Transfer (t)RNAs contain a wide variety of post-transcriptional modifications, which play critical roles in tRNA stability and functions. 3-(3-amino-3-carboxypropyl)uridine (acp\textsuperscript{3}U) is a highly conserved modification found in variable- and D-loops of tRNAs. Biogenesis and functions of acp\textsuperscript{3}U have not been extensively investigated. Using a reverse-genetic approach supported by comparative genomics, we find here that the Escherichia coli \textit{yfIP} gene, which we rename \textit{tapT} (tRNA aminocarboxypropyltransferase), is responsible for acp\textsuperscript{3}U formation in tRNA. Recombinant TapT synthesizes acp\textsuperscript{3}U at position 47 of tRNAs in the presence of S-adenosylmethionine. Biochemical experiments reveal that acp\textsuperscript{3}U47 confers thermal stability on tRNA. Curiously, the \textDelta\textsuperscript{tapT} strain exhibits genome instability under continuous heat stress. We also find that the human homologs of \textit{tapT}, \textit{DTWD1} and \textit{DTWD2}, are responsible for acp\textsuperscript{3}U formation at positions 20 and 20a of tRNAs, respectively. Double knockout cells of \textit{DTWD1} and \textit{DTWD2} exhibit growth retardation, indicating that acp\textsuperscript{3}U is physiologically important in mammals.
The emerging field of epitranscriptomics has revealed the chemical diversity and functional importance of RNA modifications. To date, about 150 species of RNA modifications have been identified in RNA molecules from all domains of life. Transfer (t)RNAs are especially heavily modified; indeed, more than 80% of RNA modifications found so far were discovered in tRNA molecules from various organisms. By stabilizing tRNA tertiary structure and fine-tuning decoding capability, these modifications ensure that tRNAs function properly.

A wide variety of tRNA modifications are found in the anticodon loop, especially at positions 34 and 37. These modifications stabilize and modulate codon–anticodon interactions on the ribosome, thereby ensuring accurate and efficient decoding during translation. The second class of modifications is clustered in the tRNA core structure formed by the D-loop, Tyc-loop (T-loop), and variable-loop (V-loop). These tRNA modifications are structural modulators that contribute to correct folding and stabilization of tRNA. Some of these modifications are also required for tRNA flexibility. The 2′-O-methyl modifications found in the D- and T-loops, in particular, 2′-O-methylguanosine (Gm) at position 18, confer conformational rigidity on the tRNA core region by fixing C3-endo ribose puckering. Gm18 stabilizes the D-loop/T-loop interaction through base pairing with pseudouridine (Ψ) at position 55 in the T-loop. Ψ55 stabilizes T-loop structure with additional hydrogen bond to the phosphate-ribose backbone. 5-methyluridine (m5U, also known as ribothymidinyl) at position 54 in the T-loop confers thermal stability to tRNA. In thermophilic organisms, additional modifications, including 5-methyl-2-thiouridine (m2′-S2U or s2T) and archaeosine (G+), are present in the tRNA core region. These modifications stabilize tRNAs, enabling cell growth at high temperature.

The modifications in the core region play crucial roles in determining not only the physicochemical properties of tRNAs but also their cellular stability. Because properly modified mature tRNAs are required for accurate and efficient translation, living organisms have evolved a control quality system that degrades hypomodified tRNAs.

3′-(3-amino-3-carboxypropyl)uridine (acp3U) is a widely conserved modification found in tRNA core region in bacteria and eukaryotes. The 3′-aminocarboxypropyl (ac) group is attached to the N3 atom of the uracil base to prevent its engaging in Watson–Crick base pairing. In Escherichia coli, acp3U is present at position 47 in the V-loop of tRNAs for Arg2, Ile1, Ile2, Ile2v, Lys, Met, Phe, Val2A, and Val2B (Fig. 1a). In eukaryotes, including human, rat and Drosophilia, acp3U occurs at positions 20 and 20a in the D-loop of several cytoplasmic tRNAs. In Trypanosoma brucei, acp3U and its dihydrouridine derivative (acp3D) are present at positions 20 and 47, respectively, in cytoplasmic tRNA\(^\text{1ys(TT)}\) (Fig. 1a). In several species of land plants, acp3U can be found at positions 20a and 20 in nuclear-encoded tRNAs, and at position 47 in tRNAs encoded in plastid and mitochondrial DNAs.

An acp3U derivative, 1-methyl-3′-(3-amino-3-carboxypropyl) pseudouridine (m1acp3Ψ), is present at the P-site of small subunit of the eukaryotic and archaeal ribosome: positions 1191 and 1248 in 18S RNA from S. cerevisiae and human, respectively. In human, m1acp3Ψ1248 is synthesized via three steps. The biogenesis is initiated by pseudouridylation mediated by HACA snoRNP bearing SNORA1325, followed by methylation catalyzed by EMGI. Finally, TSR3 transfers the acp group of S-adenosylmethionine (SAM) to form m1acp3Ψ27,28. TSR3 contains a domain similar to SPOUT-class RNA methyltransferase. The crystal structure of the archaeal homolog of TSR3 in complex with SAM revealed the molecular basis of acp3U formation, although the enzymatic activity of TSR3 has never been described. Reduction in the level of the acp modification causes accumulation of 18S rRNA precursors and a reduction in the level of 40S ribosomal subunit, indicating that this modification is involved in 40S subunit assembly.

Despite the high conservation of the acp3U modification in tRNAs from bacteria and eukaryotes, the biogenesis and physiological roles of this modification remain to be determined. The solution structure of the acp3U nucleoside does not affect its sugar conformation. Structural analysis of D-acp3U-A tri nucleotide suggested that acp3U20a binds to Mg\(^{2+}\), and this interaction may stabilize the local conformation of tRNA. Chemical acetylation of the amino group of acp3U47 in E. coli tRNA\(^{\text{Phe}}\) does not affect its binding affinity for phenylalanyl-tRNA synthetase or the ribosome. In the tertiary structure of tRNAs, the acp group of acp3U47 is oriented toward the solvent side of the tRNA structure and does not directly interact with the other residues. In 1974, enzymatic formation of acp3U in tRNA was carried out using E. coli lysate. Specifically, Nishimura’s group successfully reconstituted acp3U47 formation in tRNA\(^{\text{Phe}}\) in the presence of SAM, and demonstrated that the acp group of SAM is transferred to tRNA to form acp3U47 (Fig. 1b).

To achieve a deeper understanding of the acp3U modification in tRNAs, it is necessary to identify the enzyme responsible for generating it. For systematic search for genes responsible for RNA modifications, we developed a genetic screening method called ribonucleome analysis, which takes advantage of mass spectrometric analysis of RNA modifications. We used liquid chromatography/mass spectrometry (LC/MS) to systematically analyze total nucleosides of RNAs obtained from a series of strains harboring knockouts in uncharacterized genes. If a target RNA modification is absent in a certain knockout strain, we can identify the gene dedicated to biogenesis of the modification in a reverse-genetic manner. We have discovered dozens of genes responsible for RNA modifications in tRNAs and mRNAs. In this study, we apply ribonucleome analysis assisted by comparative genomics to identify an E. coli gene \(\text{yfIP}\) (renamed as \(\text{tapT}\)) responsible for acp3U47 formation. We successfully reconstitute formation of acp3U47 by recombinant TapT protein in the presence of SAM. Measurements of melting temperature reveal that acp3U47 stabilizes tRNA by 3 °C. We observe genome instability of the \(\Delta\text{tapT}\) strain under continuous heat stress, indicating that this modification plays a physiological role in bacteria. We also identify eukaryotic homologs of \(\text{tapT}\), including the human homologs \(\text{DTWDI}\) and \(\text{DTWD2}\), and show that they are responsible for the formation of acp3U at positions 20 and 20a of tRNAs, respectively. In human cells, double knockout of \(\text{DTWDI}\) and \(\text{DTWD2}\) causes slow growth, indicating that acp3U is also physiologically important in mammals.

Results

\(\text{E. coli yfIP}\) is responsible for acp3U47 formation on tRNAs. To identify the gene responsible for acp3U47 formation in \(\text{E. coli}\), we used comparative genomics to narrow down the candidate genes (Fig. 2a). Because little information was available about post-transcriptional modifications in the other bacterial tRNAs, we conducted nucleoside analyses of total RNAs from several bacterial species, \(\text{Acidimicrobiurn ferrooxidans, Synechocystis sp. PCC 6803, Thermus thermophilus HB27, Bacillus subtilis str. 168, and Mycoplasma mobile 163K}\), and found that none of them contained acp3U (Supplementary Fig. 1). By contrast, in protists, acp3U is present in \(\text{T. brucei}\) cytoplasmic tRNA\(^{\text{Lys(UUU)}}\), and we confirmed the presence of acp3U in the same tRNA from \(\text{Leishmania major}\). Using these data, we performed a comparative genomics analysis using the Microbial Genome Database. Among 4144 \(\text{E. coli}\) ORFs, we selected 65 genes commonly

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Fig. 1 acp3U is a prevalent tRNA modification widely conserved in eukaryotes and bacteria. a Positions of acp3U modification in tRNA (left panel). Chemical structure of 3-(3-amino-3-carboxypropyl)uridine (acp3U) (right panel). The acp group is colored in red. acp3U is present at position 20 in mammals, Drosophila and Trypanosoma, at position 20a in mammals, Drosophila and plants, and at position 20b in plants. b Biosynthetic mechanism of acp3U modifications in tRNA and rRNA. The acp group of S-adenosylmethionine (SAM) is transferred to generate acp3U modification in RNA with release of S-methyl-5'-thioadenosine (MTA).

TapT catalyzes acp3U47 formation. Next, we performed in vitro reconstitution of acp3U47 using the recombinant TapT protein. As a substrate, we isolated E. coli tRNAMet lacking acp3U from the ΔtapT strain. After the reaction, the tRNA was digested by RNase T1 and analyzed by LC/MS. We clearly detected an RNA fragment containing acp3U in the presence of both TapT and SAM (Fig. 2d). TapT efficiently introduced acp3U47 on unmodified tRNAMet transcribed in vitro (Supplementary Fig. 2), suggesting that the other tRNA modifications are not required for acp3U formation. The fragment was further probed by collision-induced dissociation, confirming that acp3U was introduced at position 47 (Fig. 2e). These data clearly demonstrated that TapT is a tRNA-modifying enzyme that catalyzes the SAM-dependent aminocarboxypropyl transfer reaction to form acp3U47.

acp3U confers thermal stability on tRNA. Given that acp3U is a modification in the tRNA core region, we investigated whether acp3U47 stabilizes the tertiary structure of tRNA. We prepared E. coli tRNAMet transcripts with or without acp3U47 and compared their melting profiles. As temperature increased, the tRNA gradually melted, and its hyperchromicity increased (Fig. 2f). The unmodified tRNA started melting around 40 °C, whereas the tRNA containing acp3U47 remained stable even at 50 °C. The hyperchromicity of the modified tRNA was lower than that of the unmodified one throughout the course of the heating process. The melting temperature (Tm) values of the tRNA with and without acp3U47 were 70.0 and 66.7 °C, respectively (Fig. 2f). Thus, a single acp3U modification thermally stabilized this tRNA by 3 °C.

Motility defect in E. coli ΔtapT strain. To investigate the physiological roles of acp3U47 in E. coli, we explored the phenotypic features of the ΔtapT strain. Because acp3U47 thermally stabilizes tRNA, we compared the cell growth of WT and ΔtapT strains cultured at different temperatures (Supplementary Fig. 3a), but observed no significant difference in cell growth even at 42 °C. However, we happened to find a morphological phenotype unique to the ΔtapT strain when cultured continuously at high temperature. After 3 days cultivation at 42 °C, small colonies suddenly appeared in the ΔtapT strain (Fig. 3a, b), although no such colonies were detected in the first 2 days. Small colonies

present in E. coli, as well as in the two protists (T. brucei and L. major), but absent in the five bacteria mentioned above (Fig. 2a, b). Based on the annotation of each gene in the EcoCyc database47, we then narrowed down the list of candidates to six genes with unknown functions. Finally, we chose yfiP as a strong candidate for acp3U formation (Fig. 2b). yfiP belongs to unknown orthologous group COG3148 that contains an uncharacterized DTW domain. According to computational analyses, YfiP has a SPOUT-like methyltransferase structure48. Moreover, COG3148 is referred to the DTWD2 family (KOG4382), and classified into the TDD superfamily, which includes the TSR3 (COG2042), DTWD1 (KOG3795), and DTWD2 families49.

To examine whether yfiP is responsible for acp3U47 formation in tRNAs, we performed LC/MS nucleoside analysis of total RNAs from E. coli WT and ΔyfiP strains (Fig. 2c). As expected, no acp3U was detected in the ΔyfiP strain, but acp3U was restored by ectopic expression of a plasmid-encoded yfiP gene. These results clearly demonstrated that yfiP is essential for acp3U formation in E. coli. Hereafter, we refer to yfiP as tapT (tRNA aminocarboxypropyltransferase).
were not detected in the WT strain, in the ΔtapT strain cultured at 37 °C, or in the ΔtapT strain complemented with plasmid-encoded tapT gene (Fig. 3c).

We isolated and characterized small and normal-sized ΔtapT colonies cultured for 4 days at 42 °C, and then examined the growth and motility of these cells. We observed no significant difference in cell growth between small and normal colonies (Supplementary Fig. 3b). Cell motility was examined based on swimming activity on soft agar plates. As shown in Fig. 3d, cells derived from normal colonies swim well, and the diameter of the halo reached 2.90 ± 0.21 cm, whereas cells derived from small colonies did not move and instead remained near the original spot (0.55 ± 0.04 cm), suggesting that the small colony phenotype of the ΔtapT strain originates from a motility defect. When we sought to reverse the phenotype by introducing plasmid-encoded tapT into cells derived from small colonies, we observed no
normal colonies on the plate (Fig. 3c). Supporting this result, lack of motility on soft agar was not restored by tapT complementation (0.54 ± 0.08 cm) (Fig. 3d). We confirmed restoration of acp³U in the small colony-derived ΔtapT strain by introduction of plasmid-encoded tapT gene (Supplementary Fig. 3c). Thus, the phenotype is caused by genomic mutations.

tRNA modification-deficient strains exhibit a moderate mutator phenotype mediated by the translational stress-induced mutagenesis (TSM) pathway. Lack of N⁶-isopentenyladenosine (i⁶A) in tRNAs in the ΔniaA strain induces transversion-type DNA mutation. If similar translation stress is induced by loss of acp³U in the ΔtapT strain, the genomic mutation linked to the small colony phenotype might be enhanced by the TSM pathway. To measure the genomic mutation rate of the ΔtapT strain, we performed a mutator assay. Specifically, we cultured the cells continuously at 42 °C and then examined their resistance to nalidixic acid (Supplementary Fig. 4). As a positive control, we used the ΔmutS strain, in which the DNA mismatch repair system is inactive; a number of small colonies arose on the plate, whereas the negative control strain (ΔyagA) showed a lower mutation rate. As reported, the ΔniaA strain exhibited a mild mutator phenotype. However, in contrast to our speculation, the mutation rate of the ΔtapT strain was in the same range as that of the control strain, suggesting that the small colony phenotype of the ΔtapT strain under heat stress cannot be explained by a mutator phenotype.

**DTWD1 and DTWD2 mediate acp³U formation in human tRNAs.** In mammals, acp³U is present at position 20 of tRNA³Tyr and position 20a of tRNA³Asn. We reanalyzed high throughput sequencing of human tRNAs and observed an apparent U-to-C conversion at positions 20 and 20a of these tRNAs, indicating that acp³U causes misincorporation during cDNA synthesis. Based on the misincorporation signatures at positions 20 and 20a of several human tRNAs, we predicted the presence of acp³U20 in two tRNAs for Cys(GCA) and Tyr, and acp³U20a in three tRNAs for Asn(GTT), Ile(AAT), and Ile(TAT). We then isolated these cytoplasmic tRNAs from HEK293T cells and analyzed the tRNA modifications by capillary LC–nanoelectrospray ionization (ESI)–MS (RNA-MS). We confirmed the presence of acp³U at positions 20 and 20a in these five tRNA species (Fig. 4a, b and Supplementary Fig. 5a–c). In addition, we found acp³D at position 20a of tRNA³Ile(AAT) (Fig. 4b).

As described above, tapT belongs to the DTWD2 family and TDD superfamily. Two paralogs of DTW-containing proteins in the TDD superfamily, DTWD1 and DTWD2, are encoded in the human genome, and we hypothesized that these proteins are responsible for acp³U at positions 20 and 20a. To examine this speculation, we knocked out each of these proteins in HEK293T cells using the CRISPR-Cas9 system. Two isoforms of human DTWD2 with different first exons are produced by alternative splicing: DTWD2L (long) and DTWD2S (short). (Supplementary Fig. 6a). To delete both isoforms, we designed sgRNAs targeting exons 2 and 3 (Supplementary Fig. 6a). We also constructed DTWD2L-specific KO cell line by targeting the exon 1 of DTWD2L. Finally, we constructed DTWD1/DTWD2 double-KO cell lines. Genotyping of each KO cell line confirmed frameshift mutations in both alleles (Supplementary Fig. 6b).

We then isolated five individual tRNAs bearing acp³U from a series of KO strains and examined their modification status by RNA-MS. In the DTWD1 KO cells, the level of acp³U20 in two tRNAs for Cys(GCA) and Tyr were drastically reduced, with the modified bases converted to D20 and U20, although a small percentage of acp³U20 remained (Fig. 4a and Supplementary Fig. 5a). We observed no effect on acp³U20a or acp³D20a in the other three tRNAs. No acp³U20 was detected in the DTWD1/DTWD2 double-KO cells (Fig. 4a and Supplementary Fig. 5a).
**Fig. 4 Human DTWD1 and DTWD2 are responsible for acp³U formation.**

**a** Mass spectrometric analyses of acp³U20 in tRNA\textsubscript{Cys(GCA)} isolated from WT (left), DTWD1 KO (left middle), DTWD2 KO (right middle), and DTWD1/DTWD2 double-KO (right) strains. XICs show corresponding negative ions of the RNase T1-digested fragments as indicated on the right-hand side of each chromatogram. Mass spectra of acp³D- or D-containing fragments overlap with the isotopic peaks of acp³U- or U-containing fragments. The peak intensities of these fragments are normalized in consideration of those isotopes. Asterisk indicates nonspecific peaks with the same m/z value.

**b** Mass spectrometric analyses of acp³U20a in tRNA\textsubscript{Ile(AAT)} isolated from WT (left), DTWD1 KO (left middle), DTWD2L KO (middle), DTWD2 KO (right middle), and DTWD1/DTWD2 double-KO (right) strains.

**c** Substrate specificity of human DTWD1 and DTWD2. DTWD1 is responsible for acp³U20 formation in tRNAs for Cys(GCA) and Tyr, whereas DTWD2 is responsible for acp³U20a formation in tRNAs for Asn(GTT), Ile(AAT), and Ile(TAT). DTWD2 also has a weak activity to form acp³U20. The sequence of tRNA\textsubscript{Cys} and tRNA\textsubscript{Asn} are shown.
Fig. 5a), indicating that DTWD1 is primarily responsible for introducing acp3U20, whereas DTWD2 plays a supportive role in acp3U20 formation (Fig. 4c). On the other hand, acp3U20a and acp3D20a were deficient, and replaced by D20a, in three tRNAs for Asn(GTT), Ile(AAT), and Ile(TAT) in the DTWD2 KO cells (Fig. 4b and Supplementary Fig. 5b, c), suggesting that DTWD2 has a strong specificity for introducing acp3U20a and acp3D20a (Fig. 4c). These data demonstrated that DTWD1 and DTWD2 have different substrate preferences for tRNAs, and are responsible for the biogenesis of acp3U20 and acp3U20a, respectively.

Regarding two isoforms of DTWD2, we isolated tRNAile(AAT) from DTWD2L KO cells, and analyzed the status of acp3U20a modification (Fig. 4b). Both acp3U20a and acp3D20a partially decreased, and instead D20a increased. These data clearly demonstrated that DTWD2L actually has an activity for acp3U(D)20a formation, and DTWD2S is redundantly responsible for the remaining modification.

Intriguingly, D20 or D20a appeared upon knockout of DTWD1 or DTWD2 (Fig. 4a, b and Supplementary Fig. 5a–c). D20 and D20a are present in other tRNA species that do not contain acp3U and have been proposed to be introduced by DUS2 and DUS4L, respectively57,58. Thus, DTWD1/2-mediated acp3U formation inhibits D20(a) formation.

**Growth reduction of DTWD1 and DTWD2 double knockout cells.** We next investigated the physiological significance of acp3U modifications in human tRNAs. In comparison with WT HEK293T cells, little growth reduction was observed in DTWD1 and DTWD2 single-KO cells (Fig. 5a), whereas the DTWD1/DTWD2 double-KO cells exhibited a severe growth phenotype (Fig. 5a), indicating that DTWD1 and DTWD2 play redundant roles in supporting normal cell growth. Given that tRNA modifications are required for the cellular stability of tRNAs35, we asked whether loss of acp3U modification affects the steady-state level of tRNAs. To this end, we performed Northern blotting to compare the steady-state levels of four tRNAs between WT HEK293T and DTWD1/DTWD2 double-KO cells (Supplementary Fig. 7a, b). No significant difference was observed for any tRNAs, suggesting that loss of acp3U does not affect tRNA stability.

Finally, we analyzed the subcellular localization of DTWD1 and DTWD2. For this purpose, FLAG-tagged DTWD1, DTWD2L, and DTWD2S were transiently expressed in HeLa cells and detected by immunostaining (Fig. 5b). DTWD1 was predominantly localized in the nucleus, suggesting that DTWD1-mediated acp3U20 formation takes place in the nucleus. By contrast, the two isoforms of DTWD2 exhibited different subcellular localization: DTWD2L was cytoplasm, whereas DTWD2S was widely distributed throughout the cells but concentrated in the nucleus. Thus, acp3U20a formation occurs in both the cytoplasm and the nucleus.

**Discussion**

In this study, we discovered that the tapT gene is responsible for acp3U47 formation in E. coli tRNAs. TapT was the last unknown tRNA-modifying enzyme in E. coli. We successfully reconstituted in vitro acp3U47 formation with the recombinant TapT protein and bacterial tRNA-modifying enzyme in E. coli. The acp moiety of SAM is also utilized for the synthesis of various biomolecules59, including bacterial betaine lipid, norcardicin, microcin C (McC1177), and diphthamide formed in EF-2. In other tRNA modification, the acp transfer reaction is involved in the synthesis of wybutosine (yW), a hypermodified nucleoside present in eukaryotic tRNAPhet16. The SAM-dependent class I methyltransferase TYW2 catalyzes the acp transfer reaction and synthesizes yW-86, a precursor of yW30,60. However, TDD superfamilies do not have sequence homology with these acp-transferases39. According to the crystal structure of archael Tsf3, which is a member of TDD superfamily protein28, it has a SPUT-class RNA methyltransferase fold. The closest structural homolog of the TDD superfamily is a RNA methyltransferase Trm10. Further structural studies of TapT and DTWD1/2 are necessary to reveal the molecular basis of tRNA substrate recognition and acp3U formation.

Measurement of melting temperature revealed that a single acp3U47 conferred thermal stability to tRNA, increasing the Tm value by 3°C. In the tertiary structures of tRNAs33–35, the acp group of the modification does not interact with any other residues but resides in close vicinity to the backbone of the T-stem. We speculated that the acp group might stabilize the local conformation of tRNA by interacting with the T-stem via magnesium ion or water31. In addition, the acp group at the N3 atom position hinders Watson–Crick base pairing and may destabilize some metastable structures so as to ensure correct folding of tRNA, similarly to the function of m1A91.

Strikingly, the E. coli ΔtapT strain exhibited a small colony phenotype associated with motility defect under continuous heat stress. Because ectopic expression of tapT did not restore the phenotype, we speculated that genomic mutations in swarming-related genes might be involved. Curiously, translation stress in E. coli strains bearing mutations in tRNA or the tRNA-modifying enzyme causes mutator phenotypes62,63. These facts prompted us to speculate that loss of acp3U in the ΔtapT strain contributes to the small colony phenotype mediated by the TSM pathway51. Unexpectedly, however, ΔtapT strain did not exhibit any mutator phenotypes under continuous heat stress conditions, indicating that neither error-prone replication nor aberrant DNA repairing is involved in this phenotype. Dysregulation of transposable elements are another possible source of genome instability64, but this should be investigated in future studies.

To investigate the phylogenetic distribution of the TDD family, we generated a phylogenetic tree of organisms possessing or lacking each homolog (Supplementary Fig. 8a). TSR3 orthologs predominated in eukaryotes and archaea, not in bacteria. Among the eukaryotes, DTWD1 orthologs are only present in vertebrates, insects, nematodes, and protists, but not in fungi and plants. DTWD2 orthologs are divided into two subfamilies, eukaryotic and bacterial (TapT). Eukaryotic DTWD2 orthologs are present in vertebrates, protists, and plants, whereas bacterial DTWD2 (TapT) orthologs are present in Bacteroidetes, Proteobacteria, Actinobacteria, Cyanobacteria, and Firmicutes, as well as in vertebrates, plants, and protists. We constructed multiple alignment of DTWD1 and DTWD2 homologs from representative organisms, and draw a phylogenetic tree using the maximum likelihood method (Supplementary Fig. 8b). DTWD1 orthologs form a small subfamily, clearly separated from the larger DTWD2 family with 100% bootstrap probability. This tree clearly reveals two major subfamilies of DTWD2: eukaryotic DTWD2, which is responsible for acp3U formation in the D-loop; and bacterial DTWD2 (TapT), which is responsible for acp3U formation in the V-loop.

T. brucei tRNAlys13 contains acp3U20 and acp3D4721. Consistent with this finding, one DTWD1 ortholog (TB09.244.2810) and one DTWD2 ortholog (TB927.3.4690) are encoded in the T. brucei genome49, indicating that these proteins are responsible for forming acp3U20 and acp3D47, respectively.

Recently, comprehensive analyses of Vibrio cholerae tRNAs identified acp3U and its derivative at positions 20b, 46, and 47. Interestingly, the Vibrio cholerae genome encodes three DTWD2
paralogs (VC1533, VC0131, and VC1980). According to the phylogenetic analysis (Supplementary Fig. 8b), VC1533 is a bacterial DTWD2 (TapT) ortholog that is assumed to be responsible for acp3U47 formation. Given that VC0131 branches from the root of the bacterial DTWD2 (TapT) subfamily, this homolog might be responsible for acp3U46 formation. VC1980 forms a unique subfamily with other bacterial orthologs near the eukaryotic DTWD2 subfamily. Considering that eukaryotic DTWD2 is responsible for acp3U20a formation, we speculated that the VC1980 subfamily is responsible for acp3U20b formation.

Pseudomonas species contain two DTWD2 paralogs (Supplementary Fig. 8b). P. aeruginosa PA3606 and PA1424 belong to the bacterial TapT and VC1980-like subfamilies, respectively, and might be responsible for acp3U20b formation.

In plants, acp3U20a and acp3U20b are found in nuclear-encoded tRNAs, while acp3U47 is found in tRNAs encoded in plastid and mitochondrial DNAs. Arabidopsis thaliana has three paralogs of DTWD2 (Supplementary Fig. 8b). AT2G41750 is a typical eukaryotic DTWD2 responsible for acp3U20a formation. AT1G03687, along with other plant orthologs, forms a subfamily closely related to the bacterial DTWD2 (TapT) subfamily, and this paralog is predicted to localize in chloroplasts, implying that it is responsible for acp3U20b formation in plastid tRNAs. AT5G54880, along with other plant orthologs, forms a subfamily near the VC1980-like subfamily, implying that this paralog is required for acp3U20b formation. Experimental verification is necessary to confirm these speculations.

Double knockout of DTWD1 and DTWD2 caused severe growth defects in culture cells, indicating the physiological importance of acp3U20b formation. Although the functional roles of this modification remain elusive, it has been suggested that acp3U20a can coordinate Mg2+, thereby stabilizing the local conformation of tRNA. The stabilization of tRNAs by acp3U20/20a may lead to efficient translation and rapid growth. According
to the Genotype-Tissue Expression project (Supplementary Fig. 9a,b)⁶⁸. DTWD1 and DTWD2 are differentially expressed in various tissues, indicating that the modification frequencies of acp³U20/20a might vary. Intriguingly, the two isoforms of DTWD2 exhibited different subcellular localization: DTWD2L and DTWD2S predominantly localized in the cytoplasm and nucleus, respectively. The expression levels of these two isoforms also varied among tissues (Supplementary Fig. 9b). Although expression of DTWD2L is low in many tissues, it is upregulated in others, including testis (Supplementary Fig. 9b). Because the different subcellular localizations of the two isoforms might affect tRNA maturation, tissue-specific isoform expression implies that acp³U modification is biologically important.

Loss of tRNA modification results in pathological consequences. We previously reported that human mitochondrial diseases are caused by hypomodification of mitochondrial tRNAs⁶⁹,⁷⁰ and proposed “tRNA modopathy” as a distinct category of human diseases⁷¹. Recent whole-exome sequencing analyses identified loss-of-function mutations in many genes responsible for tRNA modification from patients with a wide range of diseases, including neurological disorders, cancers, and diabetes⁵⁸–⁷³. DTWD1 is a target of p53 and works as a tumor suppressor gene for gastric cancer⁷⁴ and is downregulated in some cancer cell lines. HDAC3 regulates p53-mediated expression of DTWD1. In primary melanomas, high expression of DTWD1 mediated by HIF-1α correlates with poor prognosis and shorter disease-free status⁷⁵. Mutation in DTWD1 is also a risk factor for bipolar disease⁷⁶. Copy number variation in the genome region containing DTWD2 has been observed in primary open-angle glaucoma⁷⁷. Further studies will be necessary to understand the molecular pathogenesis of human diseases associated with loss of acp³U modification in tRNAs.

Methods

Bacterial strains, media, and plasmid construction. Δyfp (JW5409) or ΔyogA (JW0260) with kanamycin resistance markers (Keio collection)⁷⁸ and their parental E. coli strain BW25113 (obtained from the Genetic Stock Resource Center, National Institute of Genetics, Japan) were cultivated in LB medium with vigorous shaking. A. ferrooxidans (obtained from the Biological Resource Center, National Institute of Technology and Evaluation, Japan) was cultivated in 0.5 g/L MgSO₄·7H₂O, 0.4 g/L (NH₄)₂SO₄, 0.2 g/L K₂HPO₄, 0.1 g/L KCl, 10 mg/L FeSO₄·7H₂O, and 0.025% yeast extract (adjusted to pH 2.0 with 2 M H₂SO₄) at 45°C⁷⁹. Synechocystis sp. PCC 6803 (kindly provided by K. Sonoike of Waseda University, Japan) was cultured with BG11 medium⁸⁰ under LED light at 34°C. T. thermophila HB27 (kindly provided by N. Shig of AIST, Japan) was cultured in rich medium containing 0.8% peptone, 0.4% yeast extract, and 0.3% NaCl (adjusted to pH 7.5 with 1 M HCl) at 70°C rich medium as previously described⁸¹. B. subtilis str.168 (kindly provided by A. Soma of Chiba University, Japan) was cultured in LB medium at 37°C⁸². M. mobile 163K (kindly provided by M. Miyata of Osaka City University, Japan) was grown in Ahsatto medium (pH 7.8), consisting of 2.1% heart infusion broth (Difco), 0.56% yeast extract, 10% horse serum (inactivated at 56°C), and 0.005% ampicillin at 25°C⁸³.

To construct a plasmid encoding yfp gene for complementation, the open reading frame of yfp with promoter region was PCR-amplified from the E. coli genome using a set of primers listed in Supplementary Table 1. Amplified products were inserted into the HindIII/EcoRI sites of the pMW118 vector (Invitrogen). Human cell culture and measurement of cell proliferation. Human cell culture and measurement of cell proliferation were inserted into the plasmid pLL3.7 vectors harboring a puromycin resistance gene. One day after transfection, the cells were seeded at low density and selected with 1 μg/mL puromycin. The KO cell lines were selected by confirming the frameshift mutations in the target region. DTWD1/DTWD2 double-KO cells were generated from the DTWD1 single-KO cells.

RNA extraction and isolation of individual tRNAs. Total RNAs of E. coli were extracted as described⁸⁶. Total RNAs of A. ferrooxidans, Synechocystis sp. PCC 6803, T. thermophila HB27, B. subtilis str.168, and M. mobile 163K were extracted using the acidic phenol method as described⁷⁹,⁸⁵. Total RNAs were extracted from HEK293T cells using TriPure Isolation Reagent (Roche Life Science).

tRNA and snRNA isoforms were isolated using an LCQ off-line chromatography system as described⁸⁷ using the 5′-terminal ethylcarbamate amino-modified DNA probes (Sigma Aldrich Japan) listed in Supplementary Table 1.

Mass spectrometric analysis. For nucleoside analysis of E. coli, total RNAs (1.6 μg) were digested with 0.8 U nuclease P1 (FUJIFILM Wako Pure Chemical) at 37°C for 60 min. The digests were adjusted to 50 mM ammonium bicarbonate (pH 8.2), followed by addition of 0.2 U phosphodiesterase I ( Worthington) and left overnight at 37°C. After extraction with phenol/ chloroform (RAP, from E. coli C75, FUJIFILM Wako Pure Chemical) was added, and incubated at 37°C for 60 min, as described⁸⁶. Nucleosides were dissolved in 90% acetonitrile (10% water), and subjected to a hydrophilic interaction LC (ZIC-HILIC, 3 μm particle size, 2.1 × 130 mm, Merck) coupled with ESI-MS on a Q Exactive Hybrid Orbitrap mass spectrometer (Thermo Fisher Scientific) equilibrated with an ESI source and an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific)⁸⁸. The mobile phase consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and acetonitrile (solvent B), and the nucleosides were chromatographed with a flow rate of 100 μL/min in a multistep linear gradient; 90–40% B from 0 to 30 min, 40% B for 10 min, and then initial to 90% B. Precursors of nucleosides were scanned in a positive polarity mode over an m/z range of 103–700. Xcalibur 3.0.63 (Thermo Fisher Scientific) was used for the system operation.

Nucleoside analyses for comparative genomics were performed by reverse-phase chromatography/ESI/MS. Total RNAs (16–160 μg) were digested in a reaction mixture containing with 20 mM ammonium acetate (pH 5.3), 0.04–0.08 U BAP, and 0.05–0.1 U nuclease P1, and subjected to InertSil ODS-3 (5 μm particle size, 2.1 × 250 mm, GL sciences) or SunShell C18 (2.6 μm particle size, 2.1 × 150 mm, ChromaNik Technologies) column, then analyzed by an LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source and an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific)⁸⁹. The mobile phase consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and acetonitrile (solvent B). The nucleosides were chromatographed with a flow rate of 100 μL/min in a multistep linear gradient; 90–40% B from 0 to 30 min, 40% B for 10 min, and then initial to 90% B. Precursor adducts of nucleosides were scanned in a positive polarity mode over an m/z range of 103–700. Xcalibur 3.0.63 (Thermo Fisher Scientific) was used for the system operation.

For RNA fragment analysis, the isolated tRNAs (2 pmol) were digested with 10 U of Rnase T₁ in 20 mM ammonium acetate (pH 5.3) at 37°C for 60 min. The digests were mixed with 1/10 vol. of 0.1 M triethylamine acetate (pH 7.0) and subjected to LC/MS/MS or LC/Orbitrap Fusion (Thermo Fisher Scientific) equipped with a stainless nanoflow high-performance LC (nano-HPLC) system (DiNa, KYA Technologies) using a nano-LC trap column (C18, 0.1 × 0.5 mm, KYA Technologies) and a capillary column (HypSil C18 3.5 μm, 0.1 × 100 mm, KYA Technologies) as described⁹⁰. Digested fragments were separated for 40 min at a flow rate of 390 nl/min by capillary LC using a linear gradient from 2% to 100% solvent B (v/v) in a solvent system consisting of 0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (pH 7.0) (solvent A) and 0.4 M HIFP (pH 7.0) in 50% methanol (solvent B). The eluent was ionized by ESI source in a negative polarity and scanned over an m/z range of 600–2000. Xcalibur 2.0.7 (Thermo Fisher Scientific) was used for the system operation.

The LC/MS data were analyzed using Xcalibur Qual browser (Thermo Fisher Scientific). In nucleoside analysis, the peak of modified nucleoside was normalized by the peak area of the unmodified nucleoside. In RNA fragment analysis, the peak of each fragment was normalized by the sum of modified and unmodified fragments. Mango Oligo Mass Calculator v2.08 (https://modona.rna.albany.edu/masspec/Mongo-Oligo) was used for assignment of the product ions in CID spectra.

Expression and purification of recombinant protein. To construct an expression plasmid for TapT recombinant protein, the open reading frame of yfp was PCR-amplified from the E. coli genome using the primers listed in Supplementary Table 1. The amplified products were inserted into the NdeI/XhoI sites of the pRSET-TapT plasmid, which encodes His-tagged TapT protein. The E. coli BL21(DE3) strain was transformed with the plasmid and cultured at 37°C. When the absorbance at 600 nm (A₆₀₀) reached 0.5,
isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM for inducing expression, and the cells were cultured at 18 °C for an additional 24 h. The cells were harvested by centrifugation for the yeast target-Tag procedure. The cells were fixed with 3% formaldehyde, permeabilized with 1% Triton X-100, and blocked with 20% Ezblochekmi (ATTO). Immunostaining was performed with anti-DYKDDDDK IgG antibody (1:1000) (014–22383, Wako) for the primary antibody and anti-mouse IgG Alexa fluor 488 (1:1000) (A-11001, Envirogent) for the secondary antibody. Then, cells were stained with DAPI (1:10,000). The pictures were taken using DMI 6000 (Leica).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this Article.

**Data availability**

A reporting summary for this article is available as a Supplementary Information file. The source data underlying Figs. 2i, 3b–d, and 5a and Supplementary Figs. 3a, 4a, b, and 7a, b are provided as a Source Data file. All data supporting the findings in this study are available from the corresponding author upon reasonable request.

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In vitro reconstitution of acpP by TapT. For the substrates, we isolated tRNA^Met from the Δaapt strain by reciprocal circulating chromatography. The tRNA^Met transcript was prepared by in vitro transcription using T7 RNA polymerase, essentially as described. The synthetic DNAs used for template construction by PCR are listed in Supplementary Table 1. In vitro transcription was performed at 37 °C overnight in a reaction mixture containing 40 mM Tris-Cl (pH 7.5), 25 mM MgCl2, 5 mM DTT, 2.5 mM spermidine, 0.01% Triton X-100, 30 mM DNA template, 2 mM GTP, 2 mM CTP, 2 mM UTP, 2 mM ATP, and 10 mM GMP. Subsequently, the reaction mixture was subjected to phenol–chloroform extraction and desalted by passing through a PD-10 column (GE Healthcare). The transcribed tRNA was purified by running on 10% denaturing polyacrylamide gel electrophoresis (PAGE).

In vitro reconstitution of acpP^U by TapT. The tRNA product was digested with RNase T1 (Thermo Fisher Scientific) and subjected to LC/MS analysis as described above.

**Morphological analysis under heat-stressed conditions.** E. coli strains were cultivated in 5 mL LB medium with vigorous shaking at 37 °C or 42 °C for 16–24 h until reaching the late stationary phase. Then, cells were diluted by a factor of 10^6 or 10^7 so that the concentration was suitable for counting the colony numbers. One hundred microliters of the diluted cells were spread on LB agar plate and cultured at 37 °C overnight. In parallel, 2.5 μL harvested cells was inoculated into 5 mL fresh LB medium and cultured at the same temperature. The process described above was repeated for 5–7 days. The frequency of small colonies appearing on the plate was calculated by counting the number of normal and small colonies on each plate.

**Motility assay.** The motility assay was performed basically as described. Pre-cultured E. coli cells were diluted to 0.01 A595, and then 2 μL diluted cells was inoculated at the center of the soft agar plate (1% tryptone, 1% NaCl, and 0.3% agar). The plates were cultured at 30 °C for 16 h. The swarming diameter of each strain was averaged from five different sets of measurements.

**Mutator assay.** The mutator assay was carried out basically as described. In addition to the ΔtapT strain, ΔmutS, ΔmutA, ΔuagY strains were used as the strong mutator, mild mutator, and negative controls, respectively. E. coli cells were cultivated at 42 °C for 5 days and then plated on LB plates at every passage as described in the morphological analysis section. At every passage, adequate amounts of the harvested cells were spread on LB agar plates containing 80 μg/mL nalidixic acid and cultured at 37°C overnight. Mutation rate was calculated from the ratio of colony numbers on the LB plates with and without nalidixic acid.

**Northern blotting.** Total RNAs (4 μg) from WT HEK293T and DTDW1/DTDW2 double-KO cells were dissolved by 10% denaturing PAGE, stained with SYBR gold (Invitrogen), and blotted onto a nylon membrane (Amslermark HyBond N+; GE Healthcare) in 0.5 × TBE using a Transblot Turbo apparatus (Bio-Rad). Hybridization was performed essentially according to the manufacturer’s instructions (PerfectHyb; TOYOBO) at 42 °C with 2 μmol of 5′-32P-labeled oligonucleotides (Supplementary Table 1) complementary to each target tRNA. The membrane was washed three times with 2 × SSC, dried, and exposed to an imaging plate (BAS-MS2040; Fujifilm). Radioactivity was visualized using an FIA-7000 imaging analyzer (Fujifilm).

**Immunostaining.** Hela cells (4.0 × 10^5 cells per dish) were transfected with the pcDNA-3.1 vectors (Invitrogen) bearing the open reading frame of DTDW1, DTDW2, or DTDW2Δd, each of which expresses the C-terminally Flag-tagged protein. The cells were fixed with 3% formaldehyde, permeabilized with 1% Triton X-100, and blocked with 20% Ezblochekmi (ATTO). Immunostaining was performed with anti-DYKDDDDK IgG antibody (1:1000) (014–22383, Wako) for the primary antibody and anti-mouse IgG Alexa fluor 488 (1:1000) (A-11001, Envirogent) for the secondary antibody. Then, cells were stained with DAPI (1:10,000). The pictures were taken using DMI 6000 (Leica).
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Author contributions

M.T. and K.I. mainly performed most of experiments. S.A. and K.M. assisted genetic and biochemical works. All authors discussed the results. K.M. and T.S. designed the research. K.I., M.T. and T.S. wrote this paper. T.S. supervised all the work.

Competing interests

The authors declare no competing interests.

Additional information

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