Supplemental Material for

Extracellular *N*^{6}-isopentenyladenosine (i^{6}A) induces co-transcriptional i^{6}A incorporation into ribosomal RNAs

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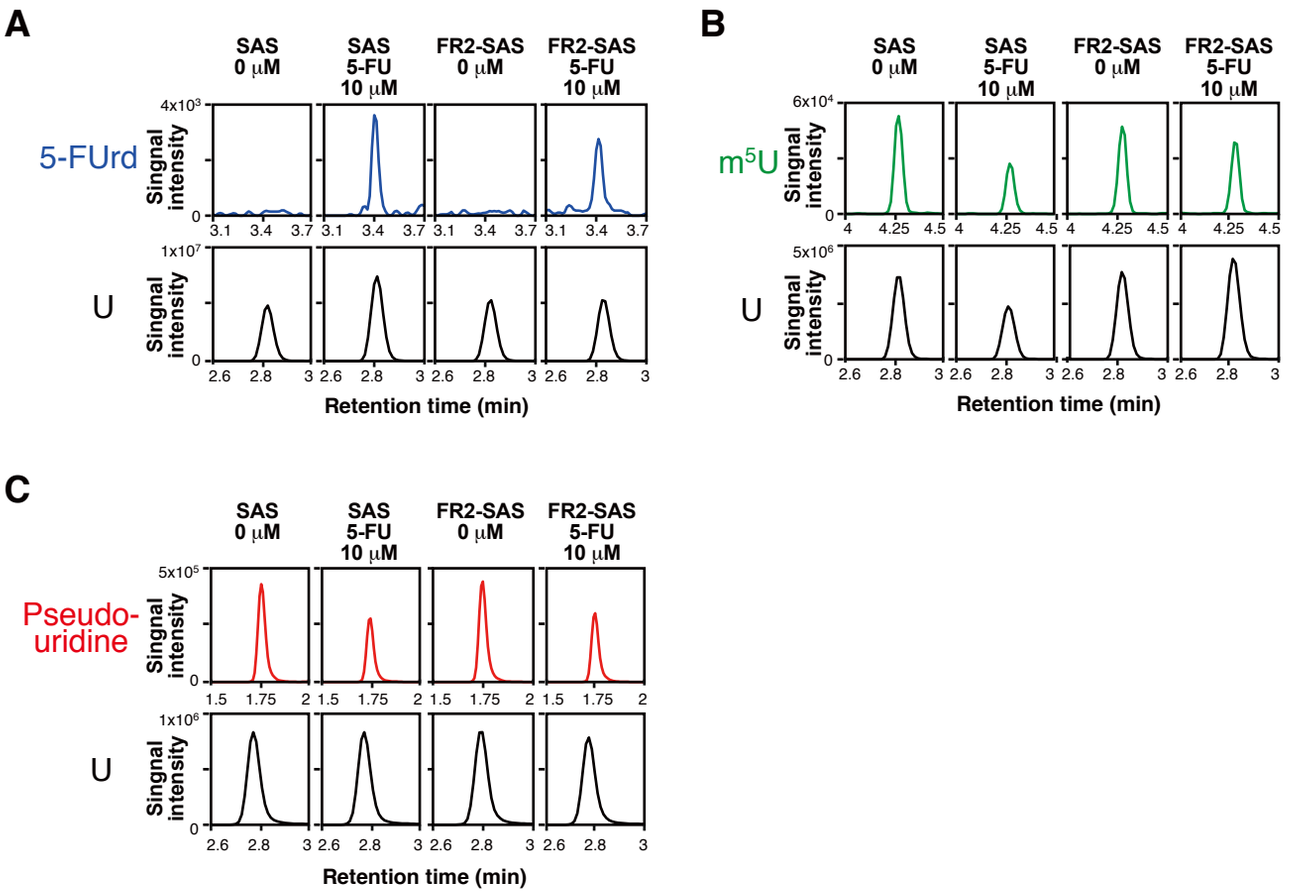
This file includes:

Supplemental Figure 1, 2, 3, 4 and their legends.

Supplemental Table 1 and 2.

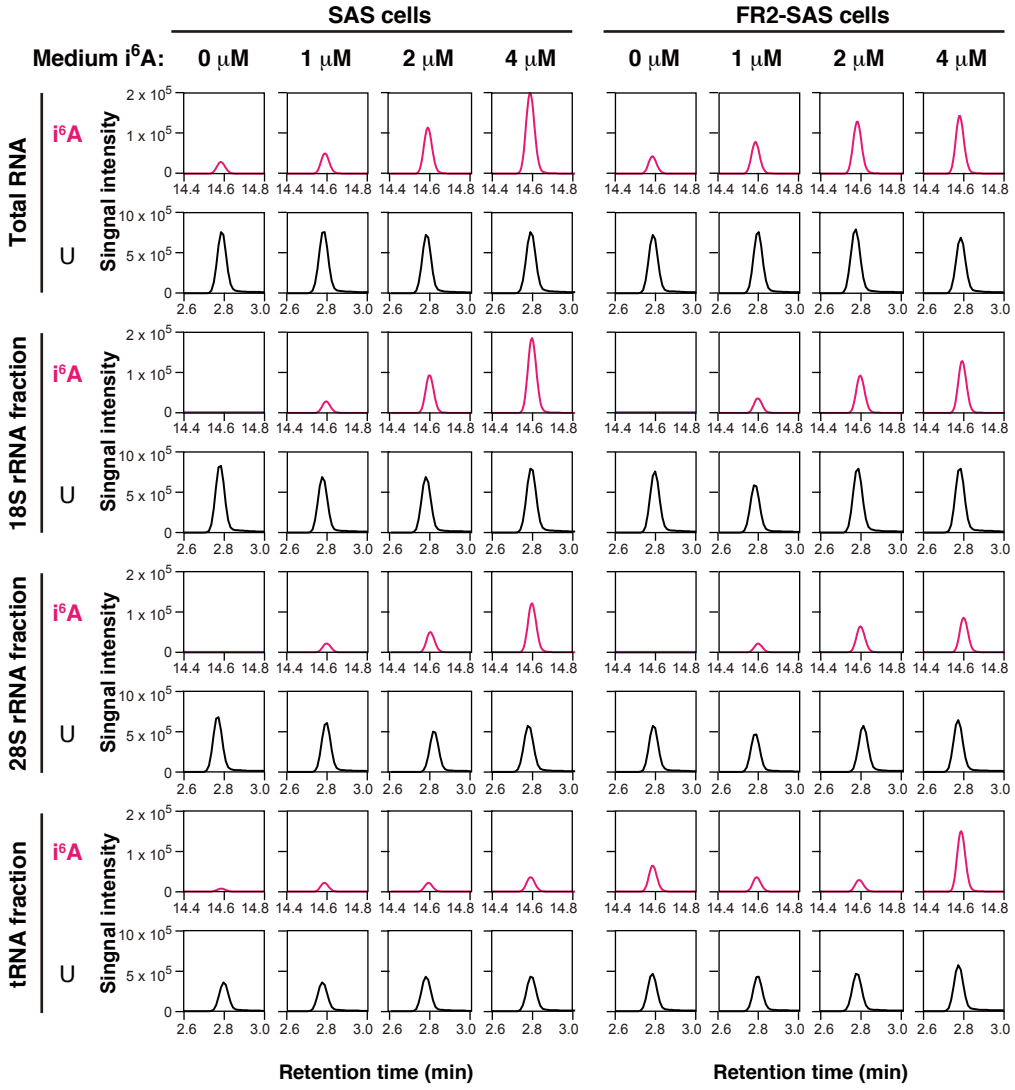
SUPPLEMENTAL FIGURE 1.
Mass chromatograms of modified nucleosides in cellular RNA upon 5-FU addition to the medium.



SUPPLEMENTAL FIGURE 1. Mass chromatograms of modified nucleosidesin cellular RNA upon 5-FU addition to the medium.

Representative mass chromatograms of 5-fluorouridine (5-FUrd) (**A**), 5-methyluridine (m^5U) (**B**) and pseudouridine (**C**) contained in the cellular total RNA after 48 hours of 0 or 10 μM 5-FU addition to the medium, corresponding to the data in Fig. 1D, F and G are shown. Uridine (U) is shown as the loading control.

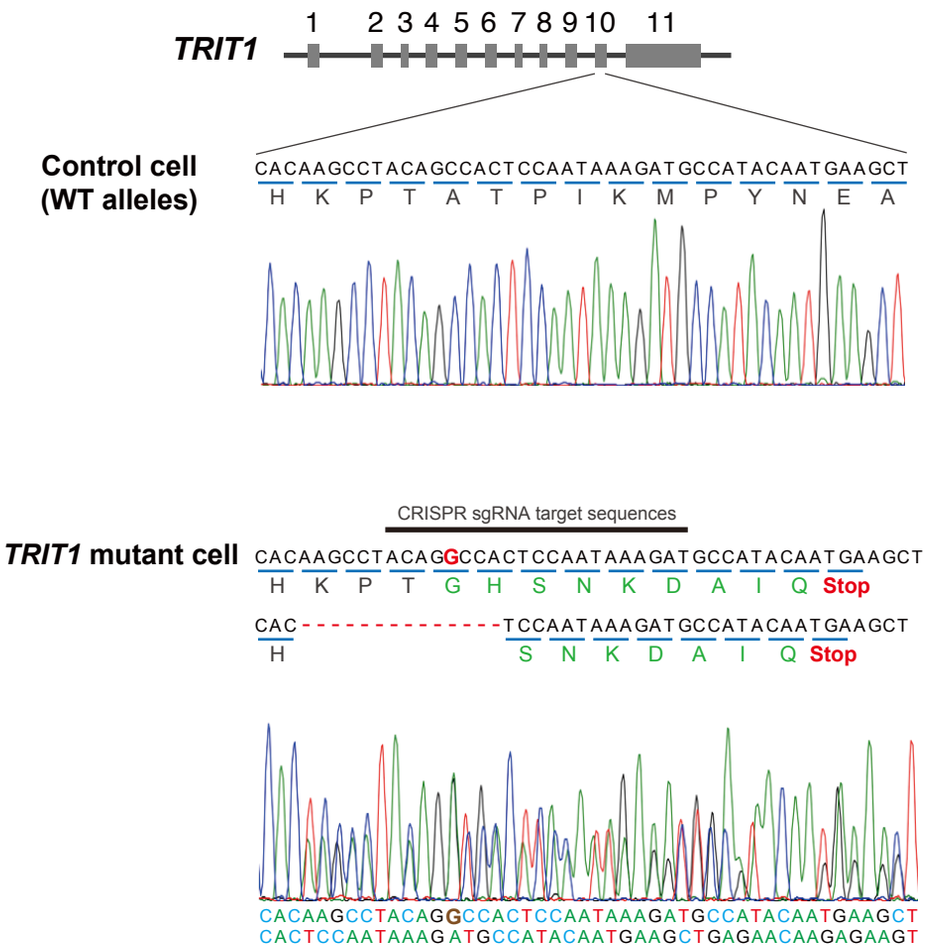
SUPPLEMENTAL FIGURE 2.
Mass chromatograms of i^{6}A in fractioned RNAs upon i^{6}A addition to the medium.



SUPPLEMENTAL FIGURE 2. Mass chromatograms of i^{6}A in fractioned RNAs upon i^{6}A addition to the medium.

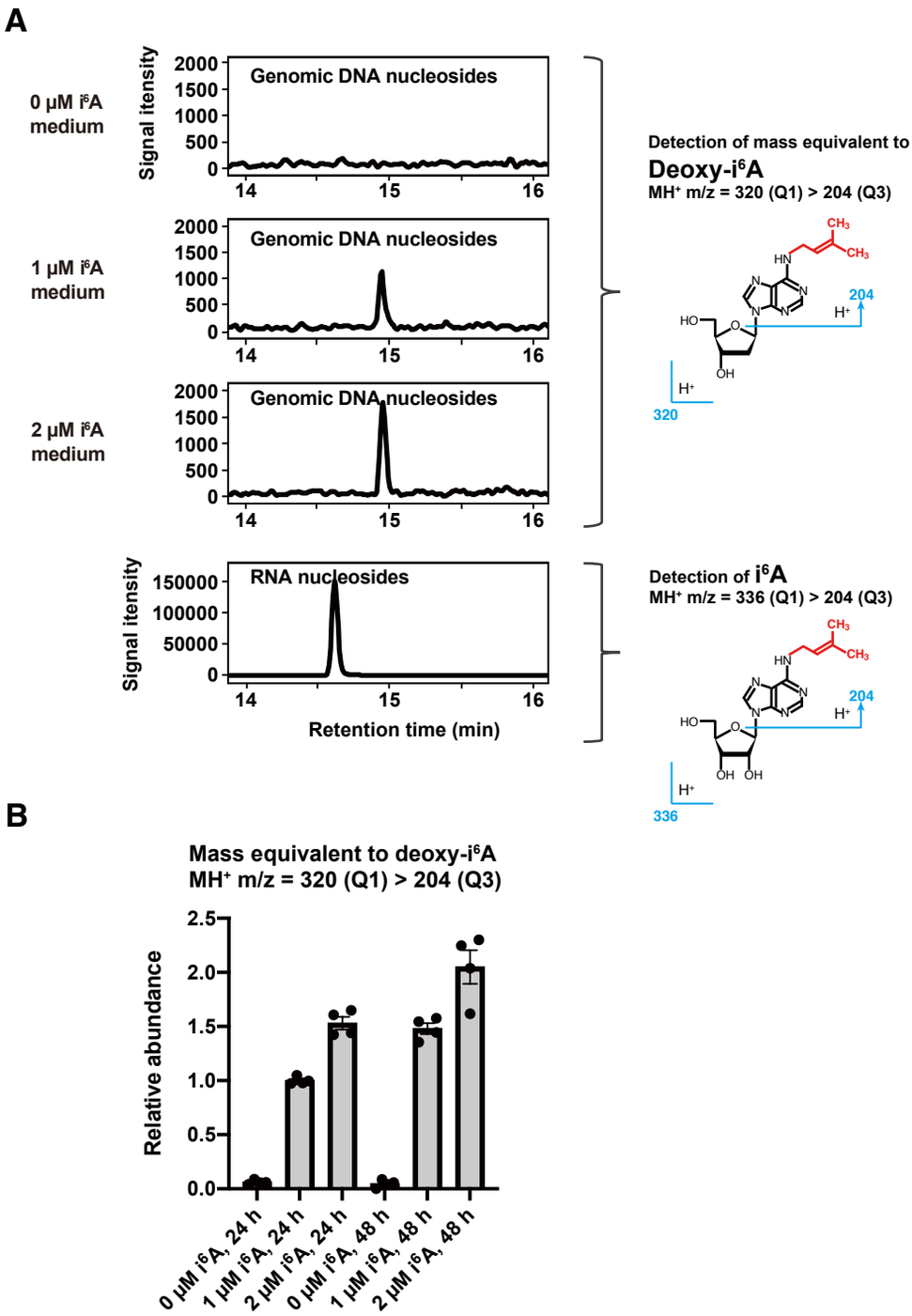
Representative mass chromatograms of the data in Fig. 4 are shown. SAS or FR2-SAS cells were cultured in the medium containing 0, 1, 2 or 4 μM i^{6}A for 24 h. After total RNA extraction, 18S rRNA, 28S rRNA and tRNA were each fractioned by gel electrophoresis and gel extraction, and subjected to digestion to nucleosides for LC-MS analysis. i^{6}A within total RNA, 18S rRNA and 28S rRNA of SAS and FR2-SAS increased upon i^{6}A addition to the medium. Whereas i^{6}A level in FR2-SAS tRNA repeatedly fluctuated in different samples (as shown in Fig. 4), i^{6}A level in SAS cell tRNA increased in proportion to medium i^{6}A concentration. Uridine (U) is shown as the loading control.

SUPPLEMENTAL FIGURE 3. Genomic sequences of *TRIT1* mutant HEK293FT cells.



SUPPLEMENTAL FIGURE 3. Genomic sequences of *TRIT1* mutant HEK293FT cells.
TRIT1 mutant HEK293FT cells or control cells were generated using CRISPR/Cas9 system using *TRIT1* targeting guide sequence or non-human DNA targeting guide sequence, respectively. After single cell cloning and expansion, genomic region around the sequences targeted by *TRIT1*-targeting guide sequence was PCR-amplified and Sanger-sequenced. In the *TRIT1* mutant cell, there is a G insertion on one allele and a 14 nt deletion on the other allele, and the overlap of these allele sequences is observed.

SUPPLEMENTAL FIGURE 4. Detection of mass equivalent to deoxy-i^{6}A within genomic DNA upon addtion of i^{6}A into the medium.



SUPPLEMENTAL FIGURE 4. Detection of mass equivalent to deoxy-i^{6}A within genomic DNA upon addition of i^{6}A into the medium.

(**A**) Mass chromatogram of peak corresponding to the same mass as deoxy-i^{6}A in genomic DNA nucleosides or i^{6}A in RNA nucleosides, after incubation of the cells in media containing 0, 1, or 2 μM i^{6}A for 48 h. In LC-MS, single proton-added positively-charged ion (MH^+) with a mass of the whole nucleoside was selected in the 1st quadrupole (Q1), followed by collision-induced dissociation, and the mass corresponding to MH^+ of dissociated modified nucleobase was selected in the 3rd quadrupole (Q3), and was detected in the final detector. The horizontal axis corresponds to LC retention time, and the vertical axis shows the signal intensity. The peak of the mass corresponding to deoxy-i^{6}A from genomic DNA appears 0.4 min after the RNA-derived i^{6}A peak. This delay may be due to lack of 2' hydroxyl group in deoxy-i^{6}A, making deoxy-i^{6}A more hydrophobic and elute at later time from the hydrophobic octa decyl silyl column used in LC.
(**B**) Quantification of the genomic DNA-digested nucleoside LC-MS peak area corresponding to the mass of deoxy-i^{6}A, after incubation of the cells in media containing 0, 1 or 2 μM i^{6}A for 24 or 48 h. Genomic DNA was extracted, and digested to nucleosides for LC-MS analysis, and the peak corresponding to the mass of deoxy-i^{6}A peak area was normalized by deoxycytidine peak area of the same sample, and the values are shown as the relative levels versus the mean of 0 μM i^{6}A cell samples.

SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1. Oligo DNAs used in this study.

RT-qPCR	
Human *GAPDH* forward	GGGAAGCTTGTCATCAATGG
Human *GAPDH* reverse	TGGACTCCACGACGTACTCA
Human *DPYD* forward	TGATGTCGTTGGTTTGGCTA
Human *DPYD* reverse	GCAGAAACGGAAGCTCCATA
Human pre-18S forward	GTCGTCCTCCTCGCTTGC
Human pre-18S reverse	AATGAGCCATTCGCAGTTTC
Human pre-28S forward	CTCTCTCCCGTCGCCTCT
Human pre-28S reverse	TGTTCACTCGCCGTTACTGA
Human 18S forward	ATTAACAAGAACGAAAGTCGGAGGT
Human 18S reverse	TTTAAGTTTCAGCTTTGCAACCATACT
Human 28S forward	CCCAGTGCTCTGAATGTCAA
Human 28S reverse	TGGGAATCTCGTTCATCCAT
Human *BIP* forward	TGCAGCAGGACATCAAGTTC
Human *BIP* reverse	GGAGCAAATGTCTTTGTTTGC
Human *CHOP* forward	GACCTGCAAGAGGTCCTGTC
Human *CHOP* reverse	CTCCTCCTCAGTCAGCCAAG
Human total *XBP1* forward	TCTGGAGCTATGGTGGTGGT
Human total *XBP1* reverse	TCTCTGGGCTGGCACCAT
Human spliced *XBP1* forward	GCTGAGTCCGCAGCAGGT
Human spliced *XBP1* reverse	CTGGGTCCAAGTTGTCCAGAAT
***TRIT1* mutant cell generation**	
sgControl top	CACCGACGGAGGCTAAGCGTCGCAA
sgControl bottom	AAACTTGCGACGCTTAGCCTCCGTC
sg*TRIT1* top	CACCGATCTTTATTGGAGTGGCTGT
sg*TRIT1* bottom	AAACACAGCCACTCCAATAAAGATC
PCR around indel site forward	CCTCCAAGGAATTTGAGCAC
PCR around indel site reverse	GGGCTGGCTGCTTTTTAATAC

SUPPLEMENTAL TABLE 2. Antibodies used in this study.

Antibody	Animal, Producer, Catalog number, Dilution
TRIT1	Rabbit, GeneTex, GTX120508, 1:1000
β-actin	Mouse, MBL, M177-3, 1:5000