Cdk5rap1-mediated 2-methylthio-\(N^6\)-isopentenyladenosine modification is absent from nuclear-derived RNA species

Md. Fakruddin1, Fan Yan Wei1,2, Shohei Emura1, Shigeru Matsuda3, Takehiro Yasukawa3, Dongchon Kang3 and Kazuhito Tomizawa1,*

1Department of Molecular Physiology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan, 2Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan and 3Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

2-Methylthio-\(N^6\)-isopentenyl modification of adenosine (ms\(^2\)i6A) is an evolutionally conserved modification that is found in transfer RNAs (tRNAs). We have recently shown that Cdk5 regulatory subunit-associated protein 1 (Cdk5rap1) specifically converts i6A to ms\(^2\)i6A at position A37 of four mitochondrial DNA-encoded tRNAs, and that the modification regulates efficient mitochondrial translation and energy metabolism in mammals. Curiously, a previous study reported that ms\(^2\)i6A is present abundantly in nuclear-derived RNA species such as microRNAs, but not in tRNA fractions. To fully understand the molecular property of ms\(^2\)i6A, the existence of non-canonical ms\(^2\)i6A must be carefully validated. In the present study, we examined ms\(^2\)i6A in total RNA purified from human and murine \(H_929\) cells, in which mitochondrial DNA-derived tRNAs were completely depleted. The ms\(^2\)i6A was not detected in these cells at all. We generated a monoclonal antibody against ms\(^2\)i6A and examined ms\(^2\)i6A in murine RNAs using the antibody. The anti-ms\(^2\)i6A antibody only reacted with the tRNA fractions and not in other RNA species. Furthermore, immunocytochemistry analysis using the antibody showed the predominant localization of ms\(^2\)i6A in mitochondria and co-localization with the mitochondrial elongation factor Tu. Taken together, we propose that ms\(^2\)i6A is a mitochondrial tRNA-specific modification and is absent from nuclear-encoded RNA species.

INTRODUCTION

In all organisms, transfer RNA (tRNA) undergoes various post-transcriptional modifications (1). To date, more than 100 species of tRNA modifications have been reported in all three domains of life (2). Most modifications have been found in bases near the anticodon region, particularly at positions 34 and 37 (3). These modifications facilitate correct codon-anticodon base-pairing, thus promoting efficient protein translation (3).

2-Methylthioation modification is an evolutionally conserved modification found across species (2). In mammalian cells, there are two forms of 2-methylthiolation: 2-methylthio-\(N^6\)-threonylcarbamoyladenine (ms\(^2\)t6A) and 2-methylthio-\(N^6\)-isopentenyladenosine (ms\(^2\)i6A) (2). Cdk5 regulatory subunit associated protein 1-like 1 (Cdkal1) converts t6A to ms\(^2\)t6A at position A37 of cytosolic tRNA\(_{\text{Lys(UUU)}}\) in mammalian cells, with a profound impact on both molecular and physiological functions (4,5). A deficiency of ms\(^2\)t6A impairs the accurate translation of the Lys codons, resulting in the production of aberrant proinsulin and the induction of aberrant glucose metabolism (5). Importantly, genetic variants of \(CDKAL1\) have been associated with the development of type 2 diabetes in humans (6). Individuals carrying risk \(CDKAL1\) mutations exhibit a reduction of ms\(^2\)t6A modification levels, which is associated with a decrease in insulin secretion (7–9).

Cdk5 regulatory subunit-associated protein 1 (Cdk5rap1) is a homolog of Cdkal1 in mammalian cells (4). Cdk5rap1 contains a mitochondria-targeting sequence at the N terminus that guides the enzyme to the inner membrane of mitochondria (10). Cdk5rap1 converts i6A37 to ms\(^2\)i6A37 in four mitochondrial DNA-encoded tRNAs, mt-tRNA\(_{\text{Trp}}\), mt-tRNA\(_{\text{Tyr}}\), mt-tRNA\(_{\text{Phe}}\) and mt-tRNA\(_{\text{Ser(UCN)}}\) (10). Similar to ms\(^2\)t6A, ms\(^2\)i6A is important...
for efficient and accurate translation in mitochondria. In Cdk5rap1-null mice, the absence of ms²i⁶A decreases the translation of mitochondrial DNA-derived respiratory subunits and impairs electron transport and aerobic respiration (10). Consequently, the cardiac function and skeletal muscle function of Cdk5rap1-null mice are significantly impaired due to insufficient energy metabolism. Importantl, the ms²i⁶A levels in these mt-tRNAs are substantially decreased in patients with mitochondrial disease. These results strongly suggested that ms²i⁶A modification of mt-tRNAs is crucial for the mitochondrial translation and that the disruption of ms²i⁶A modification is a key element of the molecular pathogenesis of the mitochondrial diseases.

While our results have clearly shown that Cdk5rap1-mediated ms²i⁶A modification occurs in mitochondrial DNA-encoded mt-tRNAs (10), a previous study reported that ms²i⁶A might exist in nuclear-encoded RNA species (11). Reiter et al. fractionated the total RNA of HeLa cells into tRNA, small RNA, polya-RNA and ribosomal RNA (rRNA) fractions and examined ms²i⁶A modification using mass spectrometry. Surprisingly, the ms²i⁶A modification was almost absent from the tRNA fraction. The ms²i⁶A modification was rather highly enriched in the miRNA and the poly-A RNA fractions. The authors hypothesized that the non-canonical ms²i⁶A modification in nuclear-encoded RNA species might be catalyzed by a splicing variant of CDK5RAP1 that lacks the mitochondria-targeting sequence. These findings challenged the current understanding of ms²i⁶A modification of the exclusive occurrence of ms²i⁶A modification in mt-tRNAs, and raised a possibility that the ms²i⁶A modification might control cellular functions through nuclear-encoded RNA species instead of mitochondrial tRNAs.

Does ms²i⁶A exist in nuclear-encoded RNA species? To answer this question, it is necessary to examine the presence of ms²i⁶A in such RNAs using carefully designed experimental approaches. It should be noted that, in growing cells, ∼80–90% of total RNA is tRNA, and 10–15% is tRNA (12). By contrast, mRNA constitutes 3–7% of total RNA, and miRNA constitutes only 0.003–0.02% of total RNA (12). Therefore, the biochemical purification of miRNA or mRNA without the contamination of mitochondrial RNA (rRNA) fractions and examined ms²i⁶A modification using mass spectrometry. Surprisingly, the ms²i⁶A modification was almost absent from the tRNA fraction. The ms²i⁶A modification was rather highly enriched in the miRNA and the poly-A RNA fractions. The authors hypothesized that the non-canonical ms²i⁶A modification in nuclear-encoded RNA species might be catalyzed by a splicing variant of CDK5RAP1 that lacks the mitochondria-targeting sequence. These findings challenged the current understanding of ms²i⁶A modification of the exclusive occurrence of ms²i⁶A modification in mt-tRNAs, and raised a possibility that the ms²i⁶A modification might control cellular functions through nuclear-encoded RNA species instead of mitochondrial tRNAs.

In the present study, we carefully investigated the presence of ms²i⁶A modification in nuclear-encoded RNA species using cell biological approaches. We provide evidence that the ms²i⁶A modification does not exist in nuclear-encoded RNA species.

**MATERIALS AND METHODS**

**Animals**

Cdk5rap1 knockout (KO) mice were generated and maintained as described previously (10). Littermates of wild-type (WT) and KO mice (8–12 weeks old) were used for experiments unless otherwise specified. Animals were housed at 25°C with 12-h light and 12-h dark cycles. All the animal procedures were approved by the Animal Ethics Committee of Kumamoto University, Japan (Approval ID: A29–016-163).

**Cell culture**

HeLa cells and B82 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. HeLa cells and B82 cells devoid of endogenous mitochondrial genomes (HeLa ρ0 cells and B82 ρ0 cells) were kindly provided by Dr Kazuto Nakata (Tsukuba University). HeLa ρ0 cells and B82 ρ0 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS, pyruvic acid (Invitrogen, final concentration 10 μM) and uridine (Sigma, final concentration 100 μg/m). Hybridoma cells that produce ms²i⁶A antibody were cultured in GIT medium (Wako) at 37°C and 5% CO₂.

**RNA purification**

Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer’s instructions. mRNA was purified using Oligotex-dT(30) mRNA purification kit (TAKARA) following the manufacturer’s instruction. The eluted mRNA was further subjected to selection for large size (> 200 nt) RNA using RNA Clean & Concentrator (Zymo Research). Size selection of mRNA was repeated twice in order to achieve maximum elimination of small RNA contamination.

**Gene expression analysis**

First-strand cDNA synthesis from total RNA was performed using the PrimeScript RT reagent Kit (TAKARA). Real-time polymerase chain reaction (PCR) quantitative analysis was performed using SYBR premix Taq (TAKARA) and the 7300 Real-Time PCR System (Applied Biosystems) following the manufacturer’s instructions. For total RNA from HeLa ρ0 cells and B82 ρ0 cells, cDNA was synthesized using the Transcriptor First-Stand cDNA Synthesis Kit (Roche Diagnostics) with reverse primer targeting mt-tRNA⁶Phe, followed by quantitative PCR using forward and reverse primers as following:

mouse mt-tRNA⁶Phe:
forward: 5′-GCTTAATAAACAAAGCAAGCAGCA
reverse: 5′-TATCCATTCAAGCATTTCATA

human mt-tRNA⁶Phe
forward: 5′-CTCCTCAAAAGCAATACACTG
reverse: 5′-AGCCCGTCTAAACATTTTCA

mouse mt-tRNA⁶Ser(UCN)
forward: 5′- CATATAGGATATGAGATTGGC
reverse: 5′- AACCCCCTTAAATTTGGTTTCA

**Modification analysis by mass spectrometry**

Twenty microliters of total RNA isolated from HeLa cells, B82 cells, HeLa ρ0 cells and B82 ρ0 cells were mixed with 1.5 μl of P1 Nuclease (WAKO, 1 μl) of alkaline phosphatase (TAKARA) and 2.5 μl of 200 mM HEPEs (pH 7.0), and the mixture was incubated at 37°C for 3 h to completely
digest RNA. The digestion products were separated on a C18 reverse phase column (GL Science), and i6A, ms2i6A and adenosine (A) were measured using a mass spectrometer (Agilent 6460) as described previously (13).

**Purification of ms2i6A antibody**

Synthetic ms2i6A was used to generate monoclonal antibody (ITM Co., Ltd. Japan). A hybridoma clone was established using a standard method described elsewhere. Hybridoma cells secreting the ms2i6A antibody were cultured in 50-ml flasks until confluent. Thereafter, 10 ml of the culture supernatant was added to a Protein-G column (GE Healthcare), and the antibody was adsorbed onto the column. Thereafter, the antibody was eluted using an elution buffer contained in the MAbTrap Kit (GE Healthcare), crosslinked with ultraviolet light and washed (2 x 10 ml each) to an Amersham Hybond-N + membrane (GE Healthcare). Finally, the eluted antibody was added to a centrifugal filter (Amicon Ultra-15, Millipore), and buffer exchange and concentration determination were performed using phosphate-buffered saline (PBS), followed by storage at −80°C until use.

**ELISA assay**

Competitive ELISA was used to validate the specificity of the ms2i6A antibody. Briefly, a 96-well plate was coated with anti-mouse Fc (Sigma) at a final concentration of 5 µg/ml at 4°C overnight. The plate was washed with PBS three times and was used for ELISA immediately. Anti-ms2i6A was diluted (1:100) with a blocking solution (1% Block Ace, DS Pharma Biomedical), and 50 µl of the diluted antibody solution was added to the 96-well plate. Next, vehicle, ms2i6A, i6A or m6A was diluted with the blocking solution to 10 µg/ml, and 50 µl of the diluted compound was added to the 96-well plate. Finally, horseradish peroxidase-conjugated ms2i6A was diluted with the blocking solution and added to the plate. The plate was incubated at room temperature for 2 h, followed by washing with PBS containing 0.1% Tween 20. O-phenylenediamine dihydrochloride (Sigma) solution was added to each well for the reaction with HRP. The reaction was stopped with 1 M phosphoric acid solution. A plate reader (WAKO) was used for the absorbance at OD492 nm.

**Dot blotting**

Total RNA derived from WT mice and total RNA derived from Cdk5rap1 KO mice (1 µg/µl) were added dropwise (2 µl each) to an Amersham Hybond-N + membrane (GE Healthcare), crosslinked with ultraviolet light and washed with 0.05% PBST. Blocking was carried out using the blocking solution (1% Block Ace, DS Pharma Biomedical) for 1 h. Thereafter, ms2i6A antibody (0.05 mg/ml) was diluted with the blocking solution (1:200) and incubated with the membrane at 4°C overnight. The next day, after washing 3–4 times with PBST, the secondary antibody (anti-mouse HRP) (1:1000 dilution) was added and incubated with the membrane for 1 h. Finally, the ECL Prime (GE Healthcare) reagent was added, and imaging was performed using an ImageQuant 400 Transilluminator (GE Healthcare).

**Isolation of mitochondria**

Mitochondria were isolated from mouse liver as described previously (10). Briefly, the liver was gently homogenized in a homogenization buffer (225 mM mannitol, 75 mM sucrose, 10 mM HEPES-NaOH at pH 7.6, 2 mM ethylenediaminetetraacetic acid). Next, the supernatant (supernatant A) was recovered by centrifugation at 800 × g for 10 min at 4°C. The supernatant was centrifuged at 7500 × g for 10 min at 4°C to obtain the crude mitochondrial fraction. The crude mitochondria were overlaid on a discontinuous gradient consisting of 1.5 M and 1 M sucrose, followed by centrifugation at 15 700 rpm at 4°C for 60 min. The purified mitochondrial fraction was homogenized in TRIzol to extract mitochondria-derived total RNA.

**Northern blotting**

Total RNA was purified from mouse liver and mitochondria isolated from mouse liver with TRIzol (Invitrogen) and then separated on a 6% TBE-Urea gel (Invitrogen). RNA was visualized by staining with SYBR Gold (Invitrogen). The RNA was then transferred to an Amersham Hybond-N+ membrane (upward capillary transfer) using the conventional capillary transfer method described elsewhere. Next, anti-ms2i6A was added to the membrane and incubated at 4°C overnight. The ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used to visualize signals corresponding to ms2i6A.

**Fluorescent immunostaining**

HeLa cells (0.75 × 10⁵ cells/ml) were seeded in a glass-bottomed dish (IWAKI) and were cultured overnight. The following day, control siRNA (siControl) and siRNA against Cdk5rap1 (siCdk5rap1) were transfected and cultured for 2 days. Next, the cells were incubated with Mitotracker Red (Molecular Probe, final concentration: 100 pM) for 30 min and then were fixed with 4% paraformaldehyde (WAKO). Cells were washed with PBS and blocked with 4% bovine serum albumin. The ms2i6A antibody was added (1:100 dilution) and reacted overnight at 4°C. The next day, Alexa488-conjugated anti-mouse secondary antibody (1: 100 dilution, Molecular Probe) was further added to the cells. Images were observed with a confocal laser scanning microscope (Olympus, FV1000). To stain the mitochondrial protein translation machinery, TUFM antibody (Abcam, 1:200) was used.

**RESULTS**

**Absence of ms2i6A modification from human and murine Rho0 (ρ0) cells**

Rho0 cells (ρ0 cells) are biochemically engineered cells, in which mitochondrial DNA are depleted completely by chemical compounds. Thus the cells do not contain mitochondrial transcripts but the nuclear-derived RNA species remain intact (14). Examination of ρ0 cell-derived total RNA would clearly verify the presence of ms2i6A derived from nuclear-encoded RNA species, if it is indeed present...
in the RNA species, without the contamination of ms2t6A-containing mitochondrial DNA-derived tRNAs. We isolated total RNA from human-derived HeLa cells and HeLa ρ0 cells, as well as mouse-derived B82 cells and B82 ρ0 cells, and examined whether ms2t6A-containing mt-tRNA (mt-tRNA^{Phe}) was depleted in the ρ0 cells by quantitative PCR. As expected, the levels of ms2t6A-containing mt-tRNA^{Phe} in HeLa ρ0 cells and B82 ρ0 cells were below the detection limit compared with those in control cells (Figure 1A). In contrast to mt-tRNAs, the expression levels of nuclear-encoded CDK5rap1 were compatible between ρ0 cells and control cells (Figure 1B). Given the successful validation of ρ0 cells, we then subjected total RNA to mass spectrometry analysis to examine the presence of ms2i6A (Figure 1C and D). The ms2i6A was clearly absent from the total RNA of HeLa ρ0 cells and B82 ρ0 cells. As a control, we examined ms2i6A modification that was derived from nuclear-encoded cytosolic tRNA^{Lys(UUC)} and found that ms2i6A modification remained intact in all cells (Figure 1C and D). These results demonstrated the absence of ms2i6A in nuclear-derived RNA species.

**Generation of ms2i6A antibody**

In addition to mt-tRNAs, mitochondrial DNA encodes two genes for mt-rRNAs and 13 genes for mt-mRNAs (10). There is a possibility that the ms2i6A modification might be present in mt-RNA and mt-mRNA. Given the technical difficulty of purifying individual mt-tRNA and mt-mRNA without the contamination of mt-tRNA, we sought to separate mt-tRNA from other mt-tRNA species by denaturing gel and then to detect ms2i6A using specific antibodies. We generated a monoclonal antibody by immunizing mice with synthetic ms2i6A. Competitive ELISA was used to validate the specificity of the ms2i6A antibody (Figure 2A). The synthetic ms2i6A abolished HRP-conjugated ms2i6A interaction with the antibody, whereas the synthetic i6A or methylated adenosine (m1A) had no effect on this interaction (Figure 2B), suggesting that the antibody specifically recognizes ms2i6A. To examine whether the antibody can recognize ms2i6A in intact RNA, the total RNA of WT and Cdk5rap1 KO mice was spotted on a membrane, fixed by UV irradiation and was then subjected to detection by conventional western blotting. The ms2i6A antibody reacted nicely with the total RNA of WT mice but not with that of Cdk5rap1 KO mice (Figure 2C). These results suggest that the antibody could detect the ms2i6A modification in intact RNA.

**ms2i6A was undetectable in mt-mRNA and mt-tRNA**

The specificity of the ms2i6A antibody prompted us to examine the presence of ms2i6A in each RNA species. Total RNA was extracted from the liver tissues of the WT and the Cdk5rap1 KO mice, followed by size-separation using denaturing Urea-TBE gel electrophoresis. In the total RNA of WT mice, an apparent single band was detected at 70–80 nt, which corresponded to the size of mt-tRNAs (Figure 3A). By contrast, no band was detected in the total RNA of Cdk5rap1 KO mice (Figure 3A). If mt-tRNA and/or

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**Figure 1.** Analysis of ms2i6A in ρ0 cells. (A) Quantification of mt-tRNA^{Phe} in the total RNA of HeLa cells, HeLa ρ0 cells, B82 cells and B82 ρ0 cells. n = 3 each. Data are the mean ± s.e.m. (B) Quantification of Cdk5rap1 transcripts in the total RNA of HeLa cells, HeLa ρ0 cells, B82 cells and B82 ρ0 cells. n = 3 each. Data are the mean ± s.e.m. (C and D) Mass spectrometry analysis of ms2i6A and ms2t6A in the total RNA of HeLa cells (C), HeLa ρ0 cells (D), B82 cells (D) and B82 ρ0 cells (D).
mt-mRNA contain the ms2i6A modification, the antibody would detect the signals in the higher molecular weight region. However, no bands were observed in the molecular weight region of >80 nt in either WT or Cdk5rap1 KO mice (Figure 3A).

The ms2i6A modification in mRNA might be difficult to detect due to the limited amount of mRNA in the total RNA fraction. We thus enriched mRNA from mouse liver total RNA using oligo(dT)-mediated affinity purification, followed by two rounds of size selection for RNA species with more than 200 nt in length (Supplementary Figure S1A). The rigorous purification resulted in a marked depletion of small size RNA species including tRNA, SS rRNA and 5.8 rRNA (Supplementary Figure S1A). Indeed, cytosolic tRNA Lys in mRNA-enriched fraction was reduced to ∼0.9% after purification (Supplementary Figure S1B and C). Surprisingly, ms2i6A-containing mt-tRNA Ser(UCN) in mRNA-enriched fraction only reduced to 23.8%, despite the apparent elimination of tRNA as observed in the denaturing gel (Supplementary Figure S1B and C). After transferring RNA to membrane, we applied the anti-ms2i6A antibody to detect the modification in mRNA-enriched fraction. However, no signals were observed in the mRNA region in both total RNA and mRNA-enriched fractions. In contrast, ms2i6A signals were clearly detected at the size corresponding to tRNA in both fractions (Supplementary Figure S1D).

The number of mitochondrial DNA-derived RNA is far less than that of nuclear-derived RNA species, the sensitivity might be insufficient when the total RNA or mRNA-enriched fractions was subjected to detection by our antibody. To enhance the sensitivity of detection, we isolated mitochondrial total RNA from a purified mitochondria fraction, and applied anti-ms2i6A antibody to the fractions. Compared with the total RNA fraction, a very strong band in the mitochondrial total RNA fraction was detected at ∼80 nt, indicating the successful concentration of mt-tRNAs. Notably, no other band was detected even in this highly purified mt-RNA fraction (Figure 3B). These results suggested that ms2i6A exists only in mt-tRNA and not in other RNA species.

ms2i6A-modified mt-tRNA is localized in the vicinity of the mitochondrial translation machinery

The superior specificity of the ms2i6A antibody prompted us to investigate the cellular localization of the ms2i6A modification by immunostaining. HeLa cells and HeLa p0 cells were stained with the antibody in the presence of MitoTracker. The spotty signals stained by the anti-ms2i6A antibody were nicely co-localized with MitoTracker in HeLa cells but had disappeared from HeLa p0 cells (Figure 4A). In addition, the mitochondrial localization of ms2i6A modification was diminished in HeLa cells when transfected with siRNA against Cdk5rap1 (Figure 4B), which further supports the idea that the modification occurs in tRNAs in mitochondria. Finally, we stained HeLa cells with the ms2i6A antibody in combination with antibody against mitochondrial elongation factor TUFM. The ms2i6A modification showed strong co-localization with TUFM (Figure 4C). Taken together, these results suggested that the ms2i6A modification exists in mt-tRNA, but not in the nuclear-derived RNA species.

DISCUSSION

The ms2i6A modification of adenosine in mt-tRNAs is critical for metabolism and energy expenditure in mammals (10). Deficiency of the modification causes malfunction of energy-consuming tissues, such as heart and skeletal muscle, and leads to the development of mitochondrial disease.
Given the important role of the ms^2i6A modification, the molecular property of the modification needs to be precisely understood.

Our data presented in this study provided strong supports to the idea that ms^2i6A is absent in nuclear-encoded RNA species. Using mass spectrometric analysis, we confirmed that in both human and mouse-derived p0 cells, the ms^2i6A was not detected in the intact nuclear-derived RNA. Furthermore, we generated a specific antibody against the ms^2i6A and used two different methods to investigate the modified bases in RNA species. Immuno-blotting with the antibody gave a specific band at the size of tRNA and the signal of the band was significantly stronger with modified bases in RNA species. Immuno-blotting with the antibody showed a high specificity and was successfully applied for ELISA and immunoblotting. Using this antibody, we presented data that strongly suggest that the ms^2i6A modification exists exclu-
Figure 4. Immunostaining of cells with the ms2i6A antibody. (A) HeLa cells and HeLa ρ0 cells were stained with Mitotracker and the ms2i6A antibody. Note that ms2i6A was co-localized with Mitotracker. Bar = 10 μm. (B) HeLa cells were transfected with control siRNA (siControl) or siRNA against CDK5RAP1. Cells were stained with Mitotracker and the ms2i6A antibody. Bar = 5 μm. (C) HeLa cells were transfected with control siRNA (siControl) or siRNA against CDK5RAP1. Cells were stained with anti-TUFM and anti-ms2i6A. Bar = 5 μm.
sively in mt-tRNAs and not in other RNA species. Furthermore, we successfully applied the antibody to immunocytochemistry. We showed that ms2\(^{16}\)A-containing mt-tRNA was predominantly localized in mitochondria and was in the vicinity of TUFM, a component of the mitochondrial translation machinery. To our knowledge, this is the first report of the spatial distribution of ms2\(^{16}\)A-containing mt-tRNA in mammalian cells. The ms2\(^{16}\)A antibody developed in this study is a great tool for studying the cellular dynamics of mt-tRNAs. Furthermore, given that the ms2\(^{16}\)A modification is involved in the development of mitochondrial disease, the antibody might be useful for clinical diagnosis in the future.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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