001017902.1; *Trypanosoma brucei*: XP 827431.1. All accession numbers are from NCBI Protein database.

A

B



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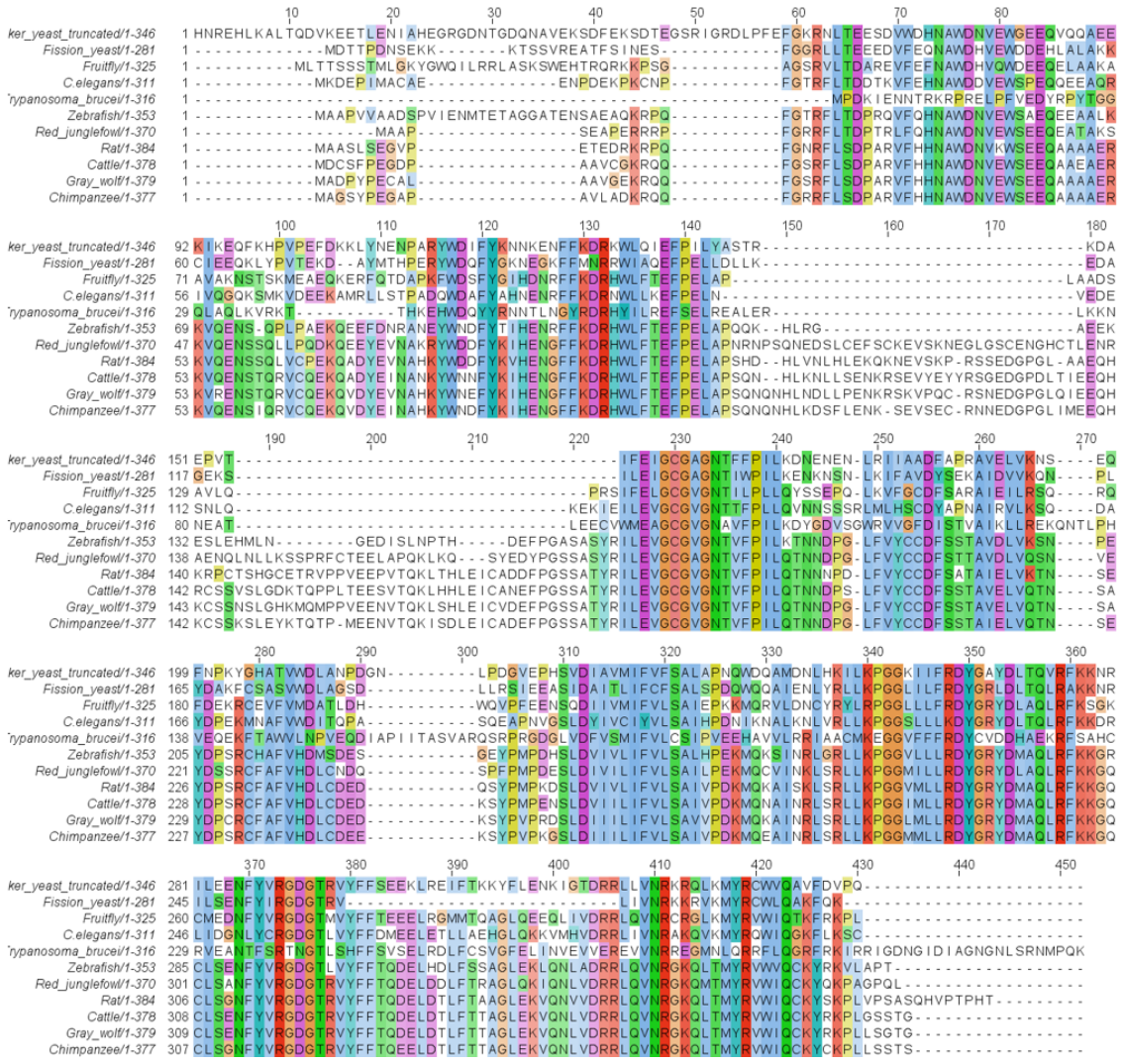
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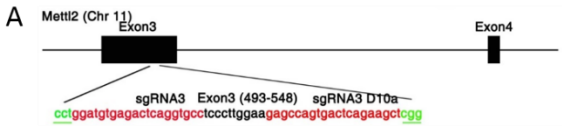
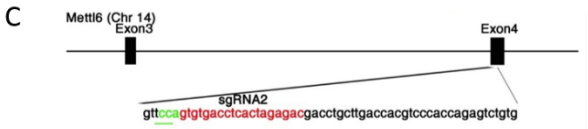
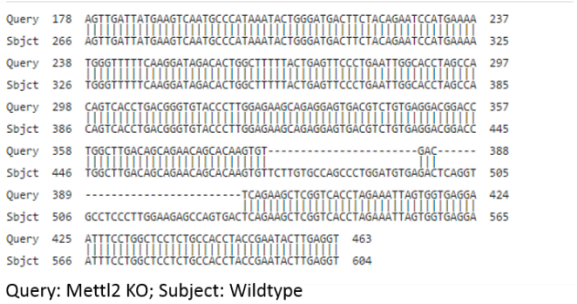
Fission_yeast/1-281
Fruitfly/1-325
C.elegans/1-311
Trypanosoma_brucei/1-316
Zebrafish/1-353
Red_junglefow/1-370
Rat/1-384
Cattle/1-378
Gray_wolf/1-379
Chimpanzee/1-377

Figure S2. CRISPR/Cas9 mediated *Mettl2*, *Mettl6* and *Mettl8* gene knockout in mouse. (A) gRNA design for targeting mouse *Mettl2* exon 3. **(B)** Sequence alignment of wildtype and clone Number 8 targeted by sgRNA3, clone number 8 is introduced a premature stop codon at *Mettl2 locus*. **(C)** gRNA design for targeting mouse *Mettl6* exon 4. **(D)** Sequence alignment of wildtype and clone Number 48 targeted by sgRNA3, clone Number 48 is verified to have premature stop codon of *Mettl6*. **(E)** Western blot for liver and brain tissue from wildtype and Mettl6 mutant mice. (Mouse Mettl6, proteintech, cat# 16527-1-AP, Vinculin antibody, CST Cat#4650). **(F)** gRNA design targeting Mettl8 mRNA coding region used to inject mouse embryo and sequencing validation of deletion mutant. Region in blue is deleted (92bp). **(G)** Western blot for liver and brain tissue from wildtype and Mettl8 mutant mice, against Mouse Mettl8 (Polyclonal, Singapore IMCB) and Vinculin (CST, Cat#4650), upper panel is the overlapping image for blot and colour picture of the member. **(H)** Deletion induced premature stop of full length protein of METTL2, 6 and 8. Amino acid highlighted in yellow are truncated protein. AA: amino acid residues





B Number 8 clone: 53bp deletion



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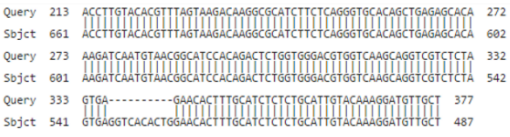
D Number 48: 10 bp deletion



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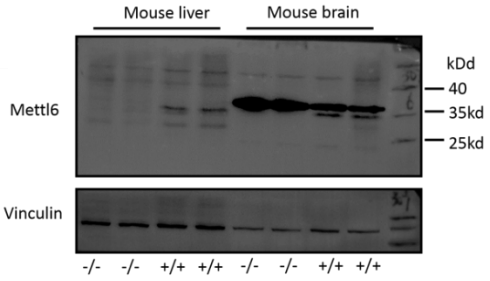
E



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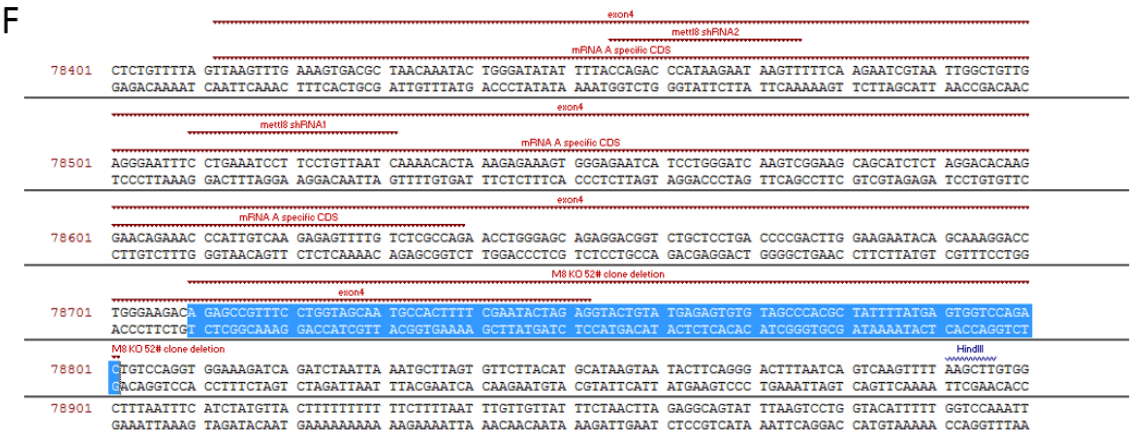

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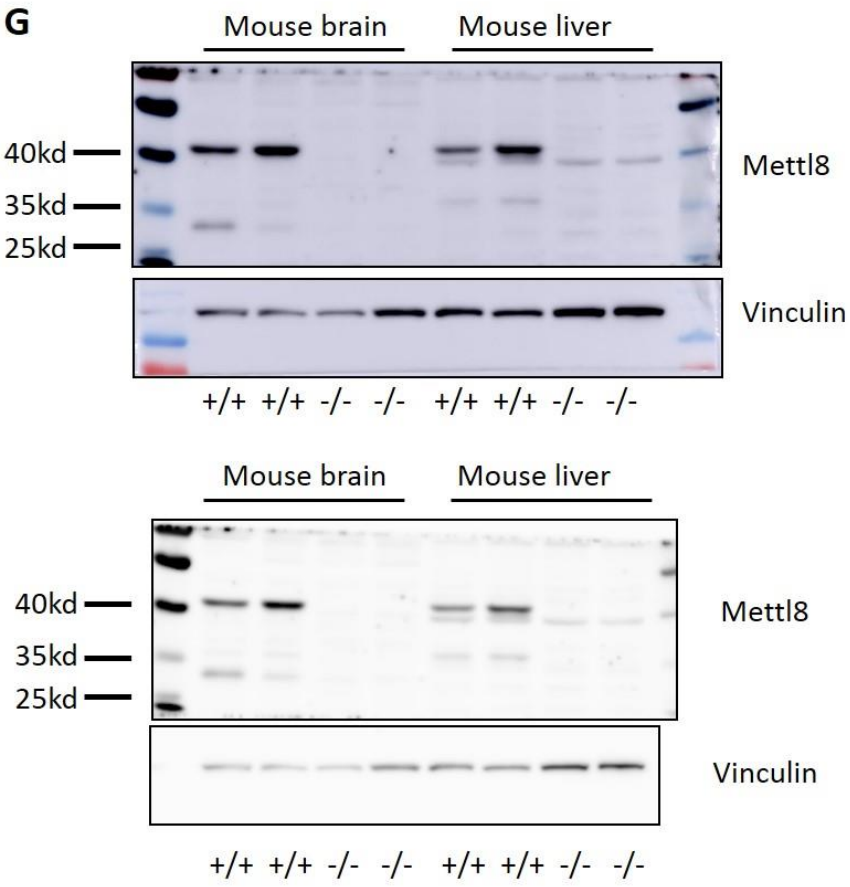

Mouse brain
Mouse liver
40kd
35kd
25kd
Mettl8
Vinculin
+/+ +/+ -/- -/- +/+ +/+ -/- -/-
Mouse brain
Mouse liver
40kd
35kd
25kd
Mettl8
Vinculin
+/+ +/+ -/- -/- +/+ +/+ -/- -/-

H

- **METTL2 full length: 378 AA**
- **Met** A A S F P E G V P E T E D G K R P Q F
  G H R F L S D P A R V F H H N A W D N V K
  W S E E Q A A A A E R K V Q E N S S P L V
  C P E K Q V D Y E V N A H K Y W D D F Y R
  I H E N G F F K D R H W L F T E F P E L A P
  S H S H L T G V P L E K Q R S D V C E D G P
  G L T A E Q H K C S C A S P G C E T Q V P P
  L E E P V T Q K L G H L E I S G E E F P G S S
  A T Y R I L E V G C G V G N T V F P I L Q T
  N N N P N L F V Y C C D F S A T A I E L L K
  T N S Q Y D P S R C Y A F V H D L C D E D
  Q S Y P V P E D S L D V I V L I F V L S A I V
  P D K **Met** Q K A I S K L S R L L K P G G
  V **Met** L L R D Y G R Y D **Met** A Q L R F K K
  G Q C L S G N F Y V R G D G T R V Y F F T
  Q G E L D T L F T A A G L E K V Q N L V D R
  R L Q V N R G K Q L T **Met** Y R V W I Q C K
  Y S K P L A L R S S Q H V P I P H A T E S S S
  H S G L L **Stop**
- **METTL2 mutant (53bp deletion): 144 AA**
  Met A A S F P E G V P E T E D G K R P Q F
  G H R F L S D P A R V F H H N A W D N V K
  W S E E Q A A A A E R K V Q E N S S P L V
  C P E K Q V D Y E V N A H K Y W D D F Y R
  I H E N G F F K D R H W L F T E F P E L A P
  S H S H L T G V P L E K Q R S D V C E D G P
  G L T A E Q H K C S E A R S P R
  N **Stop** W **Stop** G I S W L L C H L P N
  T **Stop** G W L W C R K H S L S N F T
  N **Stop** Q **Stop** P K P L R L L L **Stop** L F C H
  G Y **Stop** T A Q D K F T I **Stop** S F S L L C L
  C S **Stop** S L **Stop Stop** R S E L P S A **Stop**G
  Q S **Stop** C H R S Y I C S F S N C S R Q D A
  E S D Q Q A K P T P E A W R G D A S S R L
  W P L **Stop** H G S T S V **Stop** E R S V S I W
  K L L C E R **Stop** W H Q S L L L H T
  R **Stop** A G Y A L H R C W P G E G A E P G
  G S P L A G E S R E T A D H V P R L D S V Q
  I Q Q A S S T P L Q P T C A H S P R H R K F
  F T F G A F V

- **METTL6 full length: 282 AA**
- **Met** A S F Q R K G L Q A R I L S T E E E
  E K L K R D Q A L V S A F K Q Q K L E K
  E A Q K N W D L F Y K R N S T N F F K
  D R H W T T R E F E E L R S C R E Y E G
  Q K L T L L E A G C G V G N C L F P L L
  E E D L N L F A Y A C D F S P R A V D Y
  V K Q H P L Y N A E R C K V F Q C D L T
  R D D L L D H V P P E S V D A V T L I F
  V L S A V H P E K **Met** R L V L L N V Y K
  V L K P G R S V L F R D Y G L N D H
  A **Met** L R F K A G S K L G E N F Y V R
  Q D G T R S Y F F T D E F L A Q L F V D
  A G Y E E V V N E Y V F R E T V N K K E
  G L C V P R V F L Q S K F R K P P K D P
  A P T S D S A S L **Stop**
- **METTL6 mutant (10bp deletion):152 AA**
- **Met** A S F Q R K G L Q A R I L S T E E E
  E K L K R D Q A L V S A F K Q Q K L E K
  E A Q K N W D L F Y K R N S T N F F K
  D R H W T T R E F E E L R S C R E Y E G
  Q K L T L L E A G C G V G N C L F P L L
  E E D L N L F A Y A C D F S P R A V D Y
  V K Q H P L Y N A E R C K V F S L E T T
  C L T T S H Q S L W **Met** P L H **Stop** S L
  C S Q L C T L R R C A L S Y **Stop** T C T R
  Y **Stop** N Q A E V S Y S V T T
  G **Stop Met** I T P C L D L K L E A N L E K
  I F **Met** S G K **Met** E P D R I F L
  L **Met** N S W R S S L W **Met** Q V **Met** K
  K W **Stop** T S **Met** C F E R Q **Stop** I K K
  R A C V C L E F S F R A S S G S L R R T Q
  P L P V T L L H F
- **METTL8 full length:281 AA**
- **Met** N V I W R S C I C R L R Q G K V P H R C
  Q S G V H P V A P L G S R I L T D P A K V F E
  H N **Met** W D H **Met** Q W S K E E E D A A R
  K K V E E N S A T R V A P E E Q V K F E S D
  A N K Y W D I F Y Q T H K N K F F K N R N
  W L L R E F P E I L P V N Q N T K E K V G E S
  S W D Q V G S S I S R T Q G T E T H C Q E S
  F V S P E P G S R G R S A P D P D L E E Y S K
  G P G K T E P F P G S N A T F R I L E V G C G
  A G N S V F P I L N T L Q N I P G S F L Y C C
  D F A S E A V E L V K S H E S Y S E A Q C S A
  F I H D V C D D G L A Y P F P D G I L D V V L
  L V F V L S S I H P D R A L F I **Stop**
- **METTL8 mutant (92 bp deletion): 201 AA**
  Met N V I W R S C I C R L R Q G K V P H R C
  Q S G V H P V A P L G S R I L T D P A K V F E
  H N **Met** W D H **Met** Q W S K E E E D A A R
  K K V E E N S A T R V A P E E Q V K F E S D
  A N K Y W D I F Y Q T H K N K F F K N R N
  W L L R E F P E I L P V N Q N T K E K V G E S
  S W D Q V G S S I S R T Q G T E T H C Q E S
  F V S P E P G S R G R S A P D P D L E E Y S K
  G P G K T T P C R T F Q D P F S T A A T L P L
  K L W N L **Stop** S P T S P T A R P S V L P L F
  Met T C V T T A **Stop** P T L S Q Met G S W
  Met S F S L S L C S H L S T L T G C K L L P T
  D C P G C **Stop** S P E E C Y C F G I Met E D T
  I Met L S F V L R K G V V Y L K I F Met S E E
  Met V P E L I S L Q K G K S A V C S A R L D Y
  T K S K I W L I I A C K**Stop** T G K S K C R C T
  E C G F K E N S R N H R P G L H R V E I

Figure S3. CRISPR/Cas9 mediated gene mutations in human cell lines (A) Sanger sequencing results of several cell clones grown from single cells are aligned to the corresponding targeting region. Mutation or deletion regions are indicated in blue, human Mettl2A and Mettl2B ref_seq sequence are at the bottom of the alignment. Chromatogram of T2-1 #3 clone shows single peaks, indicating double KO of both METTL2A and METTL2B. **(B)** Equal amounts of lysates from different METTL2 knockout clones and controls were resolved on an SDS-PAGE gel and probed with HSP90 and three human METTL2A/2B antibodies, designated as anti-METTL2 abmart1, anti-METTL2 abmart2, anti-Mettl2 (in house purified, has non-specific bands besides the 42 kDa band for METTL2). **(C)** Sequencing results of METTL8 KO clone in HCT116 cell line. Genomic DNA were aligned to the respective target region of each gRNA used. Deletion were shown with gRNA underlined in red.

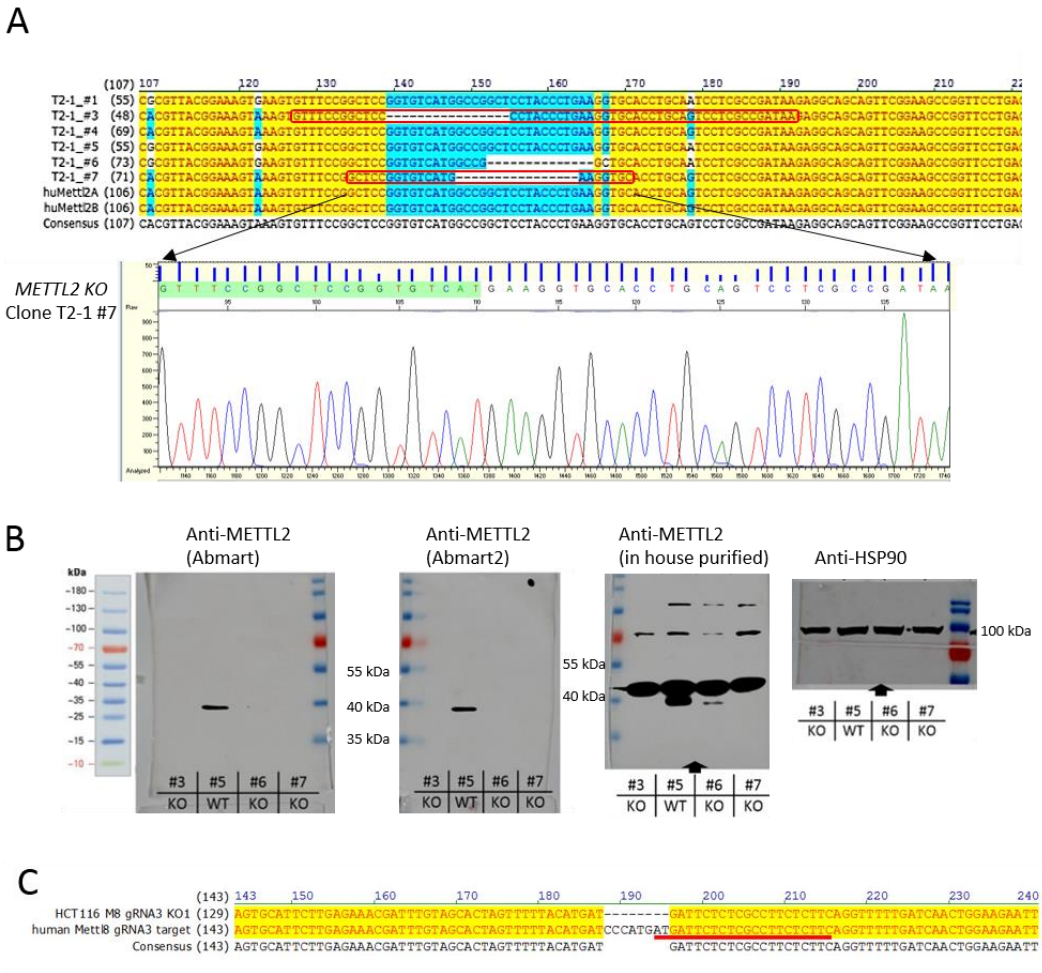
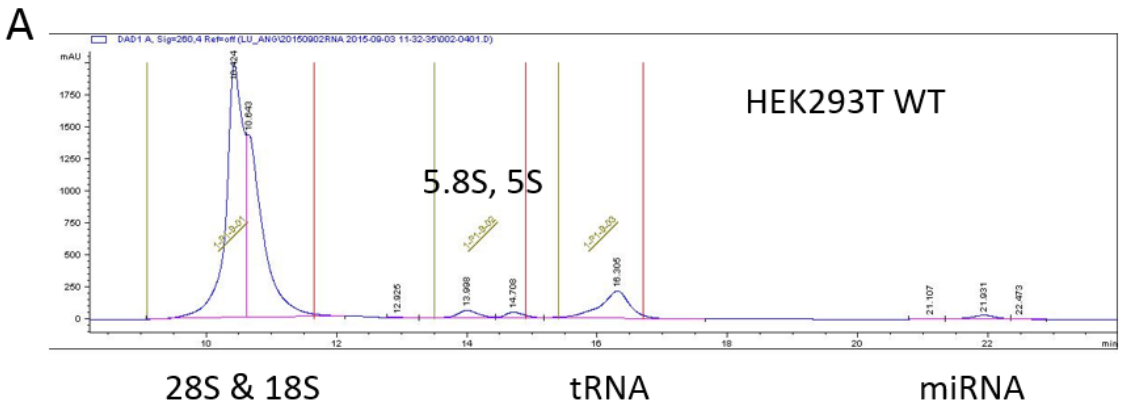
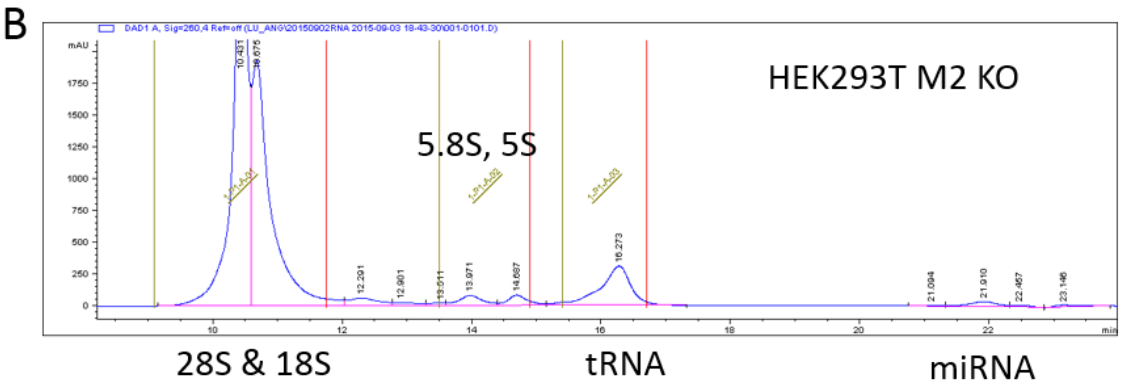
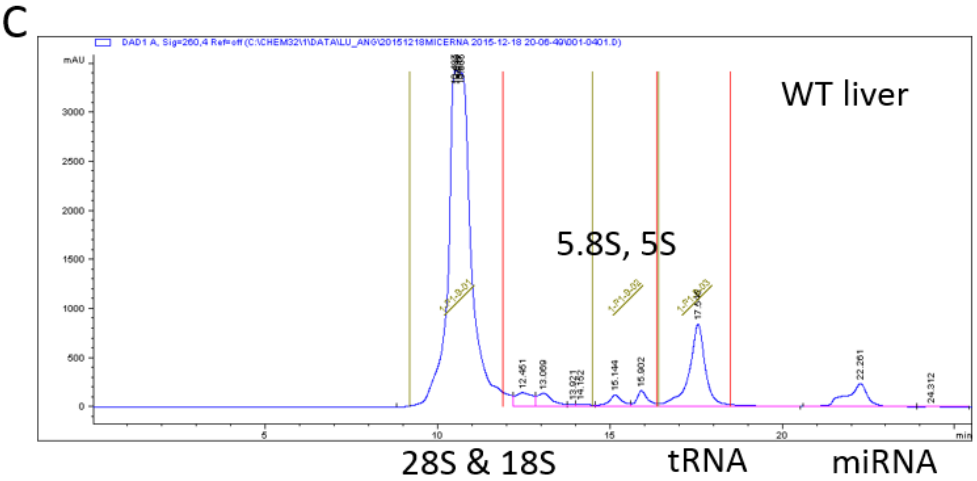


Figure S4. Size-exclusion HPLC purification of RNA species from human cells and mouse tissues. Green vertical lines in the graph: start point for collecting fractions; Red vertical lines: end of fraction collection. **(A-D)** Typical SEC3 size-exclusion HPLC chromatograms for RNA from human HEK293 WT (**A**) and METTL2 KO (**B**), and liver tissue from use WT mice (**C**) and METTL2 KO mice (**D**). **(E, F, H-J)** Typical SEC5 size-exclusion HPLC chromatograms for human HCT116 WT cells (**E**), METTL8 KO cells (**F**), liver from WT mice (**H**), METTL6 KO mice (**I**) and METTL8 KO mice (**J**); (**G**, **K**) Typical Bioanalyzer tracings of purified 28S and 18S fractions, note a contamination of 28S rRNA in 18S rRNA fraction. **(L-N)** Typical SEC3 size-exclusion HPLC chromatograms for removal of small RNA species (5.8S, 5S, tRNA) from total RNA from HCT116 WT (**L**, **N**) and M8 KO cells (**M**). RNA in **L** and **M** was previously enriched for RNA >200 nt by 35% ethanol precipitation, while RNA in N was total RNA. **(O)** Typical Bioanalyzer tracings for isolated large RNA species (> 5.8S rRNA). No small RNA is observed.







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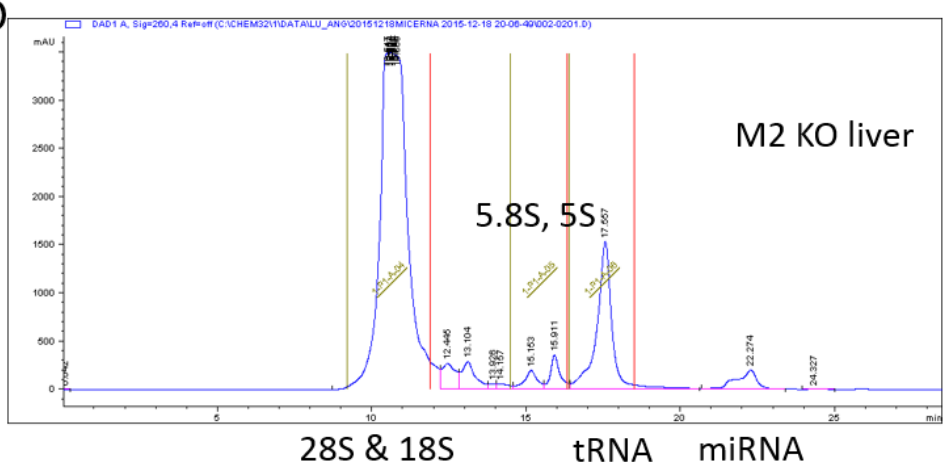
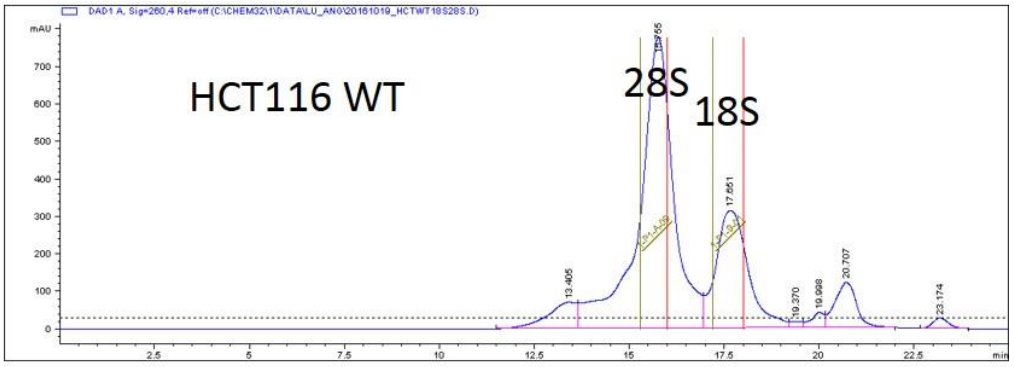
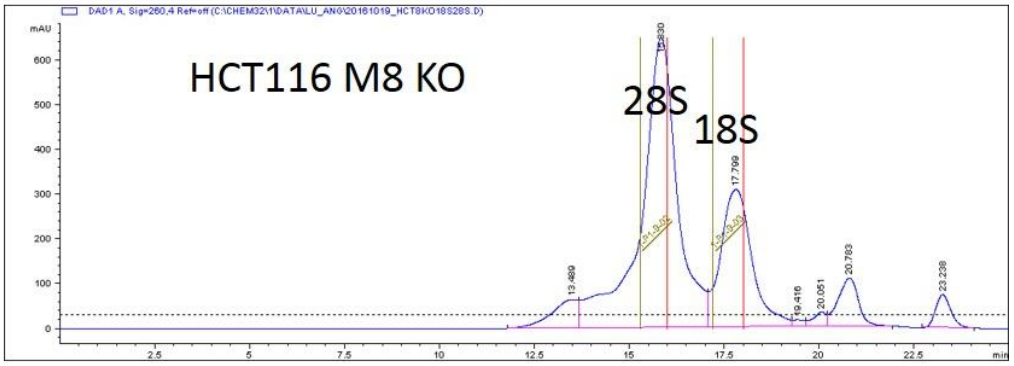

M2 KO liver
5.8S, 5S
28S & 18S
tRNA
miRNA

E


HCT116 WT
28S
18S

F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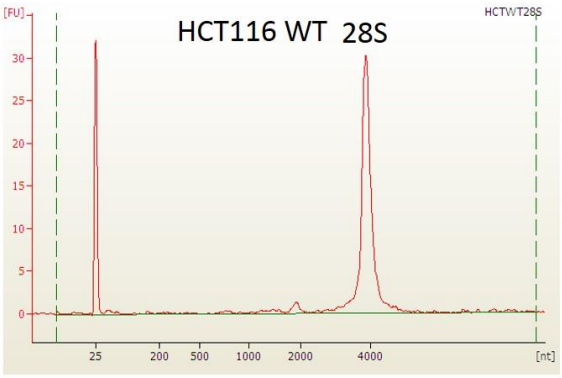
28S
18S

G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HCTWT28S
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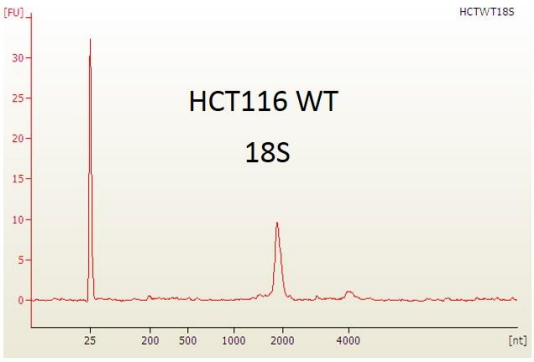
[nt]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18S
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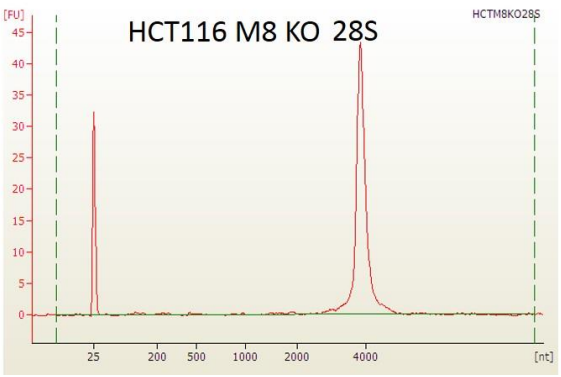
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HCTM8KO28S
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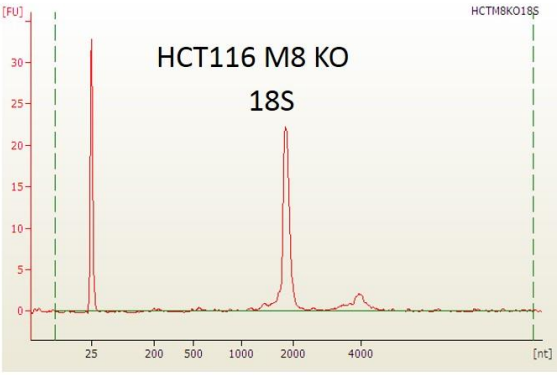
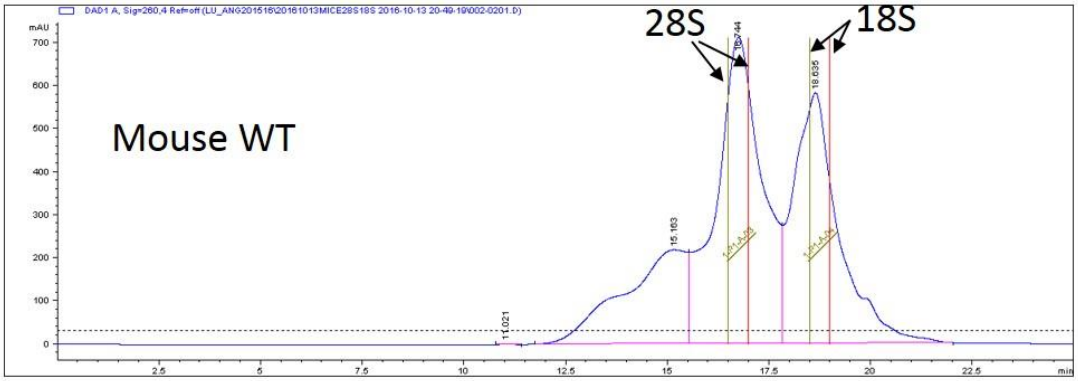
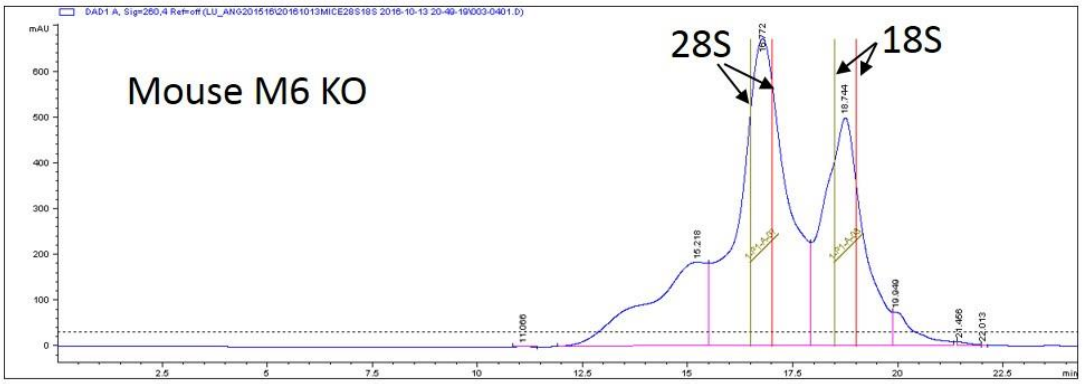

HCT116 M8 KO
18S
HCTM8KO18S
[FU]
[nt]

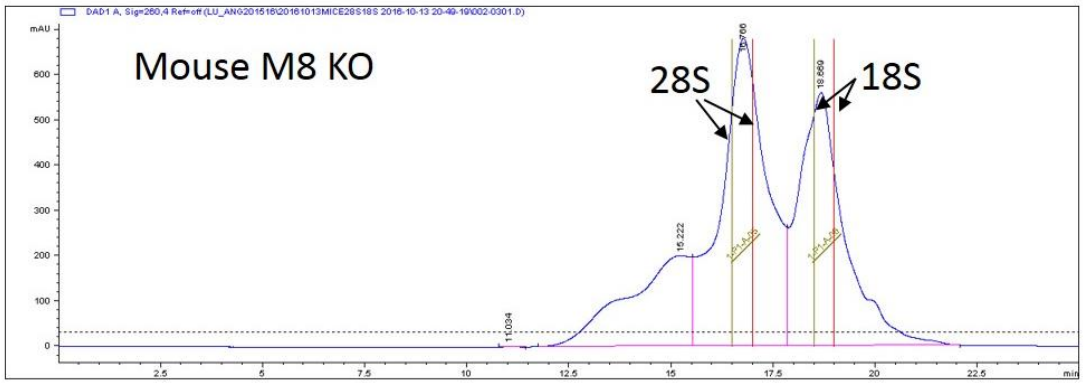
H


Mouse WT
28S
18S
mAU
min

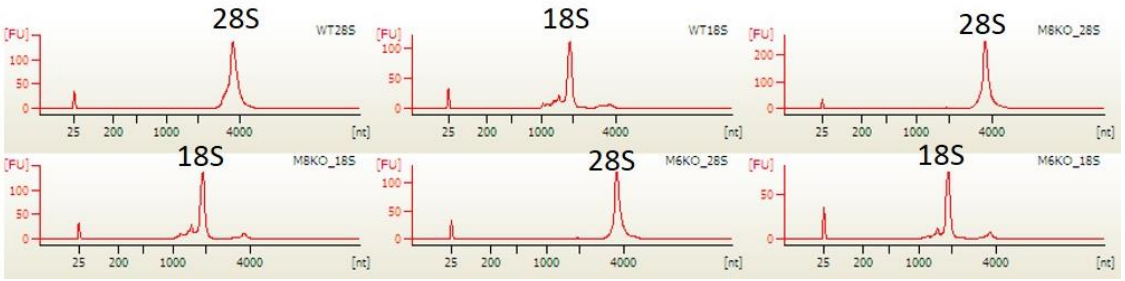
I


Mouse M6 KO
28S
18S
mAU
min

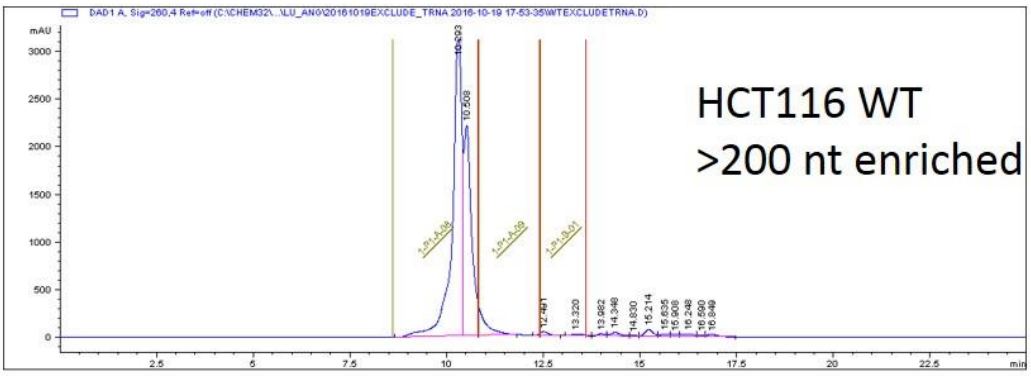
J


Mouse M8 KO
28S
18S

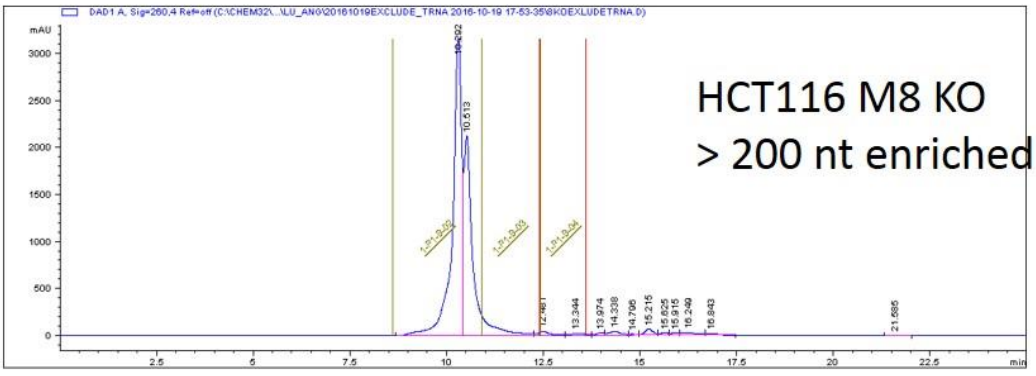
K


28S
WT28S
18S
WT18S
28S
M8KO_28S
18S
M8KO_18S
28S
M6KO_28S
18S
M6KO_18S

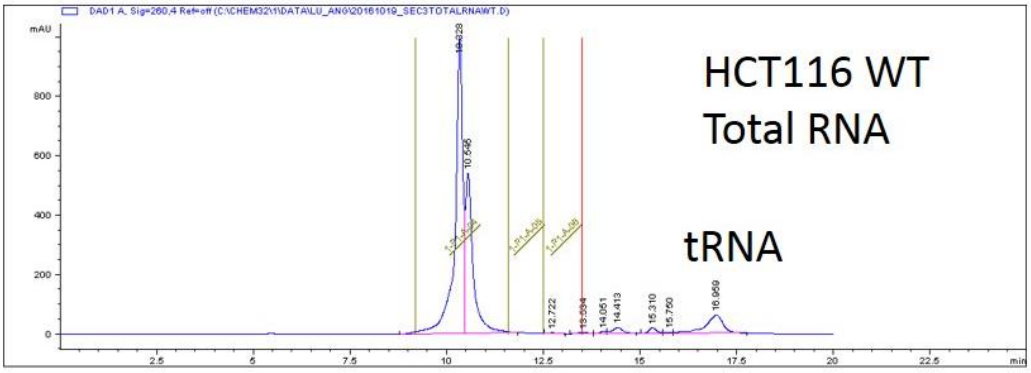
L


HCT116 WT
>200 nt enriched

M


HCT116 M8 KO
> 200 nt enriched

N


HCT116 WT
Total RNA
tRNA
mAU
min

O

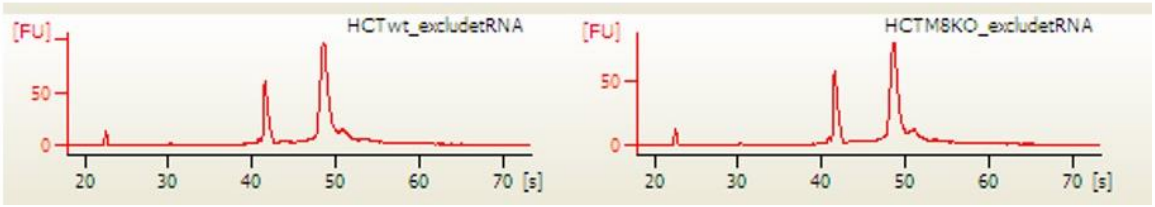
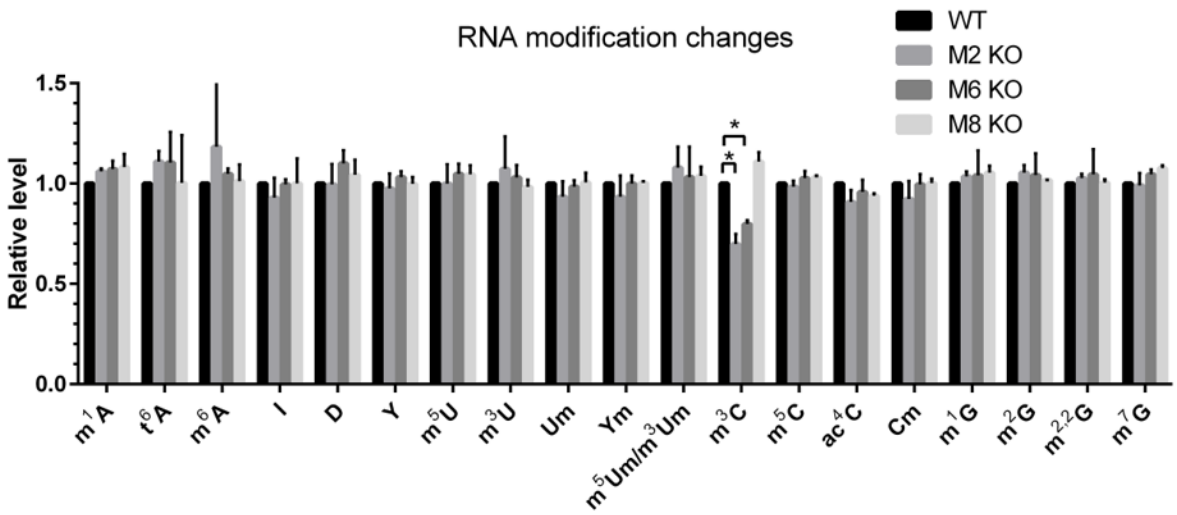

HCTwt_excludetRNA
HCTM8KO_excludetRNA
[FU]
[s]

Figure S5. (A) LC-MS/MS analysis of the levels of 19 modified ribonucleosides in tRNA from wild-type (WT) and mutant cells: *Mettl2* KO, M2 KO; *Mettl6* KO, M6 KO; *Mettl8* KO, M8 KO. **(B)** Typical LC-MS/MS chromatography for m^3C, m^4C and m^5C (258.1-126.1 m/z) chemical standards and two RNA samples (small RNA depleted) analyzed

A



B

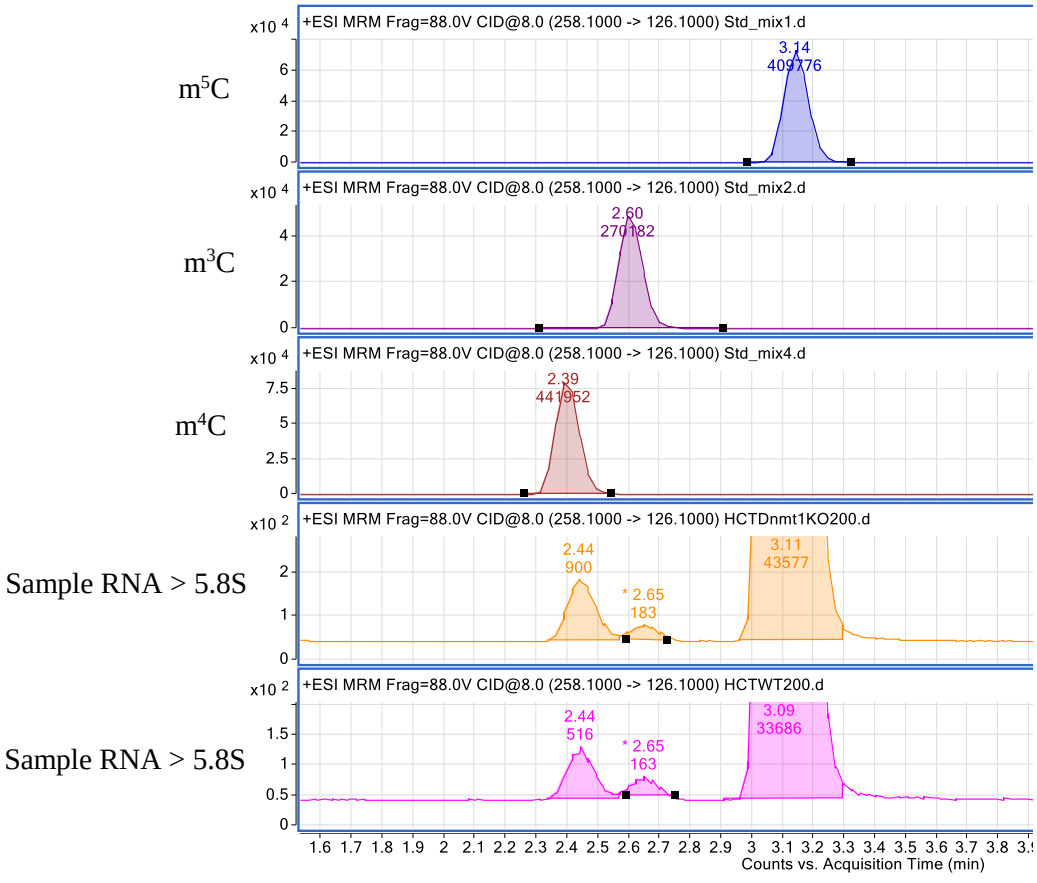


Figure S6. Primer extension analysis of human tRNAs. **(A)** Schematic showing human $tRNA^{Thr(AGU)}$ and $tRNA^{Thr(UGU)}$ isoacceptors, with red lines denoting the coverage of probes used in the primer extension assay to map polymerase-blocking modifications at position C32. **(B)** Primer extension assay using RNA from human HEK293T wildtype (WT) or *METTL2* KO (M2 KO) cells (Clone #3). In lanes 2 and 4, the two bands marked with stars represent primer extension products. The Thr^{AGU} and Thr^{UGU} probes (22nt) are used in lanes 1 and 2, and 3 and 4, respectively. Marker lane (M) contains two ssDNA probes 56 and 72 nt in length. (**C**) Primer extension for another *Mettl2* KO clone, (Clone #7), Thr^{UGU} probe was tested. (**D**) Primer extension assay with RNA isolated from M2 KO cells transfected with an empty vector (lanes 1-2) or a vector containing *METTL2* cDNA (lanes 3-4). Stars indicate bands for cDNA generated by polymerase bypass of unmodified position 32 in the tRNAs; the absence of a band indicates a polymerase-blocking modification at position 32. The marker lane (M) contains the single-stranded 56 and 72 nt DNA probes. (**E**) LC-MS/MS analysis of relative m^3C contents comparing HEK293T WT, M2 KO transfected with empty vectors or vectors with M2 cDNA for 6 or 10 days, respectively. Data represent mean ± SD for N=3 with asterisks denoting significant differences by Student's t-test, * p <0.05, ** p <0.01 (**F**) An *in vitro* methyltransferase assay performed with RNA isolated from *METTL2* KO cells incubated with recombinant human METTL2B protein. Stars indicate cDNA generated by polymerase bypass of unmodified position 32 in the tRNAs. (**G**) An *in vitro* methyltransferase assay performed with RNA isolated from *METTL2* KO cells and either WT or G3A mutant METTL2B protein.

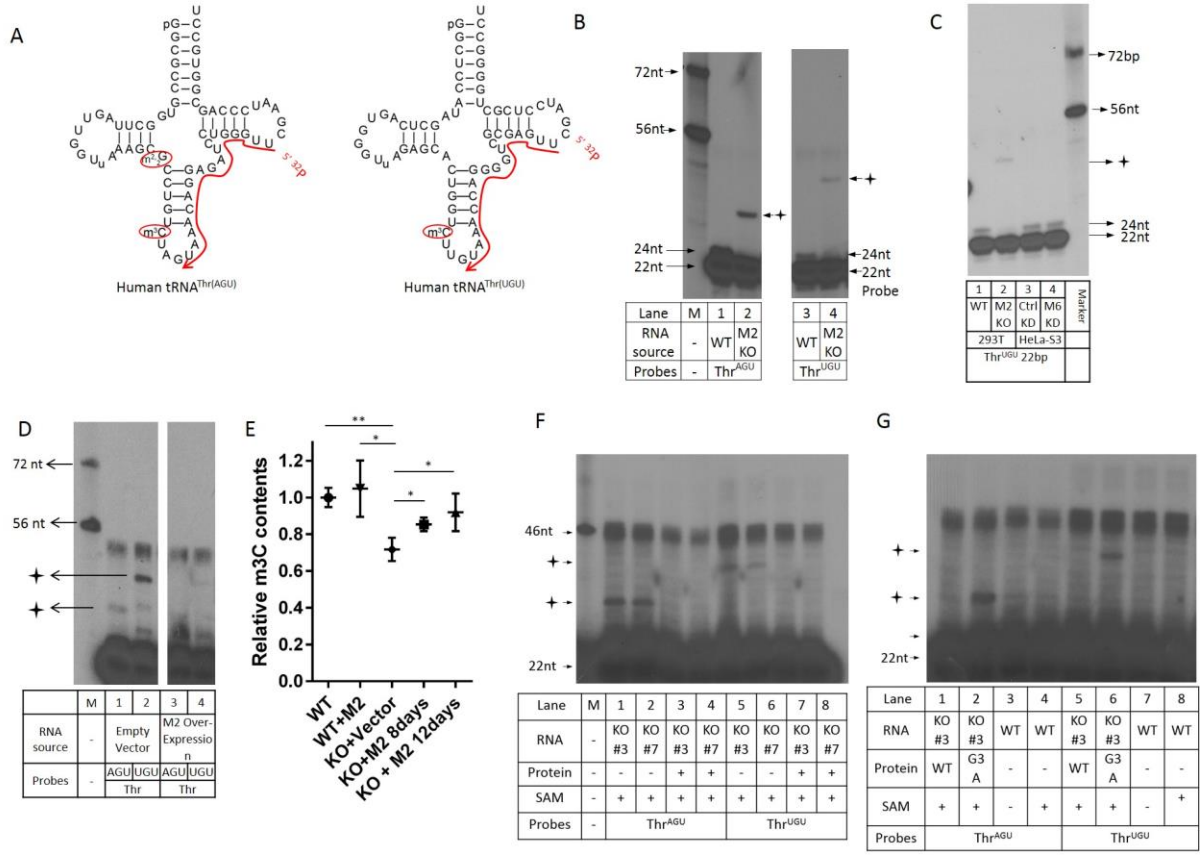


Figure S7. Sequence alignment of tRNA. Sequence alignment of mice threonine (**A**), serine (**B**) and arginine (**C**) tRNA isoacceptors. Sequences were obtained from the GtRNAdb database, aligned with Clustal O algorithm and visualize by Jalview. The annealing region of probes are highlighted in red dashed boxes. $tRNA^{Arg(UCG)}$ was not tested due to low sequence similarity.

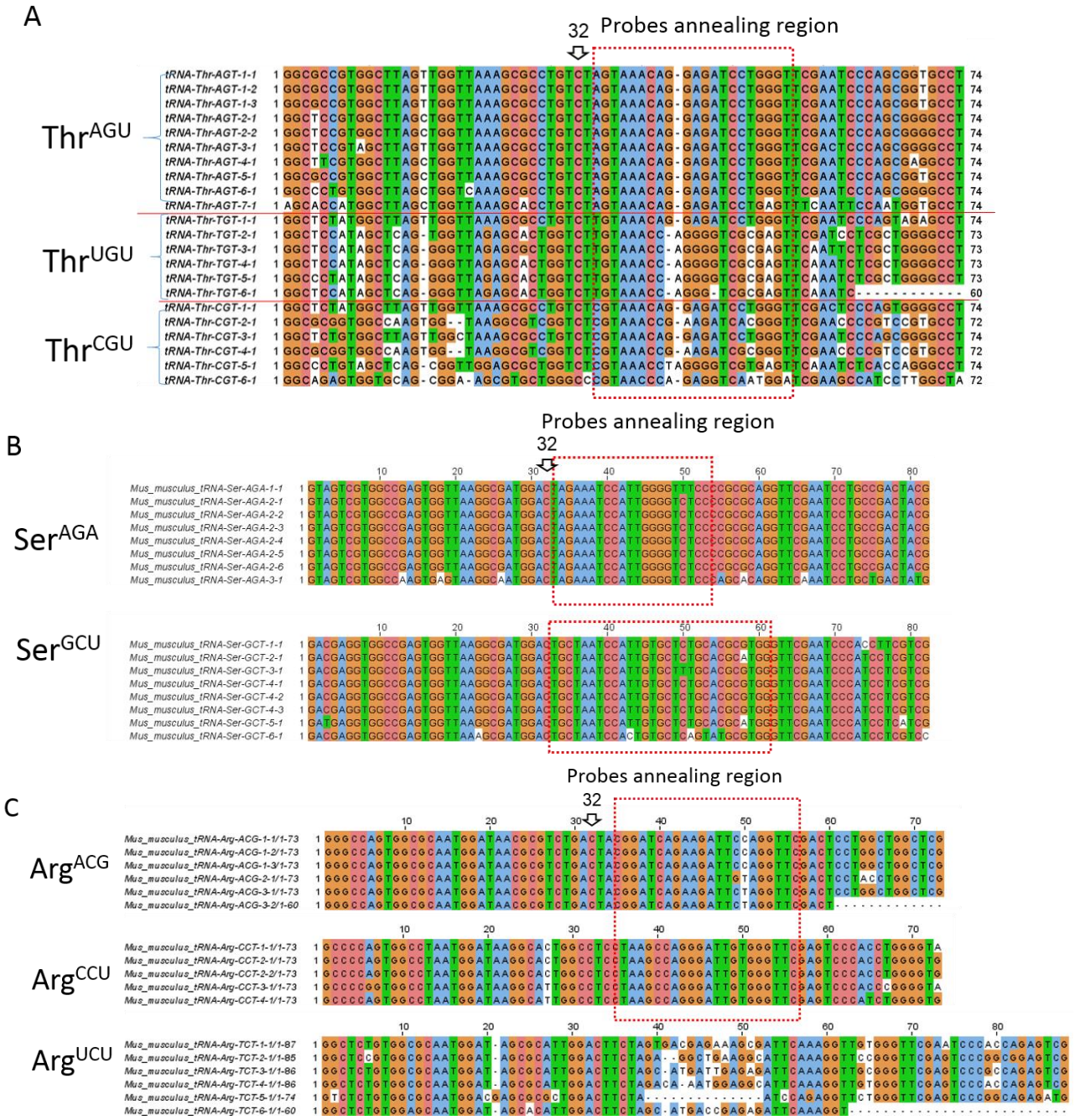


Figure S8. Primer extension analysis of mouse tRNAs. (**A**) Schematic showing mouse $tRNA^{Thr(AGU)}$ $tRNA^{Thr(CGU)}$ and $tRNA^{Thr(UGU)}$ isoacceptors and the probe design. (**B**) Primer extension assay using RNA from WT and *Mettl2*, *6* KO mice for 3 Thr tRNA isoacceptors. (**C,D**) Primer extension analysis of polymerase-blocking modifications at position 32 in $tRNA^{Ser(AGA)}$ and $tRNA^{Ser(GCU)}$ in liver tissue from WT, *Mettl2* KO (M2 KO), and *Mettl6* KO (M6 KO) mice. Stars indicate cDNA generated by polymerase bypass of unmodified position 32 in the tRNAs. (**E**) Primer extension assay for $tRNA^{Arg(ACG)}$ and $tRNA^{Arg(CCU)}$.

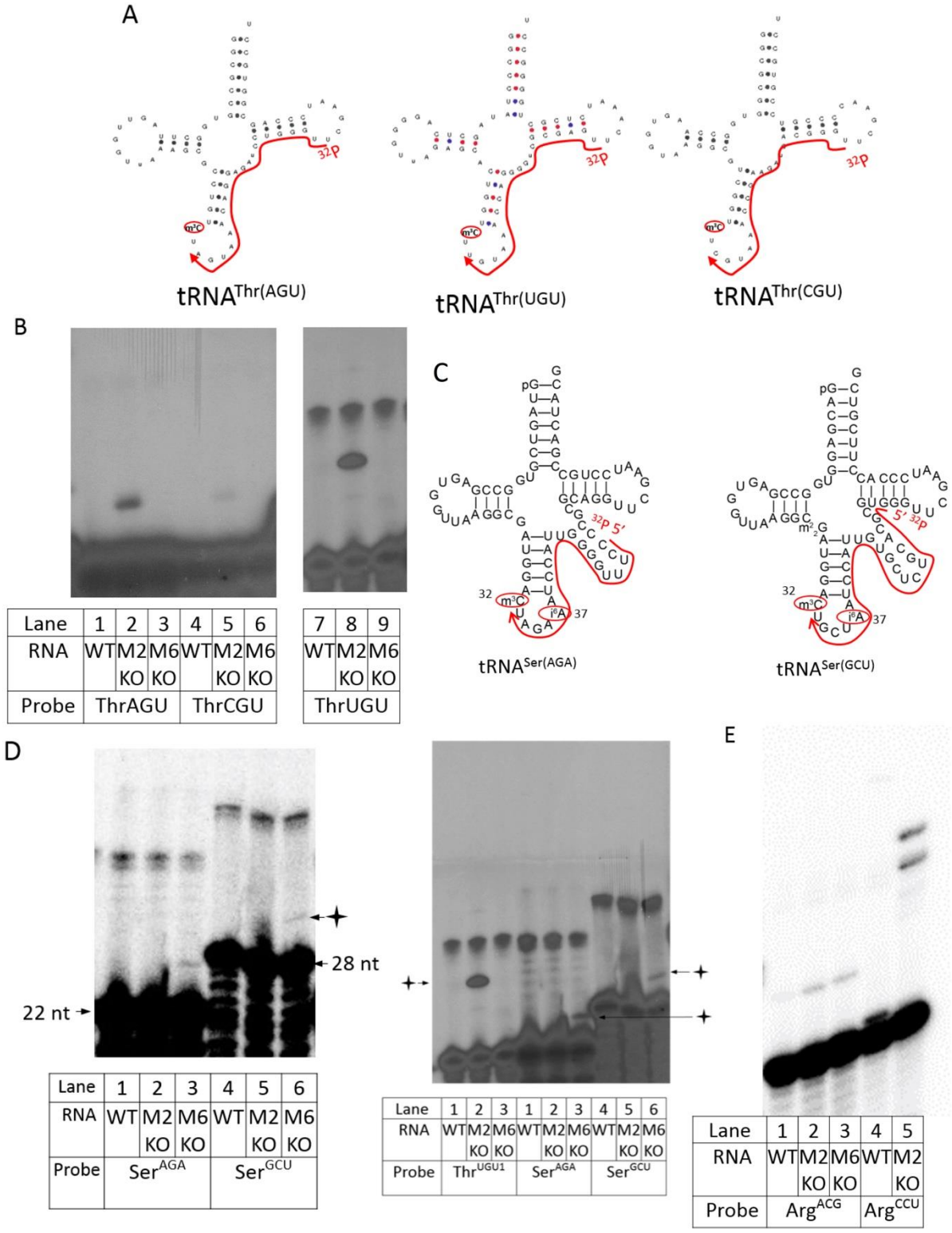
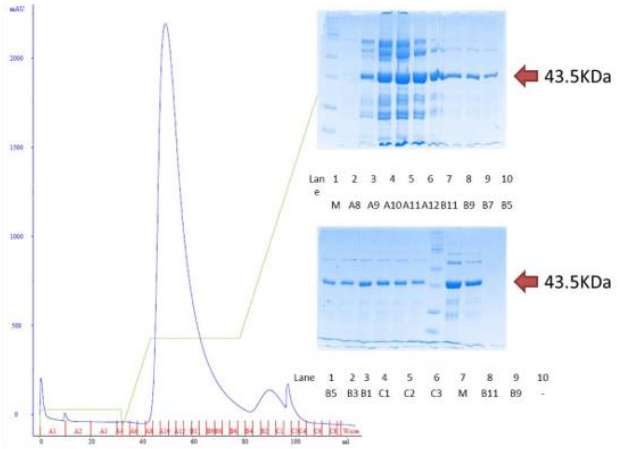


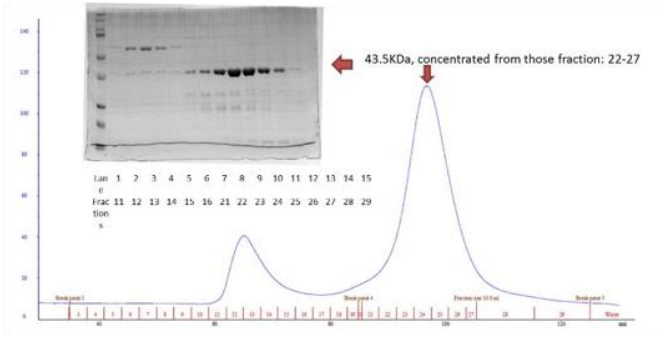
Figure S9. Purification of recombinant human METTL2B. (**A**) Nickel affinity purification of His-tagged human METTL2B. Blue: UV 280 nm absorbance. Selected fractions were resolved by SDS-PAGE and stained with Coomassie blue. (**B**) Size-exclusion purification of human METTL2B. Blue: UV 280nm absorbance. Selected fractions were resolved by SDS-PAGE and stained with Coomassie blue.

A



Affinity

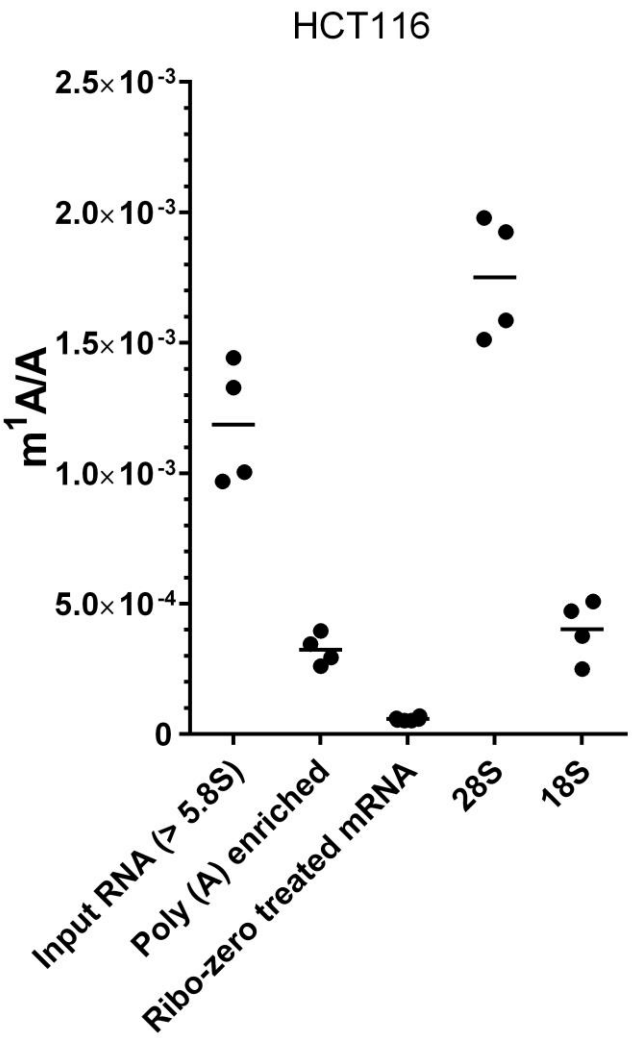
B



Gel filtration

Figure S10. Monitoring RNA modification level during mRNA purification in human HCT116 cell line. (A). m1A level during purification (B) No t^6A modification is observed in Rizo-zero treated mRNA. Top panel: t^6A elute at 22.08 min; lower panel: six samples

A



B

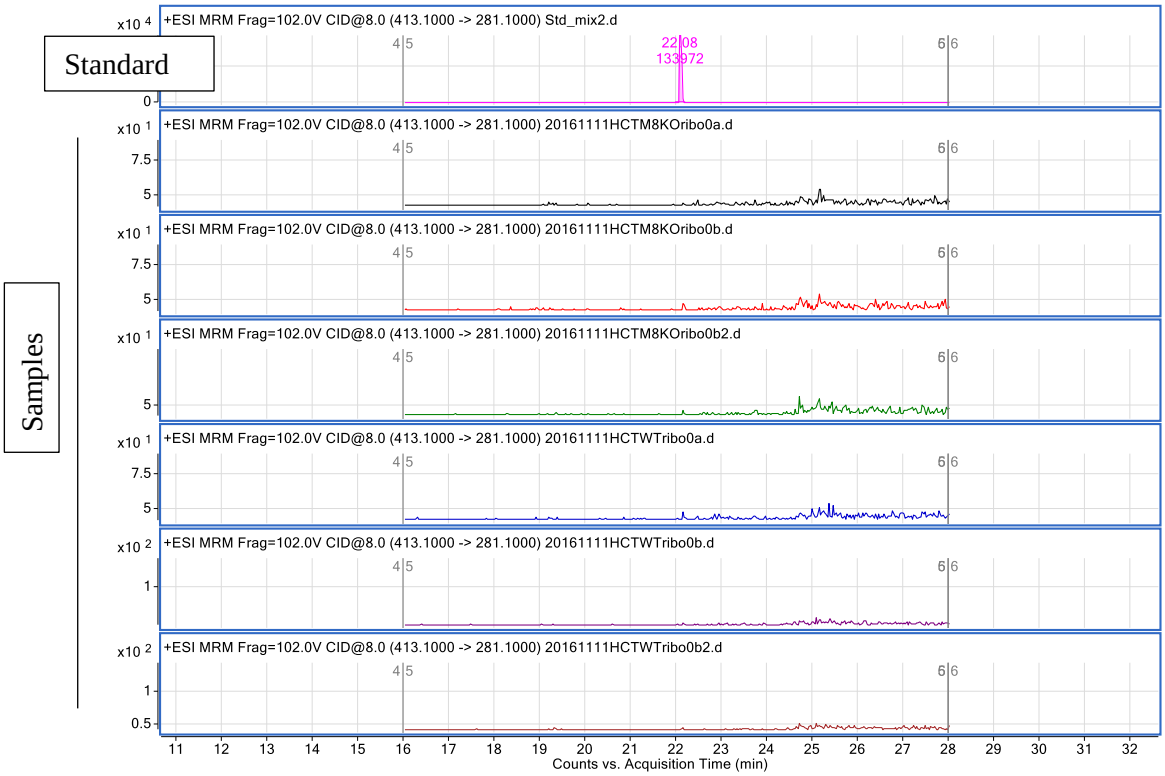


Figure S11. m^{3}C level in mRNA in HEK293T wildtype and *METTL2* KO

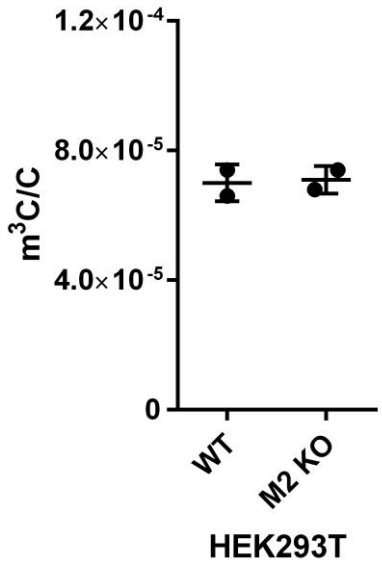


Figure S**12** MTS growth curve analysis for HEK293T WT and *METTL2* KO (**: p value < 0.01, *: p value < 0.05, student T test)

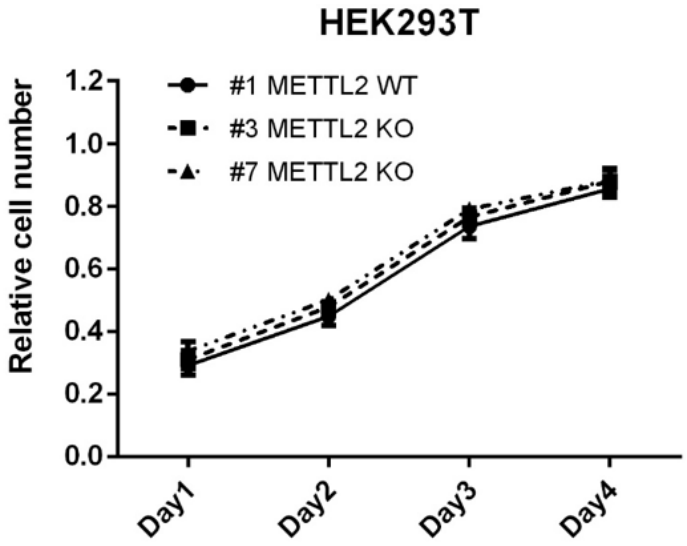


Figure S**13** MTS growth curve analysis for HEK293T WT and *METTL6* knocking-down (student T test)

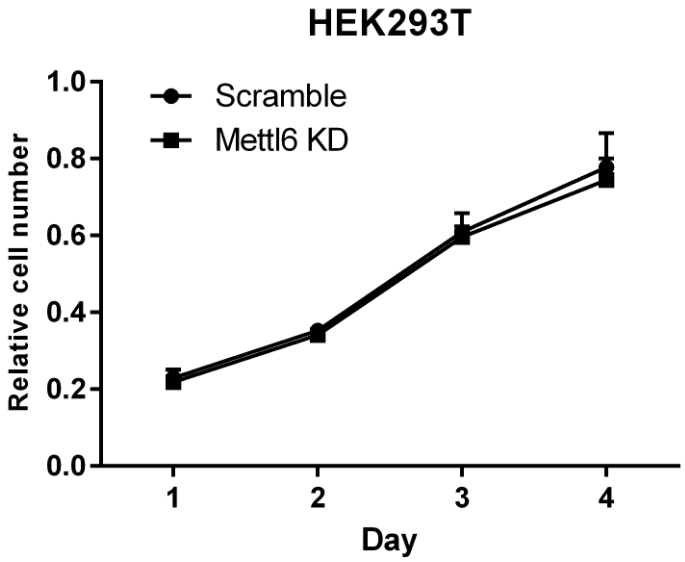
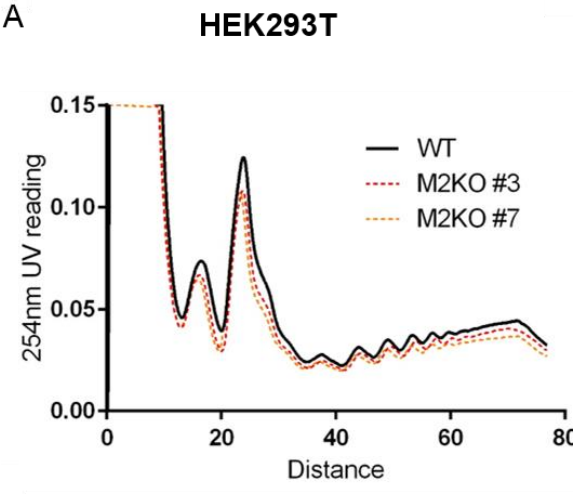
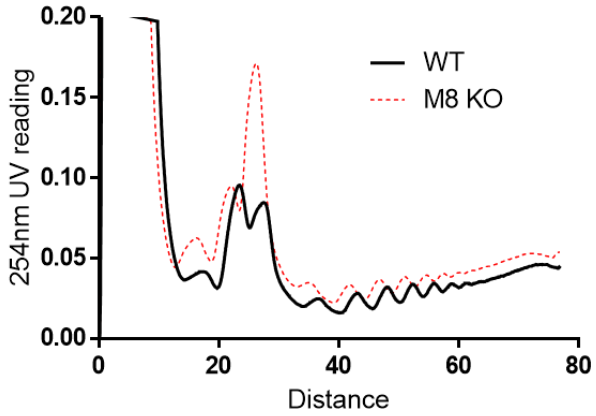


Figure S**14.** Polysome profiling comparing HEK293T WT and *METTL2* KO cell lines.



Supplementary Figure 15. Polysome profiling comparing HCT116 WT and *METTL8* KO cell lines.



Supplementary Tables

Table S1. Oligos for CRISPR/Cas9

Sequence Name	Sequence 5'-3'	Usage
U6 Mettl2 sgRNA3 up	ACCGGCACCTGAGTCTCACATCC	gRNA target mouse Mettl2
Mettl2 sgRNA For primer	CCAGTGGAGGTGTCCAGTTCTG	PCR primer for sequencing targeted loci
Mettl2 sgRNA Rev primer	CAAGGGTGACCATTCCACGCTG	PCR primer for sequencing targeted loci
U6 Mettl6 sgRNA2 up	ACCGTCTCTAGTGAGGTCACAC	gRNA target mouse Mettl6
U6/T7 Mettl6 sgRNA2 down	AAACGTGTGACCTCACTAGAGA	gRNA target mouse Mettl6
Mettl6 sgRNA2 For	CAAGTGACCATTCCGTGTCGCAG	PCR primer for sequencing targeted loci
Mettl6 sgRNA2 Rev	GATGCTCTGAAGAACAGCCACAG	PCR primer for sequencing targeted loci
U6 Mettl8 sgRNA2 up	AGTTTTGTCTCGCCAGAACC	gRNA target mouse Mettl8
Mettl8-E4 C9 For:	GCCTTTGCCTCTCAAATACTGGG	PCR primer for sequencing targeted loci
Mettl8-E4 C9 Rev:	CTTGACTGATTAAAGTCCCTGAAG	PCR primer for sequencing targeted loci
hMettl2 U6 F2	ACACCTGCACCTTCAGGGTAGGAGCG	gRNA target both METTL2A and METTL2B
hMettl2 U6 R2	AAAACGCTCCTACCCTGAAGGTGCAG	gRNA target both METTL2A and METTL2B

hMettl2_gRNAseq_F2	AGTCGGATCCGAGCGCCACCCGGACCAGACTC	Primers for seq both M2A M2B loci ,BamHI overhang
hMettl2_gRNAseq_R2	ACCGCTCGAGGTGATTACCAGGCATTGTGGTG	Primers for seq both M2A M2B loci ,XhoI overhang
hMettl8 U6 gRNA2	CTCAGCTGTGCGAGTCCTTC	gRNA target human Mettl8
hMettl8_gRNA6_F	GAAAGATGTGGTTCCTTGTCACC	PCR primer for sequencing targeted loci
hMettl8_gRNA6_R	GACTGGCATCAGGAGAGGCTAAG	PCR primer for sequencing targeted loci
hMettl6-shRNA	CCGGGATACAGAAAGATGCA	TRCN0000151394
hMettl6-shRNA2	CCGGGACCAAACTTTGGTGT	TRCN0000152989

Table S2. Oligos for primer extension assays for tRNAs from humans and mice

Probes	5' – 3'	Species
m3C-G35-Thr(AGU)	GAACCCAGGATCTCCTGTTTAC	human
m3C-G35-Thr(UGU)	GAACTCGCGACCCCTGGTTTAC	human
m3C-G35-Thr(UGU2)	GAACTCGCGACCCCTGGTTTAC	human
m3C-U39-Ser(CGA)	GAACCCGCACACCCGAAACGCGGCTCGACGGA	human
m3C-U39-Ser(UGA)	GAACCTGCGCGGGGAAACCCCAATGGA	human
m3C-U39-Ser(AGA)	GAACCTGCGCGGGGAGACCCCAATGGA	human
m3C-mito-Thr(AGU)	AAGGTTTTCATCTCCGGTTTAC	human
m3C-mito-Ser(GCT)	TAGACATGGGGGCATGAGTTAG	human
m3C-mito-Ser(TGA)	CGAACCCCCCAAAGCTGGTTTC	human
m3C-G35-Thr(CGU)	GATCCATTGACCTCTGGGTTAC	human
mThrAGU1	ACCCAGGATCTCCTGTTTACTA	Mouse
mThrUGU1	AACTCGCGACCCCTGGTTTACAA	Mouse
mSerAGA	GGAAACCCCAATGGATTTCTAG	Mouse
mSerGCU	CACGCGTGCAGAGCACAATGGATTAGCA	Mouse
mArgCCU	TCGAACCCACAaTCCCTGGCTTAG	Mouse
mArgACG	TCGAACCTGGAaTCTTCTGATCCG	Mouse

Table S3. Oligos for cDNA cloning for ectopic expression

Sequence Name	Sequence 5'-3'	Brief description
hM2_cDNA_F1	GTGAAGATCTGGCCGGCTCCTACCCTGAAGGTG	map to human *METTL2B* cDNA start with BglII overhang
hM2_cDNA_F3	CTTCGGTACCGCCACCATGGCCGGCTCCTACCCTGAAGGTG	map to h*METTL2A&B* cDNA start with kozak, ATG, KpnI in overhang
hM2_cDNA_R2	AGATAAGCGGCCGCcGCTGGTGCTGGACAGAAGGGGCT	map to human *METTL2B* cDNA end without stop codon, c added to avoid shift, NotI in overhang
hM2_cDNA_R4	AGATAAGCGGCCGCTCAGCTGGTGCTGGACAGAAGGGGCT	map to human *METTL2A* cDNA end with stop codon, (*METTL2B* has TAA as stop codon)NotI in overhang
hM8_cDNA_F2	ACGCTCGAGCCACCATGAATATGATTTGGAGAAATTCCATTTCTTGTCT	map to human Mettl8 cDNA start with overhang XhoI and Kozak sequence
hM8_cDNA_R2	TAGCACGCGTGTCTTGTGAAAGGAGTGTAGATACCATATTG	map to human Mettl8 cDNA end with overhang MluI

Table S4. Primers for mutagenesis

Sequence Name	Sequence 5'-3'
hMettl2_dSAM_F1	CTGGATCCTCAGCCACCTACCGAaacacagtctttccaattttacaaacgaacaatg
hMettl2_dSAM_R1	tcgtttgtaaaattggaaagactgtgttTCGGTAGGTGGCTGAGGATCCAGGAAACT
hMettl2_G3A_F	aacacagtctttccaattttacGcCTGTGcTGTGGcAcacctaccgaatactggaggtt
hMettl2_G3A_R	AACCTCCAGTATTCGGTAGGTGTGCCACAGCACAG GCGTAAAATTGGAAAGACTGTGTT
hMettl8_dSAM_F1	tagcaatgccactttcaggatAATAGTGTGTTTCCAATTTTGAACACT
hMett8_dSAM_R1	ttcaaaattggaaacacactatttccCCTGAAAGTGGCATTGCTACCAG

Table S5. Known or predicted m^3C sites in mammalian tRNAs

Position 32 m^3C		Position 47d m^3C	
Arg^{CCU}	METTL2	Leu^{CAG}	*
Arg^{UCU}	#	Ser^{AGA}	*
Ser^{AGA}	*(METTL6)	Ser^{CGA}	#
Ser^{CGA}	#	Ser^{UGA}	#
Ser^{UGA}	#	Ser^{GCU}	*
Ser^{GCU}	*(METTL6)		
Thr^{AGU}	*(METTL2)	Position 20	
Thr^{CGU}	*(METTL2)	Met-e	*
Thr^{UGU}	METTL2		

* Tested, either no conclusion or only read through product is observed in either Mettl6 or Mettl2 mutant cells; # not tested, either due to low number of copies of that isoacceptor or low sequence similarity among isodecoderss;

1. Zhang, D., Zhao, T., Ang, H. S., Chong, P., Saiki, R., Igarashi, K., Yang, H., and Vardy, L. A. (2012) AMD1 is essential for ESC self-renewal and is translationally down-regulated on differentiation to neural precursor cells. *Genes & development* **26**, 461-473