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# Cdk5rap1-mediated 2-methylthio-$N^6$-isopentenyladenosine modification is absent from nuclear-derived RNA species

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## ABSTRACT

**2-Methylthio-$N^6$-isopentenyl modification of adenosine ($ms^2i^6A$) 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 $i^6A$ to $ms^2i^6A$ 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 $ms^2i^6A$ is present abundantly in nuclear-derived RNA species such as microRNAs, but not in tRNA fractions. To fully understand the molecular property of $ms^2i^6A$, the existence of non-canonical $ms^2i^6A$ must be carefully validated. In the present study, we examined $ms^2i^6A$ in total RNA purified from human and murine ρ0 cells, in which mitochondrial DNA-derived tRNAs were completely depleted. The $ms^2i^6A$ was not detected in these cells at all. We generated a monoclonal antibody against $ms^2i^6A$ and examined $ms^2i^6A$ in murine RNAs using the antibody. The anti-$ms^2i^6A$ antibody only reacted with the tRNA fractions and not in other RNA species. Furthermore, immunocytochemistry analysis using the antibody showed the predominant localization of $ms^2i^6A$ in mitochondria and co-localization with the mitochondrial elongation factor Tu. Taken together, we propose that $ms^2i^6A$ 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-$N^6$-threonylcarbamoyadenosine ($ms^2t^6A$) and 2-methylthio-$N^6$-isopentenyladenosine ($ms^2i^6A$) (2). Cdk5 regulatory subunit associated protein 1-like 1 (Cdkal1) converts $t^6A$ to $ms^2t^6A$ at position A37 of cytosolic $tRNA^{Lys(UUU)}$ in mammalian cells, with a profound impact on both molecular and physiological functions (4,5). A deficiency of $ms^2t^6A$ 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 $ms^2t^6A$ 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 $i^6A37$ to $ms^2i^6A37$ in four mitochondrial DNA-encoded tRNAs, mt-$tRNA^{Trp}$, mt-$tRNA^{Tyr}$, mt-$tRNA^{Phe}$ and mt-$tRNA^{Ser(UCN)}$ (10). Similar to $ms^2t^6A$, $ms^2i^6A$ is important

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for efficient and accurate translation in mitochondria. In *Cdk5rap1*-null mice, the absence of $ms^2i^6A$ 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. Importantly, the $ms^2i^6A$ levels in these mt-tRNAs are substantially decreased in patients with mitochondrial disease. These results strongly suggested that $ms^2i^6A$ modification of mt-tRNAs is crucial for the mitochondrial translation and that the disruption of $ms^2i^6A$ modification is a key element of the molecular pathogenesis of the mitochondrial diseases.

While our results have clearly shown that Cdk5rap1-mediated $ms^2i^6A$ modification occurs in mitochondrial DNA-encoded mt-tRNAs (10), a previous study reported that $ms^2i^6A$ 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 $ms^2i^6A$ modification using mass spectrometry. Surprisingly, the $ms^2i^6A$ modification was almost absent from the tRNA fraction. The $ms^2i^6A$ modification was rather highly enriched in the miRNA and the poly-A RNA fractions. The authors hypothesized that the non-canonical $ms^2i^6A$ 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 $ms^2i^6A$ modification of the exclusive occurrence of $ms^2i^6A$ modification in mt-tRNAs, and raised a possibility that the $ms^2i^6A$ modification might control cellular functions through nuclear-encoded RNA species instead of mitochondrial tRNAs.

Does $ms^2i^6A$ exist in nuclear-encoded RNA species? To answer this question, it is necessary to examine the presence of $ms^2i^6A$ in such RNAs using carefully designed experimental approaches. It should be noted that, in growing cells, ∼80–90% of total RNA is rRNA, 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 DNA-derived tRNA is highly challenging during the validation of $ms^2i^6A$ modification in individual RNA species.

In the present study, we carefully investigated the presence of $ms^2i^6A$ modification in nuclear-encoded RNA species using cell biological approaches. We provide evidence that the $ms^2i^6A$ 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% $CO_2$. 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 $ms^2i^6A$ antibody were cultured in GIT medium (Wako) at 37°C and 5% $CO_2$.

### 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-$tRNA^{Phe}$, followed by quantitative PCR using forward and reverse primers as following:

mouse mt-$tRNA^{Phe}$:
forward: 5′-GCTTAATAACAAAGCAAAGCA
reverse: 5′-TATCCATCTAAGCATTTTCA
human mt-$tRNA^{Phe}$
forward: 5′-CTCCTCAAAGCAATACACTG
reverse: 5′-AGCCCGTCTAAACATTTTCA
mouse mt-$tRNA^{Ser(UCN)}$:
forward: 5′- CATATAGGATATGAGATTGGC
reverse: 5′- AACCCCCTAAAATTGGTTTCA

### 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 $i^6A$, $ms^2i^6A$ and adenosine (A) were measured using a mass spectrometer (Agilent 6460) as described previously (13).

### Purification of $ms^2i^6A$ antibody

Synthetic $ms^2i^6A$ 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 $ms^2i^6A$ 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). 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 $ms^2i^6A$ 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-$ms^2i^6A$ 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, $ms^2i^6A$, $i^6A$ or $m^1A$ 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 $ms^2i^6A$ 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 examination of 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 (Invitroge). The RNA was then transferred to an Amersham Hybond-$N^+$ membrane (upward capillary transfer) using the conventional capillary transfer method described elsewhere. Next, anti-$ms^2i^6A$ 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 $ms^2i^6A$.

### Fluorescent immunostaining

HeLa cells ($0.75 \times 10^5$ cells/ml) were seeded in a glass-bottomed dish (IWAKI) and were cultured overnight. The following day, control siRNA (siControl) and siRNA against Cdk5rapl (siCdk5rapl) 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 $ms^2i^6A$ 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 $ms^2i^6A$ 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 $ms^2i^6A$ derived from nuclear-encoded RNA species, if it is indeed present

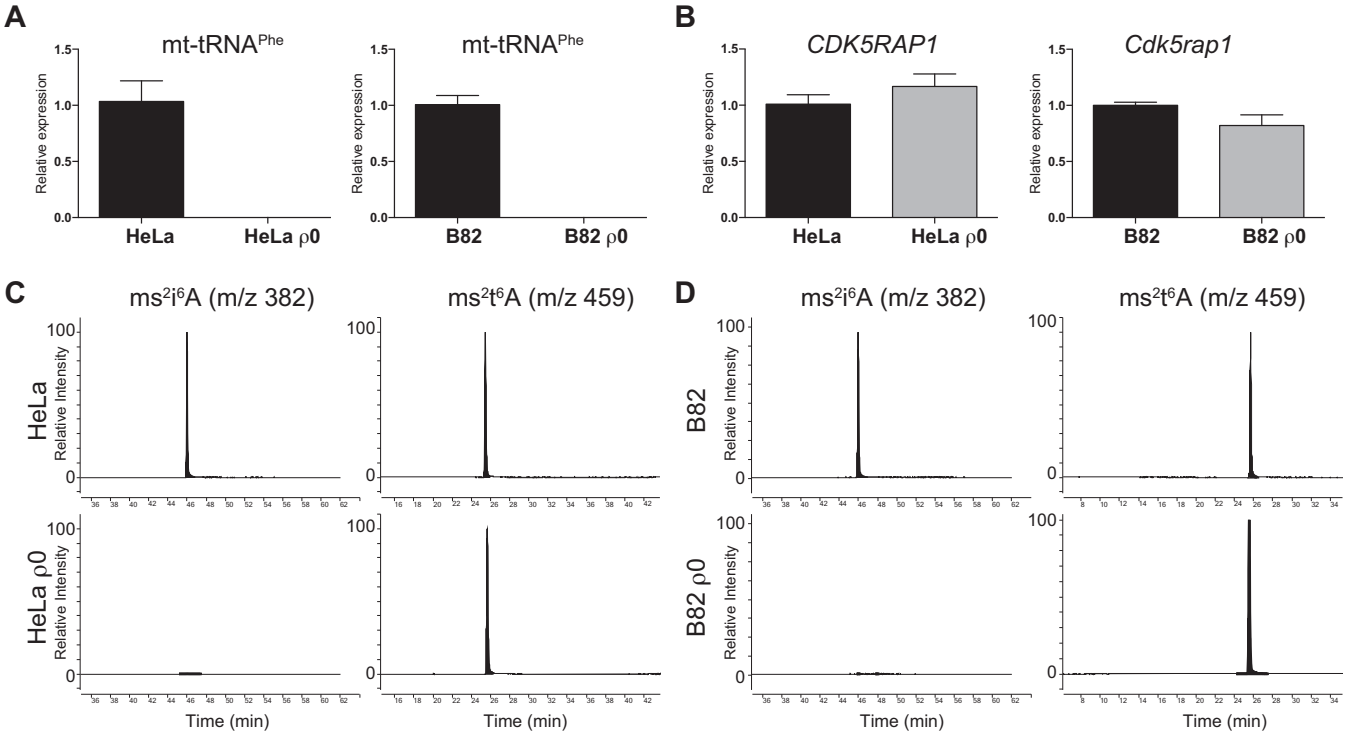


**Figure 1.** Analysis of $ms^2i^6A$ in ρ0 cells. (**A**) Quantification of mt-$tRNA^{Phe}$ in the total RNA of HeLa cells, HeLa ρ0 cells, B82 cells and B82 ρ0 cells. $n = 3$ each. Data are the mean ± s.e.m. (**B**) Quantification of *Cdk5rap1* transcripts in the total RNA of HeLa cells, HeLa ρ 0 cells, B82 cells and B82 ρ0 cells. $n = 3$ each. Data are the mean ± s.e.m. (**C** and **D**) Mass spectrometry analysis of $ms^2i^6A$ and $ms^2t^6A$ in the total RNA of HeLa cells (C), HeLa ρ0 cells (C), B82 cells (D) and B82 ρ0 cells (D).

in the RNA species, without the contamination of $ms^2i^6A$-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 $ms^2i^6A$-containing mt-tRNA (mt-$tRNA^{Phe}$) was depleted in the ρ0 cells by quantitative PCR. As expected, the levels of $ms^2i^6A$-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 $ms^2i^6A$ (Figure 1C and D). The $ms^2i^6A$ was clearly absent from the total RNA of HeLa ρ0 cells and B82 ρ0 cells. As a control, we examined $ms^2t^6A$ modification that was derived from nuclear-encoded cytosolic $tRNA^{Lys(UUU)}$, and found that $ms^2t^6A$ modification remained intact in all cells (Figure 1C and D). These results demonstrated the absence of $ms^2i^6A$ in nuclear-derived RNA species.

## Generation of $ms^2i^6A$ 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 $ms^2i^6A$ modification might be present in mt-rRNA and mt-mRNA. Given the technical difficulty of purifying individual mt-rRNA and mt-mRNAs without the contamination of mt-tRNA, we sought to separate mt-tRNA from other mt-tRNA species by denaturing gel and then to detect $ms^2i^6A$ using specific antibodies. We generated a monoclonal antibody by immunizing mice with synthetic $ms^2i^6A$. Competitive ELISA was used to validate the specificity of the $ms^2i^6A$ antibody (Figure 2A). The synthetic $ms^2i^6A$ abolished HRP-conjugated $ms^2i^6A$ interaction with the antibody, whereas the synthetic $i^6A$ or methylated adenosine ($m^1A$) had no effect on this interaction (Figure 2B), suggesting that the antibody specifically recognizes $ms^2i^6A$. To examine whether the antibody can recognize $ms^2i^6A$ 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 $ms^2i^6A$ 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 $ms^2i^6A$ modification in intact RNA.

## $ms^2i^6A$ was undetectable in mt-mRNA and mt-rRNA

The specificity of the $ms^2i^6A$ antibody prompted us to examine the presence of $ms^2i^6A$ 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-rRNA and/or

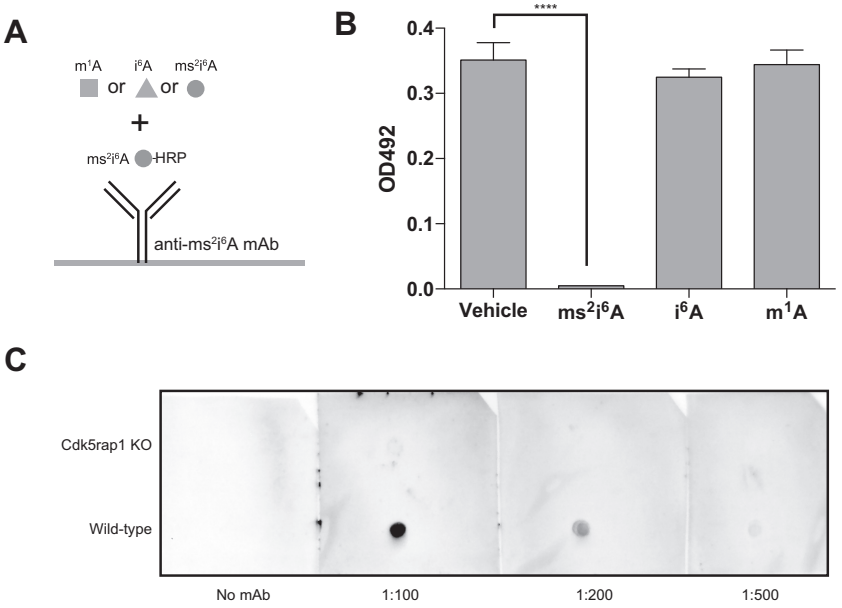


**Figure 2.** Validation of the ms$^2$i$^6$A antibody. (**A**) Schematic illustration of competitive ELISA for the validation of the ms$^2$i$^6$A antibody. (**B**) The ms$^2$i$^6$A antibody specifically recognizes ms$^2$i$^6$A but not the derivatives i$^6$A and m$^1$A. $n = 3$ for each. Data are the mean ± s.e.m. ****$P < 0.0001$ by Student's *t*-test. (**C**) Dot blot analysis of ms$^2$i$^6$A in the total RNA purified from the liver tissues of WT mice or Cdk5rap1 KO mice.

mt-mRNA contain the ms$^2$i$^6$A 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 ms$^2$i$^6$A 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, 5S 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, ms$^2$i$^6$A-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-ms$^2$i$^6$A 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, ms$^2$i$^6$A 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-ms$^2$i$^6$A 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 ms$^2$i$^6$A exists only in mt-tRNA and not in other RNA species.

### ms$^2$i$^6$A-modified mt-tRNA is localized in the vicinity of the mitochondrial translation machinery

The superior specificity of the ms$^2$i$^6$A antibody prompted us to investigate the cellular localization of the ms$^2$i$^6$A modification by immunostaining. HeLa cells and HeLa ρ0 cells were stained with the antibody in the presence of Mitotracker. The spotty signals stained by the anti-ms$^2$i$^6$A antibody were nicely co-localized with Mitotracker in HeLa cells but had disappeared from HeLa ρ0 cells (Figure 4A). In addition, the mitochondrial localization of ms$^2$i$^6$A 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 ms$^2$i$^6$A antibody in combination with antibody against mitochondrial elongation factor TUFM. The ms$^2$i$^6$A modification showed strong co-localization with TUFM (Figure 4C). Taken together, these results suggested that the ms$^2$i$^6$A modification exists in mt-tRNA, but not in the nuclear-derived RNA species.

## DISCUSSION

The ms$^2$i$^6$ 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.

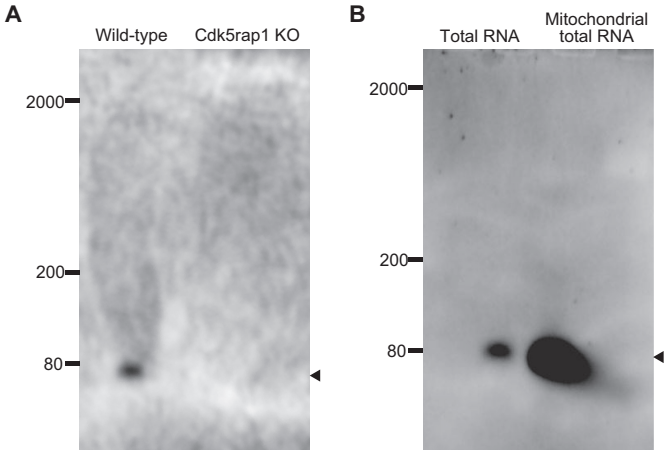


**Figure 3.** Analysis of $ms^2i^6A$ modification by northern blotting. (**A**) Total RNA purified from the liver of WT mice and Cdk5rap1 KO mice was separated by TBE-Urea gel electrophoresis, and then subjected to northern blotting with the $ms^2i^6A$ antibody. (**B**) Total RNA from the liver of WT mice and mitochondrial total RNA purified from isolated mitochondria from the liver of WT mice were subjected to northern blotting using the $ms^2i^6A$ antibody. Arrowheads indicate the position corresponding to tRNA.

Given the important role of the $ms^2i^6A$ 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 $ms^2i^6A$ is absent in nuclear-encoded RNA species. Using mass spectrometric analysis, we confirmed that in both human and mouse-derived ρ0 cells, the $ms^2i^6A$ was not detected in the intact nuclear-derived RNA. Furthermore, we generated a specific antibody against the $ms^2i^6A$ 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 mitochondrial RNA fraction than total RNA fraction. Immunostaining of cells with the antibody showed numerous punctuated foci that were localized to mitochondria and TUFM. These results thus provided strong evidence that the $ms^2i^6A$ modification is not present in nuclear-derived RNA species, at least in the samples used in this study. The defective metabolism observed in Cdk5rap1 KO mice (10) should solely be due to the defective mt-tRNA modification.

Mammalian cells contain multiple *CDK5RAP1* variants due to alternative splicing (11,15). Interestingly, one of *CDK5RAP1* variants, namely *CDK5RAP1_v2*, lacks N-terminus region that corresponds to mitochondria-targeting signal and is capable to localize in cytosol (11). Reiter *et al.* suggested that *CDK5RAP1_v2* might be responsible for the $ms^2i^6A$ modification of nuclear-derived RNA species (11). However, the cytosolic *CDK5RAP1_v2* not only lacks mitochondria-targeting signal, but also lacks an important cysteine residue in the UPF0004 domain (10). Because this cysteine residue is absolutely required for the enzyme activity of CDK5RAP1 (10), it is likely that the cytosolic CDK5RAP1_v2 cannot modify any RNA species. Indeed, we have previously shown that the absence of $ms^2i^6A$ modification in cells derived from Cdk5rap1 KO mouse was not rescued by overexpressing *CDK5RAP1_v2* (10).

Why did Reiter *et al.* observed such a large amount of $ms^2i^6A$ modification in nuclear-derived microRNA? Reiter *et al.* purified microRNA using a commercially available kit (11). However, it appears to be technically difficult to separate tRNA (∼80 nt) from microRNA (∼30 nt) using the kit as advised in the manufacturer's instructions. In fact, the size of microRNA purified by Reiter *et al.* was also almost identical to the size of tRNA (∼80 nt) (11). It is thus conceivable that the substantial amount of $ms^2i^6A$-containing mt-tRNA was co-purified with microRNA by the kit. Recently, tRNA-derived fragments (tRFs) have been emerging as a new group of functional small RNAs (16). These tRFs are ∼30 nt in length, which is the similar size of the conventional microRNAs. The tRFs can be generated through partial cleavage of full-length cytosolic tRNA and mt-tRNAs, which are widespread in cells and tissues (16). Thus, it is conceivable that the mt-tRNA-derived tRFs might also be co-purified by the microRNA purification kit. Therefore, the $ms^2i^6A$-containing microRNAs detected by Reiter *et al.* were most likely contaminated $ms^2i^6A$-containing mt-tRNA or mt-tRNA-derived fragments.

In addition to microRNA, Reiter *et al.* observed a substantial amount of $ms^2i^6A$ modification in mRNA fraction. It is likely that the '$ms^2i^6A$-containing mRNA' was also the contaminated $ms^2i^6A$-containing mt-tRNA. Indeed, a substantial amount of mt-$tRNA^{Ser(UCN)}$ remained in mRNA-enriched fraction even after extensive selection (see Supplementary Figure S1). It is worthwhile to mention that the signal of $ms^2i^6A$ in mRNA-enriched fraction was compatible to that in total RNA fraction despite the apparent decrease of tRNA in mRNA-enriched fraction (See gel image in Supplementary Figure S1). Dense and large amount of tRNA in total RNA fraction might have reduced the accessibility of anti-$ms^2i^6A$ antibody to mt-tRNA.

The unique structure of mt-tRNA might be one of the reasons that cause the selective contamination of mt-tRNA during mRNA purification. It is known that most of mammalian mt-tRNAs exhibit non-canonical cloverleaf structure (17). For example, mt-$tRNA^{Ser(UCN)}$, which contains $ms^2i^6A$ modification, exhibits relatively weak tertiary interactions between D-loop and T-loop when compared to the canonical cytosolic tRNA (18). The flexible structure might render mt-$tRNA^{Ser(UCN)}$ susceptive to denaturing condition during purification, which might cause the non-specific binding of mt-$tRNA^{Ser(UCN)}$ to oligo(dT) through A-rich region. Modifications in the residual mt-tRNAs in mRNA-enriched fraction will then give a strong background signal, which potentially leads to misinterpretation. Our study demonstrates that the residual RNA species in the purified mRNA fraction is not negligible in some cases, and these RNA species must be carefully verified by multiple approaches.

In this study, we generated a specific antibody against $ms^2i^6A$ modification. This antibody showed a high specificity and was successfully applied for ELISA and immunoblotting. Using this antibody, we presented data that strongly suggest that the $ms^2i^6A$ modification exists exclu-

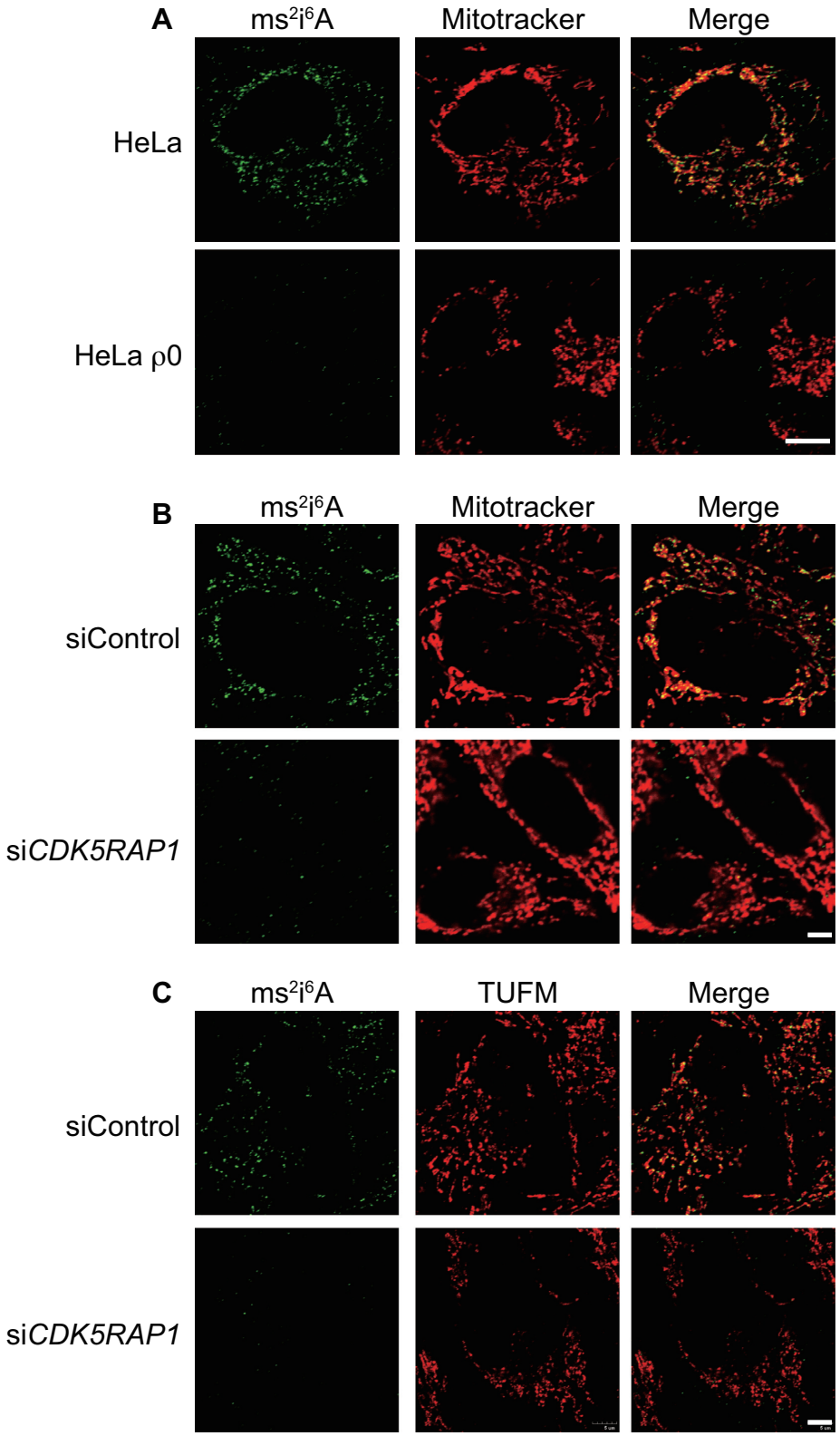


**Figure 4.** Immunostaining of cells with the $ms^2i^6A$ antibody. (**A**) HeLa cells and HeLa ρ0 cells were stained with Mitotracker and the $ms^2i^6A$ antibody. Note that $ms^2i^6A$ was co-localized with Mitotracker. Bar = 10 μm. (**B**) HeLa cells were transfected with control siRNA (siControl) or siRNA against *CDK5RAP1*. Cells were stained with Mitotracker and the $ms^2i^6A$ 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-$ms^2i^6A$. Bar = 5 μm.

sively in mt-tRNAs and not in other RNA species. Furthermore, we successfully applied the antibody to immunocytochemistry. We showed that $ms^2i^6A$-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 $ms^2i^6A$-containing mt-tRNA in mammalian cells. The $ms^2i^6A$ antibody developed in this study is a great tool for studying the cellular dynamics of mt-tRNAs. Furthermore, given that the $ms^2i^6A$ 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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