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REVIEW

The modified base isopentenyladenosine and its derivatives in tRNA

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ABSTRACT

Base 37 in tRNA, 3′-adjacent to the anticodon, is occupied by a purine base that is thought to stabilize codon recognition by stacking interactions on the first Watson-Crick base pair. If the first codon position forms an A.U or U.A base pair, the purine is likely further modified in all domains of life. One of the first base modifications found in tRNA is N^6-isopentenyl adenosine (i^6A) present in a fraction of tRNAs in bacteria and eukaryotes, which can be further modified to 2-methyl-thio-N^6-isopentenyladenosine (ms^2i^6A) in a subset of tRNAs. Homologous tRNA isopentenyl transferase enzymes have been identified in bacteria (MiaA), yeast (Mod5, Tit1), roundworm (GRO-1), and mammals (TRIT1). In eukaryotes, isopentenylation of cytoplasmic and mitochondrial tRNAs is mediated by products of the same gene. Accordingly, a patient with homozygous mutations in *TRIT1* has mitochondrial disease. The role of i^6A in a subset of tRNAs in gene expression has been linked with translational fidelity, speed of translation, skewed gene expression, and non-sense suppression. This review will not cover the action of i^6A as a cytokinin in plants or the potential function of Mod5 as a prion in yeast.

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Modification of purines at position 37 in tRNA

Among the over 100 base modifications occurring in tRNA, many target the anticodon stem loop, in particular base 34 reading the wobble base and the dangling base 37 3′-adjacent to the anticodon.[1,2] Base modifications present at base 37 include mostly bulky additions to adenosine, i.e. N^6-isopentenyladenosine (i^6A), 2-methyl-thio i^6A (ms^2i^6A), 6-hydroxy ms^2i^6A (ms^2io^6A), N^6-threonylcarbamoyladenosine (t^6A), ms^2t^6A, and modification of guanosine, to N^1-methylguanosine (m^1G) or wybutosine (yW) (Fig. 1). The i^6A modified base was one of the first hypermodified bases identified and has been found in bacteria and eukaryotes, but not in archeae.[3,4] Codon-anticodon interactions are considered weaker in the case of A.U base pairs than in case of G.C base pairs.[5] It has been argued that A.U base pairs on the first codon position (base 36 of tRNA) need a modified base 37 which would stabilize the Watson-Crick base pair by base stacking. In addition, the modification prevents illicit hydrogen bonding between U33 and A37 that would otherwise disturb the anticodon loop.[6,7] Codons starting with A are coding for Thr (ACN), Ser (AGY), Arg (AGR), Lys (AAR), Asn (AAY), Met (AUG), and Ile (AUY and AUA). Invariably, as for example in $tRNA^{Lys}$, U36 is followed by t^6A37.[2,8,9] Codons starting with U are coding for Ser (UCN), Cys (UGY), Trp (UGG), Phe (UUY), Leu (UUR), and Tyr (UAY). Accordingly, these codons are read by tRNAs having an A at position 36 that is usually followed by a modified base at position 37.[2] Modification of G37 to yW in $tRNA^{Phe}$ (UUU) is a classical example and remarkable, because yW37 is exclusively found in $tRNA^{Phe}$.[10] Most other tRNAs reading codons starting with U and bearing a sequence of A36-A37-A38 are isopentenylated, except e.g. mt-$tRNA^{Leu}$ (UUR).[8] While the AAA motif thus appears necessary, it is not sufficient to signal isopentenylation of A36 in tRNA.[11,12] Predictions on the presence and identity of A37 modifications from sequence alone are thus not possible at the moment. Therefore, a systematic study on bovine mitochondrial tRNA modification has been conducted using mass spectrometry that has identified all base 37 modifications.[8] Unfortunately, data on mammalian cytoplasmic tRNAs is still incomplete with respect to analyzed tRNAs, and has not always conclusively demonstrated the identity of the modified base.[11] The matter is further complicated by potential species differences. We have tried to summarize available data on tRNAs carrying i^6A and ms^2i^6A in *E. coli* (Table 1), the yeasts *S. cerevisiae, S. pombe* (Table 2), and mammals (Table 3). We included tRNAs that are isopentenylated in one of the species, but not in others, to highlight species differences. Data from tRNAdb was integrated.[13] In tRNA, all available data suggests that i^6A and its derivatives are exclusively found at position 37.

Isopentenyladenosine was among the first modified bases identified in tRNA, and was localized next to the anticodon in 2 Ser isoacceptors from yeast that were among the first tRNA sequenced.[16,17] The biochemical pathway leading to i^6A or ms^2i^6A formation in *E. coli* was initially discovered with the *trpX*$^-$/*miaA* mutant lacking ms^2i^6A in $tRNA^{Trp18-22}$ Subsequently, *miaB* was identified as the gene required for formation of ms^2i^6A, and *miaE* was found to catalyze ms^2io^6A in *S. typhimurium*.[3] Isopentenyladenosine is thus further modified to

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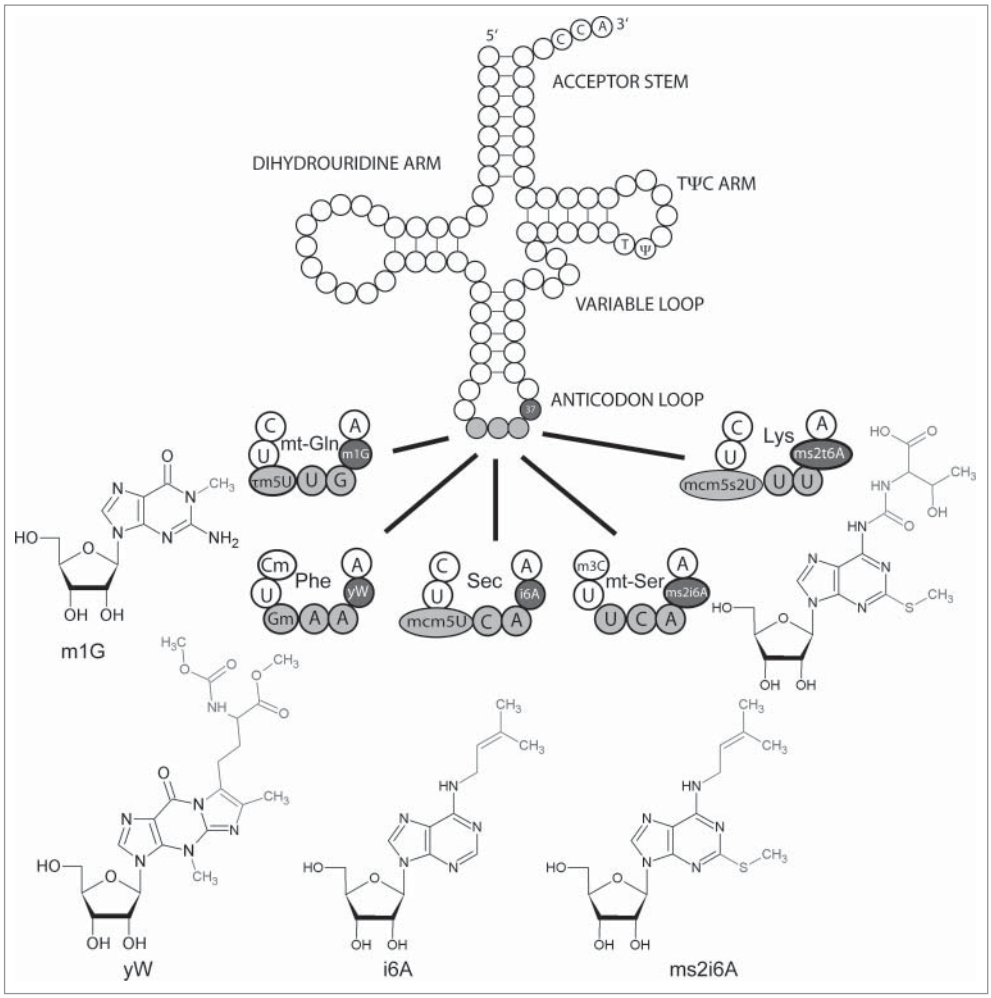

Figure 1. Canonical tRNA structure with base 37 modifications. Canonical tRNA structure and 5 individual anticodon loops of tRNAs that carry different base 37 modifications. Where appropriate, base 32 and 34 modifications are indicated as well. Structural formulae indicate the modified nucleosides at position 37. Respective drawings of base modifications have been copied from www.modomics.genesilico.pl with permission.[14,15] Cm 2′O-methylcytosine; m^1G 1-Methylguanosine; m^3C 3-Methylcytosine, τm^5U 5-taurinomethyluridine, Gm 2′O-methyl Guanosine, mcm^5U 5-Methoxycarbonylmethyluridine, mcm^5s^2U 5-Methoxycarbonylmethyl-2-thiouridine, ms^2i^6A 2-Methylthio-N^6-isopentenyladenosine, ms^2t^6A 2-Methylthio-N^6-threonylcarbamoyladenosine, yW wybutosine.

ms^2i^6A by MiaB and can be hydroxylated by MiaE to ms^2io^6A in *Salmonella*. MiaB is the founding member of a family of radical SAM enzymes involved in tRNA modification.[23] When tRNA from *mod5–1* mutants in *S. cerevisiae* was found deficient in i^6A,[24] the first eukaryotic isopentenyl transferase gene was identified. Cloning revealed that *miaA* and *mod5* are homologous genes,[21,25,26] and homology cloning led to the identification of *TRIT1* in humans,[27] suggesting that all tRNA isopentenyl transferases are homologous genes in all domains of life. The biochemistry of isopentenyl transferases will be shortly discussed toward the end of the article. In mammals, thiomethylation reactions are performed by the radical SAM enzymes CDK5RAP1 and CDKAL1 acting on i^6A37 and t^6A37, respectively.[28,29] While i^6A and ms^2i^6A have been reported in miRNA and poly A^+ RNA from HeLa cells,[28] their potential role in these RNAs and in other species is not known.

Table 1. *E. coli* tRNAs carrying i^6A37 or ms^2i^6A37.

tRNA				
Codon (5′–3′)	Anticodon (5′–3′)	Modification	Method	Reference
Cys UGC	GCA	ms^2i^6A		tRNAdb
Leu UUR	HAA	ms^2i^6A	Sanger, 2D TLC	[104]
Phe UUC	GAA	ms^2i^6A	Sanger, 2D TLC	[105]
Ser UCA	cmo^5UGA	ms^2i^6A	Sanger, 2D TLC	[106]
Ser UCG	CGA	ms^2i^6A	Sanger, 2D TLC	[106]
Trp UGG	CCA	ms^2i^6A	Sanger, 2D TLC	[107]
Tyr UAU	QUA	ms^2i^6A	Sanger, 2D TLC	[108]
Tyr UAU	QUA	ms^2i^6A	Sanger, 2D TLC	[108]
Sec UGA	UCA	i^6A	HPLC, Sanger, 2D TLC	[31]
Leu UUG	CmAA	ms^2i^6A		tRNAdb

$^{\Psi}$pseudouridine, Q queosine base, cmo^5U Uridine 5-oxyacetic acid, Sanger Sanger sequencing, TLC thin layer chromatograpy.

Delineating the biologic functions of i^6A and ms^2i^6A

E.coli (miaA) and S. typhymurium (miaA1)

Escherichia coli tRNAs decoding codons starting with U contain i^6A or ms^2i^6A at position 37, i.e., Phe UUY, Leu UUR, Cys UGY, Trp UGG, Tyr UAY, and Ser UCN[30] (Table 1). The product of the *selC* gene in *E. coli* is a bacterial $tRNA^{[Ser]Sec}$ that reads UGA codons as selenocysteine (Sec). This tRNA carries i^6A37 like its mammalian counterpart[31] (see below). In ribosome binding assays it was shown that tRNAs lacking their natural ms^2i^6A or i^6A modification had a reduced affinity to their

Table 2. Yeast tRNAs carrying i^6A37 and tRNAs that carry i^6A in other species.

Cytoplasmic					
Codon (5′–3′)	Anticodon (5′–3′)	Modification, species		Method	Reference
Cys UGC	GCA	i^6A	*S. cerevisiae*	*In vitro* isopentenylation	[60]; tRNAdb
Ser UCU, UCC	IGA	i^6A	*S. cerevisiae*	BD-cellulose chromatography; *In vitro* isopentenylation; Reversed phase chromatography	[16, 24, 49, 60]; tRNAdb
Ser UCG	CGA	i^6A	*S. cerevisiae*	BD-cellulose chromatography; TLC; PHA6 assay; *In vitro* isopentenylation; Reversed phase chromatography	[24, 49, 60, 109]
Ser UCA	UGA	i^6A	*S. cerevisiae*	BD-cellulose chromatography; *In vitro* isopentenylation; Reversed phase chromatography	[24, 49]
Tyr UAC	GΨA	i^6A	*S. cerevisiae*	Anion exchange chromatography after T1 digestion; BD-cellulose chromatography; *In vitro* isopentenylation; Reversed phase chromatography	[24, 49, 60, 110]; tRNAdb
Trp UGG	CmCA	A*	*S. cerevisiae*	RNA sequencing; Reversed phase chromatography; *In vitro* isopentenylation; PHA6 assay	[49, 60, 111]; tRNAdb
Ser UCU, UCC	IGA	i^6A	*S. pombe*	RPC-5; *In vitro* isopentenylation; PHA6 assay	[57, 60]
Ser UCG	CGA	i^6A	*S. pombe*	RPC-5; *In vitro* isopentenylation	[57, 60]
Ser UCA	UGA	i^6A	*S. pombe*	RPC-5; RNA sequencing; *In vitro* isopentenylation	[57, 59, 60]
Tyr UAC	GΨA	i^6A	*S. pombe*	RPC-5; 2D-Sanger and TLC; *In vitro* isopentenylation	[57, 60]; tRNAdb
Trp UGG	CCA	i^6A	*S. pombe*	RPC-5; *In vitro* isopentenylation; PHA6 assay	[57, 60]
Mitochondrial					
Codon (5′–3′)	Anticodon (5′–3′)	Modification, species		Method	Reference
Trp UGR	?CCA	i^6A	*S. cerevisiae*	PHA assay; Reversed phase chromatography	[49, 55]
Cys UGC	GCA	i^6A	*S. cerevisiae*		tRNAdb
Ser UCN	IGA	i^6A	*S. cerevisiae*		tRNAdb
Ser UCN	IGA	A*	*S. cerevisiae*	Reversed phase chromatography	[49]
Tyr UAC	GΨA	i^6A	*S. cerevisiae*	Reversed phase chromatography	[49]
Trp UGG		i^6A	*S. pombe*	PHA6 assay	[61]
Cys UGC		A	*S. pombe*	PHA6 assay	[61]
Tyr UAC	GΨA	i^6A	*S. pombe*		tRNAdb
Ser UCN	NGA	A	*S. pombe*	PHA6 assay	[61]

Ψpseudouridine,
*conflicting data.

cognate codons.[32] Similarly, the function of non-isopentenylated $tRNA^{Trp}$ is apparently impaired, as a mutant that was defective in attenuation of the *trp* operon, *trpX*$^-$ lacks isopentenylation of $tRNA^{Trp}$.[22] The *trp* operon is not solely transcriptionally regulated through a repressor/operator system, but also by a process called attenuation. In this regulatory process, termination of *trp* operon transcription is regulated by translation of a short translational reading frame that contains 2 consecutive UGG Trp codons. If Trp-$tRNA^{Trp}$ is plentiful in the cell, a ribosome swiftly passes through the open reading frame and disrupts by its presence a potential stem loop in the newly synthesized mRNA. Formation of an alternative stem loop induces termination of transcription. If Trp is scarce, translation through this open reading frame is slow, the terminator stem loop does not form, but another, and transcription of the *trp* operon proceeds. As *trpX*$^-$/*miaA*$^-$ cells lack isopentenylation of $tRNA^{Trp}$ (and others) and are deficient in attenuation, the hypomodified $tRNA^{Trp}$ is apparently less efficient in translation than the fully modified species. In fact, it was this publication in which the gene name *miaA* was coined as the first gene related to deficiency in $\mathbf{ms^2i^6A}$.[22] Iron restriction likewise impaired attenuation of the *phe* and *trp* operons in *E. coli*,[33] probably because the MiaB enzyme contains a [4Fe-4S] cluster needed for methylthiolation of i^6A-modified A37.[3,23] The ms^2i^6A tRNA modification appears to be harnessed as a regulatory mechanism to induce genes involved in iron accumulation in bacteria. To slow bacterial growth, bacteria-infected hosts will reduce iron available in body fluids by increasing iron-chelating protein. Pathogenic bacteria in turn will make the Fe^{3+}-chelating compound enterochelin (enterobactin) and express a membrane receptor for the Fe^{3+}-enterochelin complex to maintain their iron supply and growth.[34-36] Enterochelin biosynthesis is constitutively enhanced in *miaA S. typhimurium*. Cysteine stress and anaerobic growth likewise enhanced enterochelin along with decreased formation of ms^2io^6A.[37] Whether only the modification of tRNAs for aromatic amino acids or also $tRNA^{Ser}$ play a role in this process remained unclear.[37]

Table 3. Mammalian tRNAs carrying i^6A37 or ms^2i^6A37 and tRNAs that carry i^6A in other species.

Cytoplasmic					
Codon (5′–3′)	Anticodon (5′–3′)	Modification, species		Method	Reference
Ser UCN	IGA	i^6A	Rat liver, Morris hepatoma		tRNAdb
Ser UCN	IGA	mod	HeLa	PHA6 assay	[11]
	CGA	mod	HeLa	PHA6 assay	[84]
	UGA	mod	HeLa	PHA6 assay; *In vitro* isopentenylation	[84]
Ser UCN	UGA	mod	muscle	PHA6 assay	[84]
Sec UGA	mcm^5UCA	i^6A	Mouse, human, bovine		[74]; tRNAdb
Sec UGA	UCA	i^6A	human	*In vitro* isopentenylation	[84]
Sec UGA	UCA	i^6A	Mouse	*In vitro* isopentenylation	[67]

Mitochondrial					
Codon (5′–3′)	Anticodon (5′–3′)	Modification, species		Method	Reference
Tyr UAY	QUA	ms^2i^6A	Bovine	Mass spec	[8]
Tyr UAC	galQΨA	m^1G	bovine		tRNAdb
Tyr UAC	XYA	ms^2i^6A	Mouse liver	Mass spec	[85]
Phe UUY	GAA	ms^2i^6A	Bovine	Mass spec	[8]
Phe UUY	GAA	ms^2i^6A	rat		tRNAdb
Phe UUY	GAA	ms^2i^6A	Mouse liver	Mass spec	[85]
Cys UGY	GCA	i^6A	Bovine	Mass spec	[8]
Cys UGY	GCA	A*	Human muscle, HeLa	PHA6 assay; *In vitro* isopentenylation	[11, 84]
Ser UCN	UGA	ms^2i^6A	Bovine	Mass spec	[8]
Ser UCN	UGA	mod	HeLa	PHA6 assay	[11]
Ser UCN	UGA	mod i^6A	human	*In vitro* isopentenylation	[84]
Ser UCN	UGA	i^6A	bovine		tRNAdb
Ser UCN	UGA	i^6A	Rat liver		tRNAdb
Ser UCN	UGA	ms^2i^6A	Mouse liver	Mass spec	[85]
Trp UGA	τm^5UCA	ms^2i^6A	Bovine	Mass spec	[8]
Trp UGA	UCA	mod	HeLa	PHA6 assay	[11]
Trp UGA	UCA	ms^2i^6A	Rat liver		tRNAdb
Trp UGA	τm^5U CA	ms^2i^6A	Mouse liver	Mass spec	[85]

PHA6 assay: positive hybridization in the absence of i^6A assay [11] does not strictly differentiate between i^6A and ms^2i^6A and may only detect the presence of a modification at position 37. If the modification was not positively identified, we denote this with "mod,"
*conflicting data.
Ψpseudouridine, Q queosine base, τm^5U taurinomethyl^{5}U.

Lack of tRNA isopentenylation reduced growth rates in *E. coli* only marginally when grown in full media.[22,38] *Salmonella typhimurium miaA* mutants show slower growth rates and cellular protein production on several substrates.[39] The metabolic effects of impaired tRNA isopentenylation have been reviewed in great detail.[1,3] While modification of A37 was not demonstrated to influence tRNA charging reactions, it was shown to make decoding of UAG codons by suppressor tRNAs within a *lacI-lacZ* reporter gene more independent of codon context in *E. coli* and *S. typhimurium*.[40] The same paper also showed that near-cognate misreading of certain codons is reduced when the base 37 modification is not present.[40] Likewise, in an in vitro system of polyU translation with ms^2i^6A-dependent $tRNA^{Phe}$, decoding was more accurate in the absence of the modification,[41] possibly because near cognate decoding was less favored. The role of base 37 modifications of $tRNA^{Phe}_{GAA}$ and $tRNA^{Tyr}_{QUA}$ was dissected in *S. typhimurium miaA, miaB*, and *miaE* mutants. P-site slippage (+1 frame-shifting) was found increased 3–9-fold by tRNAs lacking the ms^2-modification, while lack of i^6A had no greater effect than lack of the thiomethylation alone.[42] In contrast, of tRNAs normally carrying m^1G37, only $tRNA^{Arg}_{CCG}$ caused a 3-fold increase in P-site slippage. Lack of modification of G37 thus seems less critical than lack of modification of A37 with respect to +1 frame-shifting.[42]

A *miaA* mutation in the pathogenic bacterium *Shigella flexneri* decreased i^6A modification of tRNAs, decreased expression of several virulence genes, but did not affect their mRNA levels suggesting that the effect was at the level of translation.[43] Complementation with a functional *miaA* gene demonstrated that the effect on virulence gene expression depended on tRNA isopentenylation.

Skewed gene expression that may be modulated by the use of rare codons or codons depending on specific modifications has been proposed for many years. It lies in the nature of such a mechanism that it is hard to prove and specificity is always an issue, but available data suggest its existence.[44,45] A particularly instructive example for both the use of rare codons and the dependence of specific genes on specific tRNA modifications is illustrated by the stationary phase sigma factor *rpoS* (σ^S) in *E. coli.* In contrast to its related gene, the vegetative sigma factor *rpoD, rpoS* contains rare codons at many positions. Replacing these codons with synonymous frequent codons reduced the levels of RpoS protein by destabilizing its mRNA[46] and it has been argued that ribosomal pausing might increase ribosomal density on the mRNA and thus protect the message from RNaseE-dependent degradation. On the other hand, one of the many mechanisms of RpoS induction in stationary growth may be increased availability of charged rare tRNAs. Recently, it was discovered that RpoS expression is reduced

in *miaA* mutant cells.[47] More recently it was shown that MiaA activity was necessary for proper decoding of UUR-Leu codons in RpoS. Exchange of the UUR (Leu) codons with CUY (Leu) codons in a *rpoS-lacZ* reporter gene alleviated the requirement for MiaA. Expression of a RpoS-lacZ reporter protein could be increased by overexpression of a *miaA*-independent $tRNA^{Leu}$.[48] Translation of RpoS is thus regulated by the availability of i^6A-modified tRNAs reading rare codons.

Mod5 in S. cerevisiae

The *mod5–1* mutation reduced the efficiency of a $tRNA^{Tyr}$ UAA suppressor. The mutant showed decreased levels of i^6A, and an altered chromatographic behavior of $tRNA^{Tyr}$ and $tRNA^{Ser}$ on benzoylated DEAE-sepharose columns.. tRNA aminoacylation and other tRNA modifications were not altered in the mutant. The lack of i^6A had almost no effect on its growth rate on several complex or simple media at either 28°C and 37°C. The only finding was that *mod5–1* homozygotes failed to sporulate.[24] Table 2 lists all yeast tRNAs that are isopentenylated. The same gene is required to modify cytosolic and mitochondrial tRNAs.[49] Alternative usage of 2 initiation codons, at positions 1 and 12, produces a longer mitochondrial isoform containing an N-terminal mitochondrial import sequence, and a shorter cytosolic isoform.[50-52] The cytosolic isoform was also found in nuclei, especially in the nucleolus, and contains a nuclear localization sequence.[53,54] Interestingly, ms^2i^6A is neither found in yeast cytoplasmic nor mitochondrial tRNAs suggesting that the respective MiaB homolog is entirely absent in yeasts[55] (Table 2).

Tit1 in S. pombe

A Tit1 mutant strain (*sin1*) exhibited a loss of tRNA suppression of the UGA codon, which was translated as serine by $tRNA^{Ser}_{UCA}$.[56-59] This tRNA was not isopentenylated. Moreover, other tRNAs were found non-isopentenylated in this strain, such as $tRNA^{Tyr}$ and $tRNA^{Trp}$.[57] As with *mod5–1*, changes in cell growth of these mutants were hardly apparent. The sequence determinants for tRNA isopentenylation are not yet fully known. The A36A37A38 sequence in the anticodon stem loop is a necessary, but not sufficient, determinant for isopentenylation as shown in *E. coli*.[12] For example, cy-$tRNA^{Trp}_{CCA}$, is isopentenylated in *S. pombe*, but not in *S. cerevisiae*. In contrast, differential modification of cy-$tRNA^{Cys}_{GCA}$ in *S. cerevisiae*, but not in *S. pombe*, is fully explained by guanosine at position 37 in the *S. pombe* $tRNA^{Cys}$. Substrates of both enzymes are cy-$tRNA^{Tyr}_{G\Psi A}$, cy-$tRNA^{Ser}_{IGA}$, cy-$tRNA^{Ser}_{CGA}$, cy-$tRNA^{Ser}_{UGA}$[60] (Table 2). The mitochondrial isoform of Tit1 is unknown. However, Lamichhane and coworkers showed mt-$tRNA^{Trp}$ was partially modified by Tit1 but not mt-$tRNA^{Cys}$ or mt-$tRNA^{Ser}$[61] (Table 2).

Rapamycin is an inhibitor of the mTOR pathway, which controls growth and proliferation. *S. pombe* is insensitive to rapamycin because of partial inhibition of TORC1 activity.[62] However, *tit1* mutants are sensitive to rapamycin and show decreased growth rate, shorten phenotype and reduced protein translation, especially of several unidentified proteins.[61] One could speculate that the lack of isopentenylation might change the amino acid composition of a component of TORC1 and make it more sensitive to rapamycin, since it was also demonstrated in the same publication that isopentenylation promoted cy-$tRNA^{Tyr}_{G\Psi A}$ misreading of a near-cognate Cys UGC codon in a ß-galactosidase reporter.

In contrast to factor *rpoS* of *E. coli*, there is no clear example of skewed gene expression in *S. pombe*. Computational analysis demonstrated enrichment of mRNAs with i^6A dependent codons among ribosomal proteins and certain mitochondrial enzymes. Accordingly, ribosomal proteins S14 and L12[61] and the elongation factor Tif512[55] mRNAs showed a reduced sucrose gradient sedimentation polysome profile in *tit1* mutant cells. One could speculate that a global reduction of protein translation shown by *tit1* mutants is due to mistranslated proteins involved in translation.

Mitochondrial dysfunction in yeasts can be unveiled by slow growth on glycerol. *Tit1* mutant cells showed this phenotype. While one might have expected that hypomodification of mt-$tRNA^{Trp}$ could explain the phenotype, it was surprisingly rescued with the cytosolic isoform of Mod5 or cy-$tRNA^{Tyr}$ overexpression. This finding is in agreement with the estimate of cy-$tRNA^{Tyr}$ as the most limiting i^6A modified cytosolic tRNA in *S. pombe*. The authors explained their finding with mitochondrial proteins involved in energy metabolism, being translated in the cytoplasm where they depend on cy-$tRNA^{Tyr}$.[55]

gro-1 in Caenorhabditis elegans

The only metazoan model organism deficient in tRNA isopentenylation is the *gro-1* (*e2400*) mutant in the roundworm *C. elegans*.[63] It was initially identified in a screen for maternally rescued mutants with extended life-span.[64] Maternal rescue designates the phenotypic rescue of homozygous mutants through a process when heterozygous mothers pass a (regulatory) factor on to their offspring through deposition of protein or RNA in eggs. The *gro-1* mutants displayed slower growth and development at several temperatures and significantly extended life-span. Since adult life-span was not increased, this means that larval development was slower. Backcrossing to the standard N2 genetic background decreased the effect on life-span.[63] The protein encoded by *gro-1* is the tRNA isopentenyltransferase homologous to MiaA, Tit1, and Mod5. Like Mod5, GRO-1 contains 2 alternative translational start sites. The *gro-1* phenotype was only rescued by a mitochondrial targeted functional GRO-1:GFP construct, while the respective cytoplasmic GRO-1:GFP construct did not rescue the growth phenotype. The mitochondrial involvement correlated with a weak and transient respiratory phenotype,[65] but is consistent with mitochondrial effects of other genes mutated in slow-growing mutants (e.g., *clk-1*). The *gro-1* mutation truncated the isopentenyltransferase by about 100 C-terminal amino acids and induced a frame-shift. Whether the maternal effect depends on the inheritance of modified tRNA or isopentenyltransferase enzyme has not been elucidated. We have not found any characterization of tRNA modification in *gro-1* worms.

TRIT1 in mammals

Human *TRIT1* was cloned by BLAST homology search based on the Mod5 sequence.[27] The mouse *Trit1* gene was simply assigned because of sequence homology. Data on the occurrence of i^6A and ms^2i^6A modifications in mammals are scattered and partially incomplete (Table 3). In contrast to yeast, most mitochondrial tRNAs that carry i^6A are further modified to ms^2i^6A in mammalian cells.[8,66] Only 2 cytoplasmic tRNAs, cy-tRNA^{Ser}(UCN) and tRNA$^{[Ser]Sec}$, are known to carry i^6A37 in human cells.[11,67] In mammals, ms^2i^6A is not reported in cytoplasmic tRNA.

Selenoprotein expression depends on tRNA$^{[Ser]Sec}$ which is reading UGA codons in mRNAs carrying a selenocysteine insertion sequence (SECIS) element in their 3′ untranslated region as sense.[68,69] Mouse models deficient in tRNA$^{[Ser]Sec}$ are not able to express any selenocysteine-containing selenoproteins,[70,71] and a hypomorphic promoter mutant expressed at reduced levels was associated with decreased selenoprotein expression and seizures.[72] A single nucleotide exchange in the human tRNA$^{[Ser]Sec}$ impairs selenoprotein expression in a patient with a complex syndrome.[73] Thus, a whole class of proteins depends on the function of one tRNA which is decoding (in all but one case) only one singular UGA/Sec codon per mRNA. Modification of tRNA$^{[Ser]Sec}$ within the anticodon loop involves i^6A37 and $mcm^5U(m)34$.[74] Mutations in the anticodon loop of tRNA$^{[Ser]Sec}$ affect selenoprotein expression.[75] Lovastatin, a lipid lowering drug, is an inhibitor of HMG-CoA reductase, the enzyme catalyzing the committed step of cholesterol biosynthesis. Inhibition of this pathway not only affects cholesterol biosynthesis, but all isoprenoids, including dimethylallylpyrophosphate (DMAPP), the substrate of tRNA isopentenyl transferases. Consistent with a role for i^6A in tRNA$^{[Ser]Sec}$, treatment of cultured cells with lovastatin reduced selenoprotein expression in a selenoprotein-specific pattern.[76] tRNA$^{[Ser]Sec}$ carrying the A37G mutation (abolishing the i^6A37 modification) supported expression of a subset of selenoproteins (including essential enzymes Txnrd1 and Gpx4), while the same tRNA$^{[Ser]Sec}$ seemed to act as a dominant negative on translation of a subset of stress-related selenoproteins, including Gpx1. In a more recent study, the effect of overexpression of A37G tRNA$^{[Ser]Sec}$ on selenoprotein expression was investigated by ribosomal profiling demonstrating the transcript-specific effects of the tRNA$^{[Ser]Sec}$ mutation.[77] Another mutant, U34A, was most likely edited *in vivo* to inosine (I34), and was able to sustain low levels of MsrB1 (and traces of Gpx4), while Gpx1 translation was apparently not supported by this tRNA.[75] The inosine is expected to read pyrimidine bases at the wobble position and adenosine, but not guanosine. Some selenoproteins were made at lower efficiency in the presence of this tRNA. It remained unclear whether the I34 in tRNA$^{[Ser]Sec}$ promoted Sec incorporation at UGY/Cys codons in other proteins.[75] We probed the role of i^6A in tRNA$^{[Ser]Sec}$ by showing that reduced expression of Trit1 in cells reduces the expression of selenoproteins.[67] Structural studies suggested that the i^6A37 modification in tRNA$^{[Ser]Sec}$ clearly promotes ordering of the anticodon stem loop. *In vitro* transcribed tRNA$^{[Ser]Sec}$ crystallized in 2 aberrant conformations - both incompatible with reading the codon, because A37 embarked on illicit intermolecular base-pairing interactions and the characteristic U-turn 3′ of the anticodon did not form[78] (Fig. 2). In similar nuclear magnetic resonance studies on oligonucleotides representing the anticodon stem loop structure of *E. coli* tRNA^{Phe}, i^6A alone was not able to induce a U-turn and order the anticodon,[79] suggesting that other modifications might contribute to anticodon structuring.

Proposed functions for TRIT1 not associated with tRNA isopentenylation include tRNA gene mediated transcriptional silencing[81] and a role for TRIT1 as a tumor suppressor.[82]

Base 37 modification in human disease

Defects in tRNA modification lead to several human disorders, including mitochondrial diseases (Table 4).[83] In a family with clinical symptoms of mitochondrial disease, 2 patients carrying the homozygous p.Arg323Gln mutation in TRIT1 were

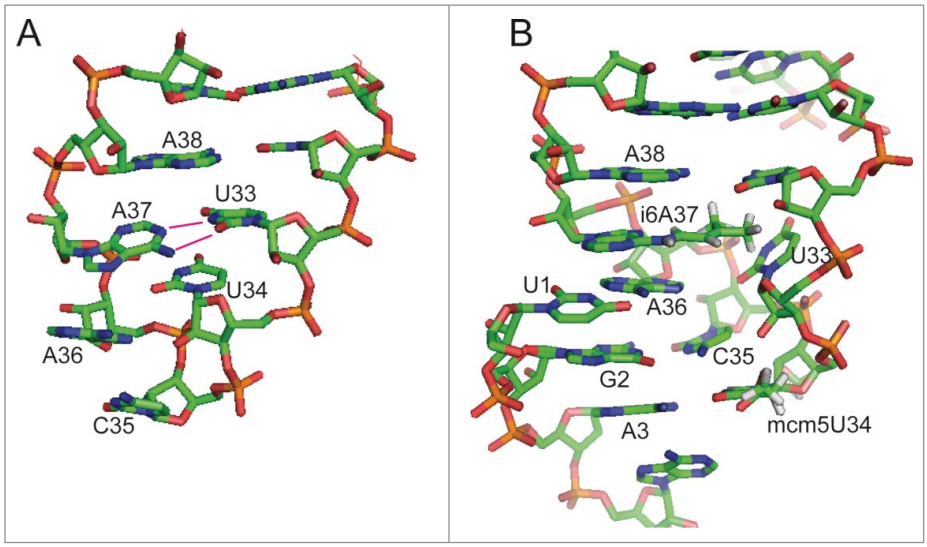

Figure 2. Effect of base 37 modification in tRNA^{Sec}. (A) Unmodified murine tRNA^{Sec} displays a disordered anticodon loop. Instead of forming a U-turn, U33 base pairs with unmodified A37 leading to an anticodon incapable of interaction with the codon.[78] (B) Model of fully modified tRNA^{Sec} decoding the codon in the ribosome. The model was generated by the authors using MacPyMOL (Schrödinger) software and is based on PDB 2UUC containing a bacterial tRNA^{Val} carrying 5-Oxoacetic acid-modified U34 and N^6-Methyladenosine at position 37.[80]

Table 4. Genes involved in tRNAs modification at position 37 in eukaryotes and associated phenotypes.

Modification	Enzyme/s	Function in translation	References
i^6A	MiaA, Mod5, ***TRIT1***	Promotes fidelity at cognate codons but misreading at non-cognate codons. Necessary for proper decoding of UUX-Leucine codons in rpoS and iraP translation	48, 61, 84
ms^2i^6A	MiaB, ***CDK5RAP1***	Improves translation efficiency of trp and nonsense codon UGA and minimizes codon context sensitivity of tRNAs. Prevents misreading of Phe codons	85, 112, 113
t^6A	Threonyl-carbamoyl Transferase Complex, TCTC (named before KEOPS or EKC complex) is made of Tcs3p (Kae1p/ ***OSGEP***), Tcs5p (Bud32p/ ***PRPK***), Tcs6 (Pcc1/ ***LAGE3***), Tcs7 (Cgi121/ ***TPRKB***) and Tcs8 (Gon7/ ***C14ORF142***)	Prevents mistranslation of near-cognate Met codons to restrict translation initiation to AUG codon. Modulates elongation rate. Lack of t^6A produces increased frameshifts, read-through of stop codons, increased initiation upstream non-AUG codons	114
ms^2t^6A	***CDKAL1***	Decreases anticodon stacking of $tRNA^{Lys}$ Mistranslation of Lys codon in proinsulin	88,115
m^1G	TrmD, ***TRM5***	Prevents +1 frameshift	116 117
yW	TYW1, TYW2, (also named ***TRMT12***) TYW3 and ***LCMT2*** (also named TYW4)	Prevents ribosome slippage on Phe codons UUU and UUC Prevents -1 frameshift	118 83, 119

Gene names in bold face: human genes

identified.[84] Severe combined mitochondrial respiratory chain defects mainly affecting complex I and complex IV were found in skeletal muscle biopsy. *In vitro* experiments indicated reduced expression of ND1 and ND5 of complex I and COXI and COXII in complex IV. All these proteins are mitochondrial encoded and it was concluded that mitochondrial translation was affected. Since TRIT1 acts on mitochondrial tRNAs, recombinant enzyme was found to have reduced ^{14}C-DMAPP: tRNA isopentenyl transferase activity toward oligonucleotides mimicking mt-tRNA substrates. The authors further showed that the m.7480A>G mutation that changes base A38 to G in mt-$tRNA^{Ser}$(UCN) abolishes isopentenylation of the tRNA, because it disrupts the AAA motif in TRIT1.

Most mammalian mt-tRNAs carrying i^6A are further modified to ms^2i^6A (Table 3). Wei et al. have proposed that oxidative stress caused by the mt-DNA mutation m.3234A>G (affecting taurinomethylation of τm^5U34 in mt-$tRNA^{Leu}$) interferes with the function of [4Fe-4S] clusters in CDK5RAP1, the enzyme needed for methylthiolation of isopentenylated mt-tRNAs, and thus further decreases mitochondrial protein expression and activity leading to mitochondrial encephalopathy lactic acidosis and stroke-like episodes (MELAS).[85]

Mutations in an intron of *CDKAL1*, encoding the enzyme mediating methylthiolation of t^6A to ms^2t^6A in mt-tRNAs, have been associated with lowered insulin secretion and development of type 2 diabetes mellitus.[86,87] Accordingly, in a pancreatic β-cell specific *Cdkal1*-knockout mouse model, aberrant proinsulin biosynthesis and impaired glucose metabolism were described.[88] Since the glucose sensor in pancreatic β-cells is in essence an ATP sensor, mitochondrial dysfunction may play a role in decreased insulin secretion. The authors have proposed another potential pathomechanism, i.e., the misreading of Lys (AAA and AAG) codons in insulin by cytoplasmic $tRNA^{Lys}$ (UUU). Indeed, proteolytic processing of proinsulin depends on Lys at the cleavage site between the A chain and the C-peptide of proinsulin. ^{14}C-Lys incorporation and C-peptide abundance were reduced in *Cdkal1*-deficient pancreatic β-cells explaining the hyperglycemia of the knockout mice.[88] Human mutations affecting formation of the t^6A modification have not yet been identified, although the enzymes responsible for the modification are present in metazoans, including humans.[89]

A biosynthetic defect in wybutosine formation has not been detected, possibly implying that this modification of $tRNA^{Phe}$ at base 37 is essential. Amplification of one of the genes involved in wybutosine formation (*TRMT12*) has been reported in breast cancer.[90] Given the large number of genes involved in tRNA base 37 modification (Table 4), it can be expected that more patients are discovered carrying inborn errors in base 37 modification.

The isopentenyl transferase reaction

As mentioned above, all tRNA isopentenyl transferases are encoded by homologous genes. Mod5 is highly homologous to MiaA from *E. coli* with the notable exception that eukaryotic isopentenyl transferases contain an additional zinc-finger domain at their C-terminus, which is not present in bacteria (Fig. 3). To clarify the mechanism of tRNA isopentenyl transferases, bacterial MiaA and yeast Mod5 have been crystallized as apo enzymes as well as in several conformations bound to tRNA substrates and DMAPP.[91-93] In general, isopentenyl transferase binds the anticodon stem loop of tRNA and distorts it flipping out bases U33, G34, C35, and A37 from the anticodon loop of tRNA. Remarkably, A36 and A38 of the AAA motif engage in specific interactions within the tRNA, i.e., the AAA sequence motif is not read by the protein, but by the tRNA.[53] The substrate base A37 is specifically bound in a channel within the enzyme that leads to the DMAPP binding site. Here, N^6 of A37 is activated by Asp46 and attacks the DMAPP molecule with pyrophosphate as excellent leaving group in a nucleophilic substitution (S_N2-) reaction. The DMAPP binding site forms only upon tRNA binding (and includes direct interaction with the purine ring of A37) supporting the ordered mechanism observed before in kinetic experiments.[94] There exist several lines of evidence, which indicate that the C-terminal domain is necessary for substrate discrimination. First of all, the C-terminal domain

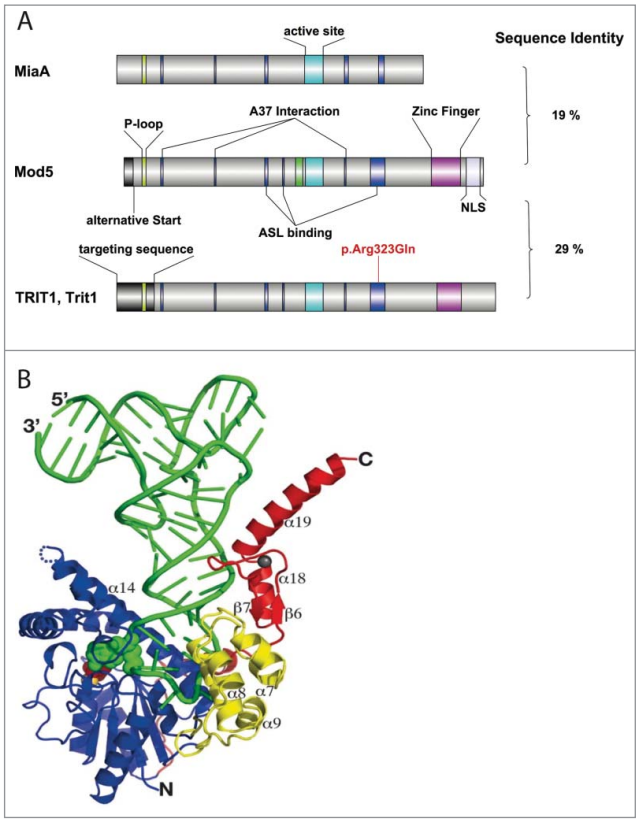

Figure 3. Structure of isopentenyl transferases and mode of substrate binding. (A) Comparison of isopentenyl transferases from E. coli (MiaA), baker's yeast (Mod5), and mammals (TRIT1, Trit1). Stretches of sequences with amino acid identity are highlighted in color. The sequence shown in green represents the P-loop involved in DMAPP binding. The turquoise sequence is the core of the active site. The blue sequences refer to amino acids interacting with the tRNA substrate (A37 binding or anticodon stem loop (ASL)-binding). The zinc-finger present in eukaryotes is depicted in violet. NLS, nuclear localization sequence. Overall sequence identity is shown on the right. The pathogenic mutation in human TRIT1 is indicated in red. The figure was prepared with IBS software.[95] (B) Crystal structure of Mod5- tRNA-$^{Cys}_{GCA}$ complex. The tRNA is shown in green. The DMAPP-binding and catalytic center in green balls, the zinc-finger domain is shown in red. Reproduced with permission from Zhou C and Huang RH. Crystallographic snapshots of eukaryotic dimethylallyltransferase acting on tRNA: insight into tRNA recognition and reaction mechanism. Proc Natl Acad Sci U S A 2008; 105:16142–7.

interacts with other nucleotides in the anticodon stem loop of the tRNA, which are not involved in catalysis as shown in the Mod5-tRNA$^{Cys}_{GCA}$ crystal structure. One of these nucleotides is G34.[93] Second, this interaction is necessary for enzyme activity as removal of this domain compromises Tit1 activity.[60] Third, mutation of C at position 34 of the non-Mod5 substrate, cy-tRNA$^{Trp}_{CCA}$, to G converts it into a Mod5 substrate. Fourth, mutation of the G34 binding loop of Mod5, which disrupts the contact with the wobble base, selectively impairs isopentenylation of cy-tRNA$^{Tyr}_{G\Psi A}$ and cy-tRNA$^{Cys}_{GCA}$, over tRNA$^{Ser}_{NGA}$, which does not carry G34.[60] Therefore, the wobble base is a determinant for Mod5 activity in both tRNAs. However this rule is no applicable to cy-tRNA-$^{Ser}_{NGA}$.[60] A shown in Fig. 3, the Zn-finger domain also interacts with the D-loop of substrate tRNA.

DMAPP, an intermediate of the isoprenoid and sterol biosynthetic pathways, is the isopentenyl donor substrate used by tRNA isopentenyl transferases. Isotope labeled DMAPP can be used to directly demonstrate isopentenylation of tRNAs or oligonucleotides resembling tRNA anticodon stem loops. Competition for DMAPP between tRNA modification and sterol biosynthesis *in vivo* became apparent, when Erg20 (farnesyl diphosphate synthetase) overexpression decreased isopentenylated tRNAs, which were restored by overexpression of Mod5.[96,97] Statins are drugs widely used to decrease cholesterol biosynthesis in patients with hypercholesterolemia. As statin treatment of cells decreased selenoprotein expression,[76] it was speculated that statin toxicity may be mediated by a specific lack of selenoprotein N (SELENON),[98] a gene known to be mutated in a spectrum of congenital myopathies, selenoprotein N-related myopathy.[99-101] That statins, applied in therapeutic dosage, impair tRNA isopentenylation has not been demonstrated.

It is interesting that apart from the function of Mod5 as an isopentenyl transferase, other functions have been discovered recently. It was suggested that the longer eukaryotic C-terminal domain might be involved in these and thus would be exclusive to eukaryotic organisms. One of these functions is the involvement of the nuclear fraction of Mod5 in tRNA-mediated transcriptional silencing.[81,102] In addition, Mod5 can form prions, of which the full biologic role is not clear. Formation of *MOD*$^{+}$ prions, however, reduced i^{6}A in tRNA.[103] As both functions are not clearly linked to tRNA isopentenyl transferase activity of Mod5, they are beyond of the scope of this review.

Conclusion

Isopentenyladenosine is one of the longest known tRNA modifications and occurs only at one specific position in tRNA. More than 40 y of study in several model organisms have accumulated a wealth of data on i^{6}A, ms^{2}i^{6}A, and other modifications specific for base 37 in tRNA. All these modifications are of ancient origin and are retained in prokaryots and eukaryots. Clearly, i^{6}A and ms^{2}i^{6}A are functionally important for mammals, including humans. All isopentenyl transferase genes known are homologous. The database on tRNAs that carry i^{6}A and its hypermodified derivatives is almost complete and will certainly soon be complete with ever better mass spectrometric analyses. In a next step, it would be desirable, that i^{6}A and ms^{2}i^{6}A is studied in all standard organisms of molecular biology (at least *E. coli*, the 2 yeasts, and human and mouse) using the same technology and independent of synthetic reporter genes. Then, the effects of loss of all base 37 modifications should be studied with the same technology in the same organism. As a result of this review, we realized that not even for i^{6}A, t^{6}A, and related modifications there are 2 global analyses of gene expression in the same organism. Such data, in our view, would be required to fully appreciate the biologic roles of these modifications. Structural data on all relevant modified tRNAs, ideally in complex with mRNA in the context of the ribosome might finally reveal subtle differences between e.g., ms^{2}i^{6}A and ms^{2}t^{6}A modifications. The application of recently developed powerful technology like ribosomal profiling might reveal on a global, yet gene-specific, scale why these modifications have been retained through evolution.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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