



# TRMT2A is a novel cell cycle regulator that suppresses cell proliferation

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## ABSTRACT

During the maturation of transfer RNA (tRNA), a variety of chemical modifications can be introduced at specific nucleotide positions post-transcriptionally. 5-Methyluridine ( $m^5U$ ) is one of the most common and conserved modifications from eubacteria to eukaryotes. Although TrmA protein in *Escherichia coli* and Trm2p protein in *Saccharomyces cerevisiae*, which are responsible for the 5-methylation of uracil at position 54 ( $m^5U54$ ) on tRNA, are well characterized, the biological function of the U54 methylation responsible enzyme in mammalian species remains largely unexplored. Here, we show that the mammalian tRNA methyltransferase 2 homolog A (TRMT2A) protein harbors an RNA recognition motif in the N-terminus and the conserved uracil-C5-methyltransferase domain of the TrmA family in the C-terminus. TRMT2A predominantly localizes to the nucleus in HeLa cells. TRMT2A-overexpressing cells display decreased cell proliferation and altered DNA content, while TRMT2A-deficient cells exhibit increased growth. Thus, our results reveal the inhibitory role of TRMT2A on cell proliferation and cell cycle control, providing evidence that TRMT2A is a candidate cell cycle regulator in mammals.

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## 1. Introduction

Transfer RNA (tRNA) is the key adaptor molecule responsible for the delivery of amino acids to the ribosome, where tRNA participates in the decoding of mRNA template into protein. Eukaryotic cytoplasmic tRNA is transcribed by RNA polymerase III as a precursor molecule in the nucleus. Functional maturation of pre-tRNA involves a series of processing events, including numerous post-transcriptional modifications. To date, more than 100 chemically distinct post-transcriptional modifications have been reported for

tRNAs [1]. These modifications play essential roles in the fidelity of translation, the folding and stability of tRNA [2–4] and the recognition of tRNA by the translation machinery [5].

One of the most abundant modifications found in tRNA is 5-methyluridine ( $m^5U$  or rT, ribothymidine). The ubiquitous presence of  $m^5U$  at position 54 in the T $\psi$ C stem-loop of eubacteria and in eukaryotic elongator tRNAs implies the pivotal role for this modification. In *Escherichia coli*, the enzyme that catalyzes the formation of  $m^5U54$  is TrmA [6], while in *Saccharomyces cerevisiae*, the enzyme responsible for this modification is Trm2p [7]. Both TrmA and Trm2p belong to the S-adenosyl-methionine (SAM)-dependent methyltransferase family, catalyzing methyl group transfer from SAM to the C5 of U54 of tRNA [7–9]. Previous studies have shown that the presence of  $m^5U54$  increases the fidelity and efficiency of protein synthesis by stabilizing the three-dimensional structure of tRNA *in vitro* [10,11]. In mammals, TRMT2A and TRMT2B are considered to be homologs of yeast Trm2p [12]. The

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predicted methyltransferase domain of TRMT2A shows a higher identity (36%) with that of Trm2p. Similar to Trm2p, TRMT2A harbors a putative RNA recognition motif (RRM) in the N-terminus. By contrast, the shared identity between TRMT2B and Trm2p is low (28%), and the TRMT2B protein does not have an identifiable RRM motif. This evidence indicates that TRMT2A is the likely mammalian homolog of Trm2p, for which the biological function remains largely unexplored.

Here, we investigated the effects of TRMT2A on the growth in mammalian culture systems. TRMT2A-overexpressing HeLa cells exhibit decreased cell growth and altered cell cycle profile, while Trm2a knock-out (KO) mouse embryonic fibroblasts (MEFs) show elevated cell growth. Our results provide evidence that TRMT2A exerts an inhibitory effect on cell proliferation and is a promising cell cycle regulator in mammals.

## 2. Materials and methods

### 2.1. Plasmids

The coding region of human *TRMT2A* was PCR-amplified from cDNA extracted from HeLa cells using the primers F 5'-CTGAATTCGATGAGTGAGAACCTCGACAAC-3' and R 5'-CGGGATCCCTAGGATGAGGGGAAGTCCCAG-3'. EcoRI and BamHI restriction sites were added to the primers to facilitate directional cloning into the pEGFP-c1 (BD Biosciences) vector for expression as an EGFP-tagged fusion protein. Sequences were verified by Sanger sequencing using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and were analyzed on a 3500 Genetic Analyzer sequencing machine (Applied Biosystems). Expression of GFP-TRMT2A in HeLa cells was confirmed by immunoblotting and immunofluorescence assay.

### 2.2. Transfection and generation of stable cell lines

HeLa cells and derived cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 8% fetal bovine serum (Sigma). To establish HeLa cells stably expressing GFP (GFP-HeLa) and GFP-TRMT2A (GFP-TRMT2A-HeLa), the cells were transfected with the indicated expression plasmids with Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco) according to the manufacturer's instructions and selected with G418 (Gibco) at a concentration of 1 mg/ml for 1 month. Positive clones with more than 95% GFP (clone 1) and GFP-TRMT2A (clone 1) expression verified by immunofluorescence assay and flow cytometry analysis were selected for further experiments.

### 2.3. Immunoblotting

Proteins from whole cell lysates prepared using NP-40 lysis buffer (1% NP-40, 50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) with complete protease inhibitor (Roche) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide gel) and were transferred to polyvinylidene difluoride (PVDF) membrane (Merck Millipore). After blocking, the primary antibodies used were TRMT2A (Santa Cruz, sc-86496) and  $\beta$ -actin (MBL, PM053-7). The immunoblots were developed using LAS-3000 (FujiFilm).

### 2.4. Immunofluorescence

Slide-cultured cells were harvested at 24 h post-plating. After air-drying, slides were fixed with 4% paraformaldehyde at room temperature for 20 min and washed with PBS for 5 min. Cells were permeabilized with 0.1% Triton X-100 for 5 min and subjected to

DNA staining with Hoechst 33342 (Molecular Probes) in Fluoromount medium (COSMO BIO). Slides were observed by Bioevo BZ-9000 fluorescence microscopy (Keyence), and images were acquired using BZ-II Analyzer software (Keyence).

### 2.5. Cell proliferation and survival

Cells were seeded in 24-well plates ( $2 \times 10^4$  per well) in triplicate. At 24, 48, 72, and 96 h post-plating, cells were trypsinized and stained with trypan blue. Live cells with positive trypan blue staining were counted using a hemocytometer. The cell survival rate was confirmed using a TC10 automated cell counter (Bio-Rad).

### 2.6. Clonogenic assay

Cells were seeded in 6-well plates (100 cells per well) and cultured for 14 days. Colonies formed were fixed with 100% cold methanol for 30 min and then stained with 0.5% crystal violet (in 20% methanol) for 1 h. After air-drying, colony numbers were counted. Plate efficiency (PE) reflected the ratio of the number of colonies to the number of cells originally seeded.

### 2.7. Cell cycle analysis

Cells were seeded in 6-cm plates at  $4 \times 10^5$  cells per dish. At 24 h after cell seeding, culture media were harvested to collect mitotic cells, which had rounded and were easily floating. Attached cells were trypsinized and resuspended in 1 ml of complete DMEM culture medium. DNA was stained with 5  $\mu$ M Vybrant DyeCycle Violet (Invitrogen) at 37 °C for 30 min. Cell cycle distribution according to DNA contents was determined by CytoFLEX (Beckman Coulter) and analyzed using CytoExpert software (Beckman Coulter).

### 2.8. Preparation of mouse embryonic fibroblasts (MEFs)

Primary mouse embryonic fibroblasts (MEFs) were prepared from 13.5-day-old (E13.5) embryos derived from WT or Trm2a KO C57BL/6j mice. Isolated embryos were dissected to remove the heads and internal organs. Tails were cut to isolate DNA for genotyping. Embryo trunks were minced and digested with 0.25% trypsin at 37 °C for 30 min. Cells were dispersed and applied to a 70- $\mu$ m cell strainer (BD Biosciences). After centrifugation, cells were resuspended in MEF medium (DMEM with 10% FBS, 200 mM L-Glutamine (Gibco), 100 U/ml penicillin/streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 0.1 mM MEM non-essential amino acids (Gibco) and 0.1 M 2-mercaptoethanol (Wako)) and were plated in a 10-cm dish precoated with 0.1% gelatin (Sigma).

### 2.9. Total RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA from MEFs was extracted with an RNA isolation kit (MACHEREY-NAGEL) and reverse transcribed to cDNA using a QuantiTect Reverse Transcription kit (Qiagen). Real-time PCR was performed using SYBR Green PCR master mix (TAKARA) in a Thermal Cycler Dice Real Time system (TAKARA). Experiments were conducted in duplicate, with two independent experiments performed. The primers used for TRMT2A detection were F 5'-GGCTCCGAAGGTGAAGAGAG-3' and R 5'-CCTGCCA-CAGTGGAACTCAA-3'. The primers used for HPRT, which served as a normalization control, were F 5'-TTGTGTGGATATGCCCTTGACTA-3' and R 5'-AGGCAGATGGCCACAGGACTA-3'.

2.10. Statistics

All data are presented as the means ± SEM. P-values were calculated using a two-tailed Welch's *t*-test. P-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. TRMT2A is conserved with TrmA protein

The human TRMT2A gene is located on chromosome 22q11.2 and is predicted to encode a 625-amino-acid protein with unidentified function. Amino acid sequence prediction based on NCBI conserved domain search [13] revealed that TRMT2A is a homolog of TrmA of *E. coli* and Trm2p of *S. cerevisiae*, containing an N-terminal RNA recognition motif (RRM) responsible for RNA binding and a C-terminal class I S-adenosyl methionine (SAM) methyltransferase domain conserved across the TrmA family (Fig. 1A). When aligned with TrmA and Trm2p, both human and mouse TRMT2A show the conserved SAM binding motifs and a strong homology between the C-terminal ends (Fig. 1B). The identity between human and mouse TRMT2A protein is 81%, implying the conserved function in mammals. In addition, sequence analysis using NLStradamus [14] revealed a nuclear localization signal (NLS) located after the RRM in Trm2p and in mouse and human TRMT2A proteins, suggesting the nuclear localization of the eukaryotic homologs (Fig. 1A).

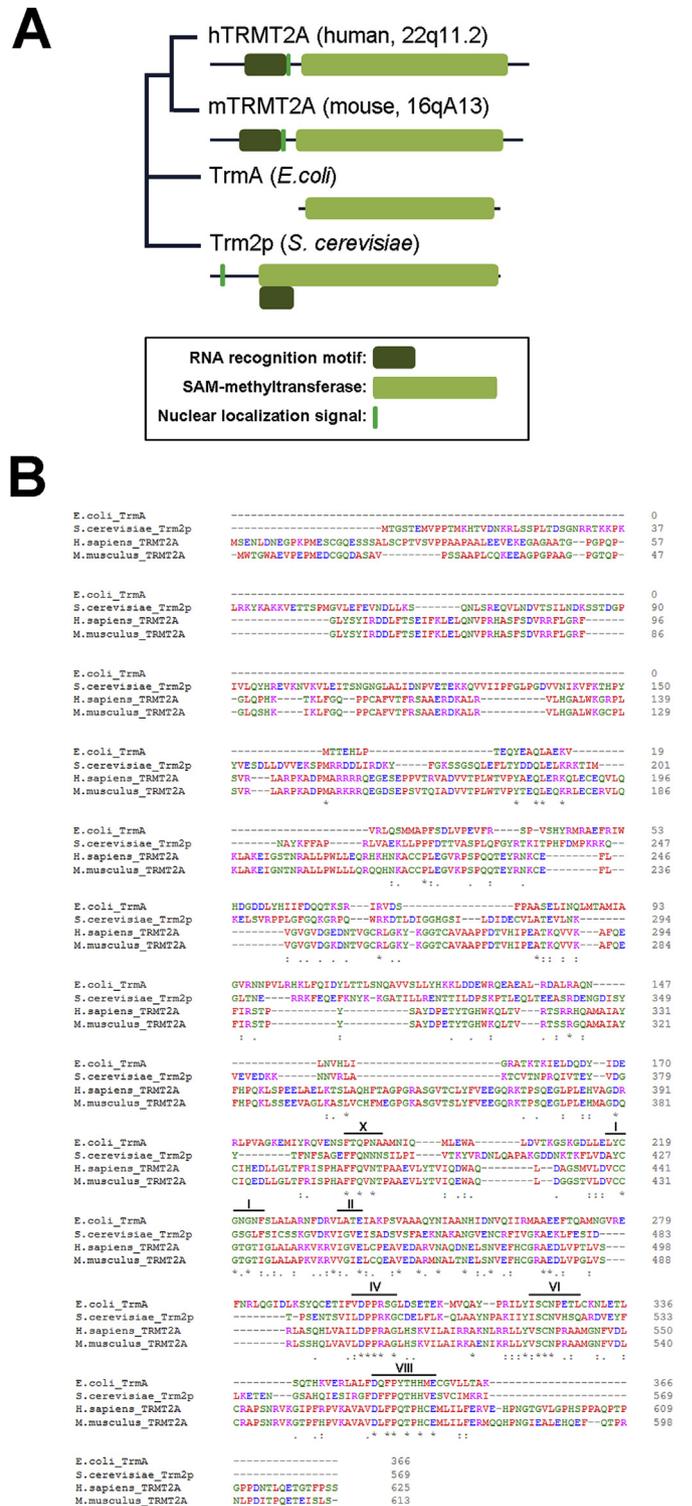
3.2. TRMT2A mainly localizes to the nucleus

To analyze the biological function of TRMT2A, an expression construct of N-terminal GFP-tagged TRMT2A was generated. The construct was transfected into HeLa cells, and GFP-TRMT2A-HeLa cells were generated to elucidate the effects of increased TRMT2A expression on cell properties. GFP-HeLa cells stably expressing GFP vector were also established as a control. The expression of GFP and GFP-TRMT2A was confirmed by immunoblotting using TRMT2A antibody (Fig. 2A) and immunofluorescence (Fig. 2B).

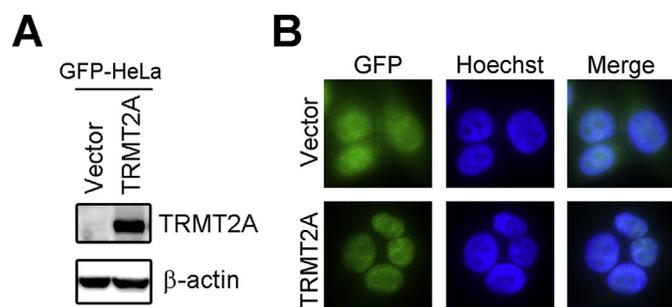
In *S. cerevisiae*, tRNA U54 methylation was reported to be an early event in tRNA processing because unspliced pre-tRNA has been shown to carry the m<sup>5</sup>U nucleoside [15,16]. In vertebrates, the intron-containing pre-tRNA is restricted to the nucleus [17], implying that TRMT2A resides and catalyzes the m<sup>5</sup>U modification in the nucleus. The subcellular localization of TRMT2A in HeLa stable cells was confirmed by immunofluorescence assay (Fig. 2B). TRMT2A exhibited a homogeneous pattern in the nucleus by immunofluorescence microscopy, while the GFP control alone showed a diffuse cytosolic and nuclear localization. Accordingly, the existence of a putative NLS and the observed localization of TRMT2A indicate a role in pre-tRNA methylation in the nucleus.

3.3. TRMT2A exerts an inhibitory effect on cell growth

A previous study revealed that the transcriptional kinetics of mouse TRMT2A gene oscillate during the progression of the cell cycle, peaking in S-phase and decreasing during mitosis [18]. This is a typical expression pattern for many known cell cycle regulators, such as MCM and Cdk1, implying that TRMT2A might be a novel cell cycle-regulating protein. To examine whether TRMT2A influences cell growth, cell proliferation was monitored in GFP-TRMT2A-HeLa cells. Ectopic expression of TRMT2A significantly retarded cellular proliferation (Fig. 3A). The decrease in cell number was not due to the increase in cell death because viability was confirmed to be



**Fig. 1. Predicted domains and sequence alignment of TRMT2A and its homologs.** (A) Annotated phylogenetic tree and predicted domains of human TRMT2A and its homologs from *E. coli*, *S. cerevisiae* and mouse. (B) Amino acid sequence alignment of TRMT2A and its homologs. The conserved motifs of the SAM binding site within the methyltransferase domain are noted over the sequence alignment. The gene tree and sequence alignment were predicted and constructed using the online program Clustal Omega.



**Fig. 2. Subcellular localization of TRMT2A.** (A) GFP-HeLa and GFP-TRMT2A-HeLa were seeded and harvested at 24 h post-plating. The protein expression levels were resolved by 10% SDS-PAGE.  $\beta$ -actin served as a loading control. (B) Individual cell expression of GFP and GFP-TRMT2A in stable cell lines was confirmed by immunofluorescence. DNA was stained with Hoechst 33342.

more than 95% by trypan blue staining in both cell lines (Fig. 3B). To verify the inhibitory influence of TRMT2A on cell growth over a longer time period and to monitor the growth capacity at the single cell level, a clonogenic assay was performed. A plating efficiency of approximately 80% was observed in GFP-HeLa control cells, while only 60% of plated GFP-TRMT2A-HeLa cells formed colonies after 14 days of culturing (Fig. 3C). In addition, colonies of GFP-TRMT2A-HeLa cells displayed a marked reduction in size (Fig. 3D), implying that the increased expression of TRMT2A is adverse to cell proliferation.

#### 3.4. Overexpression of TRMT2A leads to enrichment of the G2/M phase population

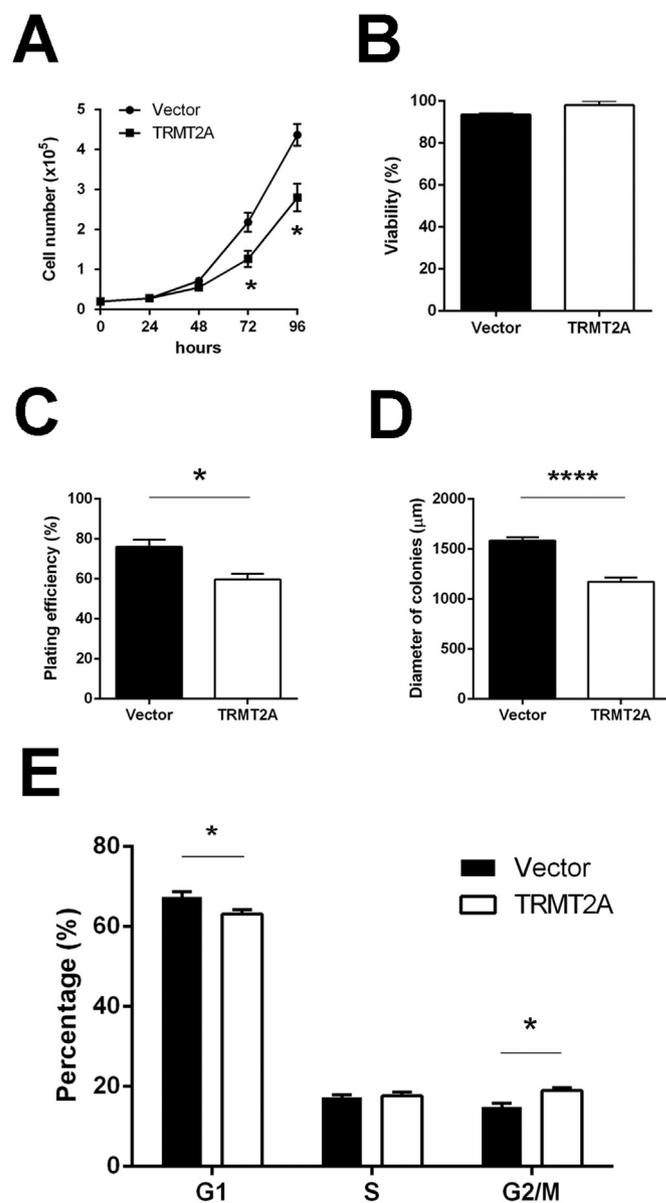
Reduced proliferation might be due to the perturbation of the cell cycle. To examine whether overexpression of TRMT2A in HeLa cells causes alterations in the cell cycle, the cellular DNA content was quantified by flow cytometry (Fig. 3E). The results showed that the G1 population decreased while the G2/M population increased in GFP-TRMT2A-HeLa cells, indicating the slower mitosis upon ectopic expression of TRMT2A.

#### 3.5. *Trmt2a* KO MEFs exhibit increased cell proliferation

To confirm the influence of TRMT2A on cell proliferation, mouse embryonic fibroblasts (MEFs) were generated from *Trmt2a* knock-out (KO) mice established in our laboratory. The KO of *Trmt2a* was confirmed at the transcript (Fig. 4A) and protein (Fig. 4B) levels by RT-qPCR and immunoblotting, respectively. Contrary to the effects of overexpression observed in GFP-TRMT2A-HeLa cells, *Trmt2a* KO MEFs exhibited significantly increased cell growth compared with the WT control (Fig. 4C). Taken together, these results suggest that TRMT2A negatively regulates cell proliferation in mammalian cells.

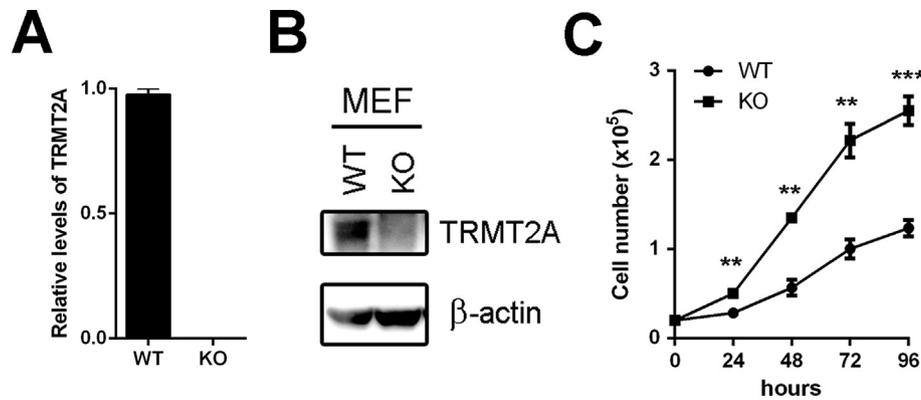
## 4. Discussion

Post-transcriptional modifications of tRNA occur across all kingdoms of life. These modifications have been shown to play essential roles in the structural stability of tRNA and the functional regulation of translation. The 5-methyluridine is one of the most common and conserved modified nucleosides from most eubacteria to eukaryotes in the majority of tRNA species. Despite intensive efforts to understand the roles of  $m^5U$  and the corresponding methyltransferases in *E. coli* and *S. cerevisiae*, it is still difficult to define the biological functions due to the variation in reported phenotypes. For example, an *E. coli* point mutant strain completely



**Fig. 3. TRMT2A overexpression in HeLa cells results in reduced cell proliferation and an altered cell cycle profile.** (A) (B) GFP-HeLa and GFP-TRMT2A-HeLa cells were seeded into 24-well plates in triplicate, and cell numbers were counted at 24, 48, 72, and 96 h in culture. One representative result is shown from three independent experiments. Cell viability was examined by using the trypan blue exclusion method. (C) (D) GFP-HeLa and GFP-TRMT2A-HeLa cells were seeded into 6-well plates in triplicate. After 14 days of culture, the number of colonies formed was counted, and the size of randomly selected colonies ( $n > 30$ ) from each cell was measured by using BZ-II Analyzer software. One representative result is shown from four independent experiments. (E) Asynchronous GFP-HeLa and GFP-TRMT2A-HeLa cells were seeded. At 24 h post-plating, cells were harvested and stained with Vybrant DyeCycle Violet. The cell cycle profiles were analyzed using flow cytometry. Percentages of the cell cycle profiles of four independent experiments are shown. \* $p < 0.05$ ; \*\*\*\* $p < 0.001$ . Differences were compared with the GFP control.

lacking  $m^5U54$  only showed a mild reduction in growth, while strains with disrupting insertions in relatively early *trmA* genes are not viable, suggesting that the TrmA protein possesses other crucial functions in addition to methyltransferase activity [19,20]. In *S. cerevisiae*, mutation or deletion strains of *Trm2* are viable and exhibit no obvious phenotype [7,21]. This is not to say that the physiological role and functional significance of this methylation and the responsible enzymes in mammals have remained



**Fig. 4. Trmt2a KO MEFs exhibit increased cell proliferation.** Trmt2a KO mouse embryonic fibroblasts (MEFs) were derived from KO mice. The knock-out efficiency was confirmed at the (A) transcript and (B) protein levels. (C) WT and Trmt2a KO MEFs were seeded into 24-well plates in triplicate, and cell numbers were counted at 24, 48, 72, and 96 h in culture. One representative result is shown from two independent experiments. \*\* $p < 0.01$ ; \*\*\* $p < 0.005$ . Differences were compared with the WT control.

unverified. Here, we showed that TRMT2A is the putative homolog of the TrmA family and is a promising cell cycle-regulating protein. Overexpression of TRMT2A leads to reduced cell proliferation and an increase in the G2/M population, implying that increased TRMT2A expression is disadvantageous to cell growth and cell cycle progression. The inhibitory effect of TRMT2A on cell proliferation is confirmed by the observation in which Trmt2a KO MEFs exhibit faster cell growth than WT cells. Together, these results provide evidence for the conserved function of mammalian TRMT2A in cell proliferation. In addition, we demonstrated that TRMT2A genes in mammals are dispensable for survival since KO cells were viable.

Although the m<sup>5</sup>U54 is a universal modification in *E. coli*, the m<sup>5</sup>U54 contents in eukaryotic tRNA vary. In mammals, including humans, many tRNAs have been reported to lack the 5-methyluridine at position 54, showing only approximately 50% of modified uridine in total tRNA [22,23]. For example, in the initiator tRNA<sup>Met</sup>, 5-methyluridine is completely replaced by adenosine, while in tRNA<sup>Val</sup> from mouse myeloma, unmethylated uridine is present instead of methylated uridine [24,25]. In some other tRNAs, including tRNA<sup>Phe</sup>, partial conversion of uridine to methyluridine has been revealed [26,27]. We also observed incomplete 5-methyluridine modification because the m<sup>5</sup>U levels in HeLa cells are much less methylated than control tRNAs extracted from *E. coli* by tRNA hydrophilic interaction liquid chromatography (HILIC) analysis (methods modified from Ref. [28], data not shown). It is interesting that partially methylated uridine in tRNAs purified from human placenta and rat liver can be fully modified *in vitro* by an excess amount of TrmA of *E. coli* [29]. Hence, we hypothesized that overexpression of TRMT2A in HeLa cells might lead to increased levels of 5-methyluridine. However, the m<sup>5</sup>U levels in the entire pool of tRNAs extracted from GFP-TRMT2A-HeLa cells were methylated and comparable with those of GFP control cells, as demonstrated by tRNA HILIC analysis (data not shown), implying that increased amounts of TRMT2A do not affect the homeostasis of m<sup>5</sup>U modification in HeLa cells. On the other hand, our unpublished results demonstrated that 5-methyluridine levels in tRNAs extracted from Trmt2a KO mice were completely lost. Hence, further experiments to elucidate the specific tRNA targets of TRMT2A in mammalian cells will be required to understand the significance of 5-methyluridine and TRMT2A.

In the past few decades, observation of the elevated levels of tRNA methyltransferases in cancer cells led to the hypothesis that overmethylation of tRNA is associated with carcinogenesis [30]. However, the roles and mechanisms behind these connections remain to be explored. A study analyzing tRNA base composition from Morris hepatomas and rat liver revealed less 5-methyluridine

in hepatomas than in normal liver tissue. However, the comparison of tRNAs from two tumors could not provide evidence for a distinct correlation between degrees of tRNA modification and growth rates and the histological characteristics of the tumors [31]. Another study profiling modified nucleosides showed elevated m<sup>5</sup>U54 levels in the cell culture supernatants in MCF-7 breast cancer cells compared with nontumorigenic MCF-10A mammary epithelial cells [32]. One of the reasons for the differences observed between these experiments may result from the origins of cancer and the cellular compartments where tRNA was extracted. Recent clinical screenings have suggested overexpression of TRMT2A as a novel biomarker in HER2<sup>+</sup> breast cancer patients, with a higher risk of recurrence [33]. Of note, the TRMT2A expression pattern reported is a cytoplasmic form. Since the U54 methylation was reported to occur in the nucleus, and we observed a uniform nuclear localization of GFP-tagged TRMT2A, it is possible that cytoplasmic expression of TRMT2A is an aberrant form and perturbs the HER2 signaling pathway independent of its methyltransferase activity.

Taken together, systematic analysis and comprehensive mapping of individual tRNA species and proteins altered by aberrant m<sup>5</sup>U54 and TRMT2A are required. Understanding the role of TRMT2A-catalyzed m<sup>5</sup>U54 will uncover the target proteins and the underlying signaling pathways of TRMT2A in both physiological and pathological conditions.

#### Conflicts of interest

The authors declare no competing financial interests.

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