Review Article

The Evolution and Implications of the Inosine tRNA Modification ☆

Peter T. S. van der Gulik [1,*], **and Wouter D. Hoff** [2]

*1 - **Algorithms and Complexity Group,** Centrum Wiskunde & Informatica, P.O. Box 94079, 1090 GB Amsterdam, the Netherlands*
*2 - **Department of Microbiology and Molecular Genetics,** Oklahoma State University, Stillwater, OK 74078, USA*

Correspondence to Peter T.S. van der Gulik: *CWI, Amsterdam, The Netherlands. Peter.van.der.Gulik@cwi.nl (P. T.S. van der Gulik)*
https://doi.org/10.1016/j.jmb.2025.169187
Edited by Ute Kothe

Abstract

Ever since the legendary publication by Francis Crick in *JMB* introducing the wobble hypothesis in 1966, inosine has been a permanent part of molecular biology. This review aims to integrate the rich array of novel insights emerging from subsequent research on the adenine-to-inosine modification of tRNA, with an emphasis on the results obtained during the last 5 years. Both the grand panorama of 4 billion years of evolution of life and the medical implications of defects in inosine modification will be reviewed. The most salient insights are that: (1) inosine at position 34 (the first position in the anticodon) is not universally present in the tree of life; (2) in many bacteria just a single homodimeric enzyme (TadA) is responsible for both tRNA inosine modification and mRNA inosine modification; (3) rapid progress is currently being made both in the molecular understanding of the heterodimeric ADAT2/ADAT3 enzyme responsible for inosine modifications in eukaryotes and in experimental capabilities for monitoring both the cytoplasmic tRNA pool and their modifications; (4) for selected tRNAs, inosine modification at position 37 has been demonstrated but this modification remains under-studied; (5) modification of tRNAs known to contain inosine can be incomplete; (6) the GC content of the T-stem is of great importance for wobble behavior, including wobbling behavior of inosine; and (7) the tRNA inosine modification is of direct relevance to human disease. In summary, research on inosine continues to yield important novel insights.

Introduction

While inosine (I) was discovered at the very start of the field of tRNA biology, it remains one of the most interesting tRNA modifications. Contrary to many other tRNA modifications, the inosine modification does not involve the addition of a new side-group to the nucleotide; instead, a side-group is taken away during the A-to-I modification (Figure 1). The mechanism of A deamination involves an oxygen atom from water taking the place of the amino-group of A. This oxygen atom is connected to the ring-system via a double bond; it is a keto-group. Often, the process of modifying A to I is called “editing” because, contrary to other modifications, I is seen as one of the main nucleotides in addition to U, C, A and G.

Because inosine is only known from three positions in the tRNA molecule, the classic cloverleaf representation of tRNA structure (Figure 2) suffices for the purpose of this review. In this representation, a tRNA molecule has an acceptor-stem and three arms consisting of a stem and a loop: the D-arm, the anticodon-arm and the T-arm (often still called “TΨC-arm”). The

☆ This article is part of a special issue entitled: ‘tRNA modifications (2025)’ published in Journal of Molecular Biology.

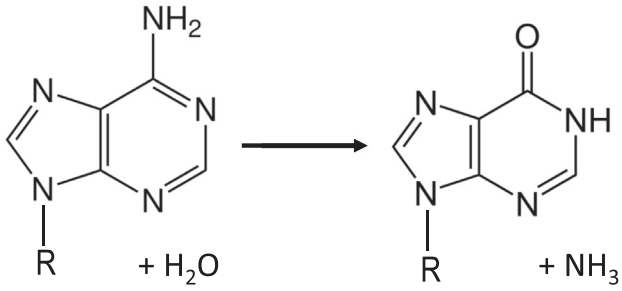

Figure 1. Hydrolytic deamination of Adenosine to Inosine. The amino group important for Watson-Crick pairing is removed, with a keto-group appearing instead. Please note that a double bond in the ring-system is removed during the process.

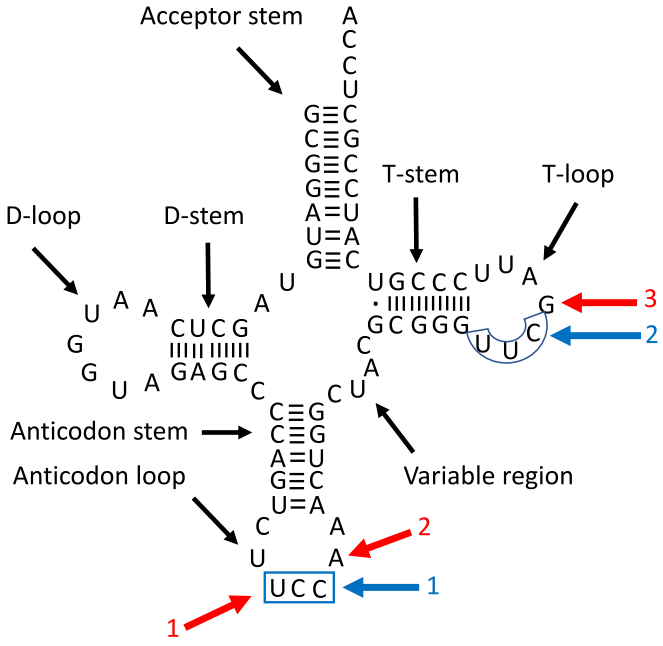

Figure 2. Essential tRNA structure (cloverleaf depiction) highlighting relevant features. Important Aspects of the tRNA structure. The three red arrows indicate the three positions in the tRNA structure for which presence of inosine is known: 34, 37 and 57. The two blue arrows indicate important boxed sub-sequences in tRNAs: the anticodon (positions 34–35–36) and the T-Ψ-C sequence (positions 54–55–56). Note that some tRNA sequences (e.g. the depicted one) are shorter than 76 nucleotides and Sprinzl numbering is not consecutive in such cases. In the depicted tRNA positions 17 and 46 are absent. Black arrows indicate important conserved parts of tRNA molecules.

locations where the inosine modification is found in tRNA are the anticodon-arm and the T-arm, and more specifically: the anticodon-loop and the T-loop. In the below manuscript we will follow notations and nomenclature for tRNAs that are widely used in the field. For example, a tRNA that transfers serine will be denoted as $tRNA^{Ser}$. For most amino acids, several tRNA genes are present which may or may not differ in sequence. When two tRNA molecules for the same amino acid differ in anticodon sequence, these two tRNA molecules are isoacceptors of each other.

The inosine modification can occur both in type I and type II tRNAs. The distinction of tRNA molecules in these two types goes back all the way to 1976, when Brennan and Sundaralingam wrote: "*For simplicity, we have categorized them into two classes depending on the size of the variable loop alone. tRNAs having a short variable loop of 4 or 5 nucleotide residues are classified as type I, and those having a long variable loop, with a double helical stem of 3 to 7 base pairs and a loop of 3 to 5 residues, as type II*" [1]. Below we refer to this region of tRNA molecules as the variable region instead of variable loop because it can exist both as a short loop or as an extra arm. Type II tRNAs are mainly all $tRNA^{Ser}$ molecules, the most $tRNA^{Leu}$ molecules (see [2] for $tRNA^{Leu}$ molecules

which are Type I tRNAs, more of such $tRNA^{Leu}$ molecules can be found in e.g. cyanobacteria as can be seen in GtRNAdb [3,4]), and in bacteria all $tRNA^{Tyr}$ molecules. In Type II tRNAs the variable region (which is located between the anticodon-arm and the T-arm) takes the form of an extra arm. The extra arm is relevant for the present paper because $tRNA^{Ser}$ molecules and $tRNA^{Leu}$ molecules belong to the group of tRNAs in which modification of A_{34} to I_{34} is found in eukaryotes (see [5] and references therein). The Type I tRNAs (which have a short loop instead of this extra arm) in which modification of A_{34} to I_{34} is found, are $tRNA^{Ile}$, $tRNA^{Val}$, $tRNA^{Pro}$, $tRNA^{Thr}$, $tRNA^{Ala}$ and $tRNA^{Arg}$ (see [5] and references therein).

The natively folded tRNA molecule has the form of a letter "L", with the CCA-terminus to which the amino acid is attached at one of the two extremities and the anticodon at the other extremity. The region in the middle (in the "elbow" of the letter "L") is referred to as the core of the tRNA. In this region, the D-loop and the T-loop touch each other, and interactions between nucleotides in the D-loop and nucleotides in the T-loop are important for the stability of the tRNA. Often, a contrast is made between modifications at nucleotides in the anticodon-arm and modifications at nucleotides in the rest of the tRNA; these modifications in the rest of the tRNA are then referred to as "core modifications". Modifications at nucleotides in the acceptor-stem would thus be called "core modifications", but this potentially problematic use of the term "core modifications" is in practice not an issue because there are only very few modifications found at nucleotides in the acceptor-stem.

In an initially unsettling finding it was discovered that "*the majority of tRNA modifying enzymes have been found to be nonessential for cells grown in ideal laboratory conditions*", as mentioned in [6]. Subsequent work showed that under stress conditions, the tRNA modification knock-out cell lines showed clear phenotypes ([7] and references therein for examples). Despite that fact, the observation that mutants lacking tRNA modifications are viable could shed some doubt on the importance of these modifications. Two considerations dispel such doubts. First, many tRNA modifications have been shown to persist in major taxonomic lineages over very long periods of evolutionary time. Secondly, ideal laboratory conditions in general are not a good proxy for the evolutionary pressures that organisms experience in nature. Saleh and Farabaugh [8] make a convincing case that modifications in the core of the tRNA molecule modulate translational misreading errors. Under ideal laboratory conditions, the organisms tolerate non-ideal functioning of the translational apparatus. However, when organisms are subjected to natural selection in the wild, tRNA modifications become essential for maintaining translation at a level that ensures competitive survival.

Of great interest here is the finding that the nonessentiality (in laboratory settings) of some tRNA modification enzymes does not extend to the A-to-I editing enzymes for position 34 of tRNA. Organisms containing this A_{34}-to-I_{34} editing are always found to be unable to survive without it. In this light, it is very interesting to note that heat maps of lost genetic information in certain cancer cell lines [9] suggest that these cells in fact do survive without an essential part of the A-to-I editing complex (see below).

Compared with U, C and G, what stands out about A is the very sparse use of A as a first nucleotide of an anticodon (except for the use in fourfold-degenerate codon boxes in eukaryotes where the A is modified to I). In most cases when the gene of a tRNA with an A-starting anticodon is present, this A is modified to I in the mature tRNA (not only in eukaryotes but also for the ACG anticodon in many bacteria). Why the presence of *unmodified* A as the first nucleotide of an anticodon is so extremely rare is one of the great unsolved mysteries in evolutionary biochemistry. Misreading of codons in the codon box other than the U-ending codon is known already for a long time (see e.g. [10–12]). Referring to [13], the statement "*Unmodified A can decode all four synonymous codons of a family box*" is made in the landmark article [5], but this statement of general "superwobble" (see also the section on wobble) for unmodified A is likely to be too simplistic. Wobble by unmodified A is a complex subject, and a thorough analysis of this topic is beyond the scope of this review.

The logical flow of this review is as follows. We start with an overview of the AUN codon box in which inosine plays a supporting role, and an overview of the presence of inosine in the tree of life. Next, the presence of inosine in archaea and bacteria is reviewed. This is followed by a discussion of the presence of inosine in eukaryotes at position 34 and position 37. Then follow two sections on specialized topics: incomplete deamination of tRNAs with A-starting anticodons, and the wobbling behavior of inosine. These are followed by an overview of the disease relevance of the inosine modification, and a concluding section on perspectives and future directions.

The AUN codon box of the genetic code

The pattern of occurrence of the inosine modification is complex with respect to taxonomic distribution and regarding the codon boxes in which it occurs. It is found in 8 different codon boxes: in 7 of the 8 fourfold-degenerate codon

boxes (the Glycine codon box is the exception), and in the AUN codon box. In view of the complexities involved in the readout of the AUN codon box and the involvement of the inosine anticodon modification in this codon box, we review it here.

The Genetic Code consists of 16 codon boxes in which the first and the second position of the codon are shared. The four codons in such a codon box differ only in the third codon position. An example of a codon box is the UUN codon box in the top left-hand side of the genetic code table, where Phe and Leu are the amino acids encoded by the codons. In the Standard Genetic Code the codons UUU and UUC are coding for Phe and are read by a tRNA with a G-starting anticodon, while the codons UUA and UUG are coding for Leu and are read by two tRNAs: one with a U-starting anticodon and one with a C-starting anticodon. Thus, readout of the UUN codon box in general is performed by three different tRNAs with three different anticodons. In exceptional cases the tRNA with the C-starting anticodon is absent. Examples of such absent tRNAs with C-starting anticodons, e.g. in *Methanococcus vannielii* SB (GCA_000017165.1), can be readily found in GtRNAdb, a database with tRNA sequences for hundreds of genomes covering all cellular life [3,4].

Decoding of a codon box with three kinds of tRNAs is the rule. The first base of the anticodon of the three tRNAs involved varies depending on the codon box and on the taxonomic group being studied. In many eukaryotes, in 7 of the 8 fourfold-degenerate codon boxes the three kinds of tRNAs are one with an A-starting anticodon, one with a U-starting anticodon and one with a C-starting anticodon. The A in these cases is modified to I. Above, we have seen the example of a split codon box where the three tRNAs are one with a G-starting anticodon, one with a U-starting anticodon and one with a C-starting anticodon. Thirteen of the sixteen codon boxes in the Standard Genetic Code follow the pattern of three tRNAs with three different anticodons decoding the codon box; there are three codon boxes in the Standard Genetic Code which do *not* follow this pattern. In [5], these three codon boxes are referred to as "*Special Decoding Boxes*". Two of them do not show the general pattern due to the presence of stop codons: the UAN codon box and the UGN codon box. In the Standard Genetic Code, only a single tRNA is present in the UAN codon box: the tRNA with anticodon GUA which is reading the codons UAU and UAC as Tyr. The codons UAA and UAG are both stop codons. For the UGN codon box, two tRNAs are present in the Standard Genetic Code: the tRNA with anticodon GCA which is reading the codons UGU and UGC as Cys, and the tRNA with anticodon CCA which is reading the codon UGG as Trp. The codon UGA is a stop codon.

The last codon box of the Standard Genetic Code involves the highest degree of complexity. This is the AUN codon box, which is shared between Ile and Met. In bacteria this codon box is decoded by four kinds of tRNAs. The tRNA with a G-starting anticodon decodes the AUU and AUC codons as Ile. In addition to this tRNA, *three* tRNAs with a C-starting anticodon are found. These three C-starting anticodons are a source of considerable confusion in literature, particularly when these are incorrectly treated as one category of tRNA sharing the same decoding behavior. The first issue is that all organisms contain two functionally distinct tRNAs that transfer methionine: one for inserting Met during the elongation process of mRNA translation, and the other reading the start codon during the initiation of translation. Thus, there are two tRNAs for Met with a CAU anticodon which are emphatically not the same kind of tRNA. The word "isoacceptor" is misleading in this case, as the initiator tRNA is the most divergent of the canonical tRNAs because it is the only one not interacting with an elongation factor but with an initiation factor. A special aspect of bacteria is that the methionine on the initiator-tRNA is formylated to formylmethionine. This is not the case in archaea and eukaryotes. On top of this complex situation of two different tRNAs with anticodon CAU acylated with Met, a third tRNA with anticodon CAU is present. This tRNA is not transferring Met but Ile. It is the tRNA reading the codon AUA. The unusual readout properties of this tRNA to recognize the AUA codon but not the AUG codon are caused by a modification of the C in the CAU anticodon. The resulting special nucleotide is called lysidine because the C is modified with a lysine amino acid. The nucleotide resulting from this noteworthy use of an amino acid to modify a nucleotide in the anticodon of a tRNA is usually referred to as L. Interestingly, as long as the modification has not been added, the methionyl-tRNA synthetase (MetRS) of the bacterial cell recognizes this tRNA as a substrate [14] and acylates it with Met. Only when the CAU has become a LAU, the tRNA has turned into a real $tRNA^{Ile}$. When analyzing tRNA sets in an organism it therefore is of great importance to distinguish between the three functionally quite distinct tRNAs that share the CAU anticodon.

In archaea, we find basically the same situation, with four tRNAs for the AUN codon box: one with a G-starting anticodon, and three with a C-starting anticodon. An important difference between archaea and bacteria is that the modification for the $tRNA^{Ile}_{CAU}$ is not with lysine but with agmatine [15]. Because this small molecule does not contain an acid group, it is not an amino acid. The modified nucleotide is called agmatidine. The enzyme modifying the C in the first position of the anticodon with agmatine is an entirely different enzyme compared

to the bacterial enzyme modifying the C with lysine [16]. This situation points to convergent evolution to accurately read AUA as Ile in archaea and bacteria [17].

Recently a specialized group of bacteria was discovered which further developed the modification for $tRNA^{Ile}_{CAU}$ [18]. Here, the modification is not with lysine, but with aminovaleramide. This $tRNA^{Ile}_{CAU}$ modification is also found in chloroplasts and in plant mitochondria [18]. Indications have been found that the aminovaleramide modification has "*better steric complementarity to the cleft between rRNA and mRNA than the L side chain*" [18]. Lysidine is a step on the way to aminovaleramide [18] and therefore what we encounter here is, in our opinion, not convergent evolution but a further elaboration of the lysidine system.

In animal mitochondria, the situation of the AUN codon box is very different. AUA is not decoded as Ile but as Met. One $tRNA^{Ile}$ is present, with an anticodon GAU, and one $tRNA^{Met}$ is present, with an anticodon CAU, which is modified to 5-formylcytosine (f5C). This last tRNA functions both as initiator and Met-elongator, and is able to read both AUA and AUG (see [19] and references therein). Please note that this situation with a single $tRNA^{Met}$ functioning both in initiation and elongation is something which is unique for the mitochondrial system and is unknown in the cytosolic system of *all* organisms.

When turning away from the mitochondrial tRNAs, and focusing on the tRNAs decoding the AUN codon box in eukaryotes, four tRNAs are present: $tRNA^{Ile}_{AAU}$, $tRNA^{Ile}_{UAU}$, the initiator $tRNA^{Met}_{CAU}$, and the elongator $tRNA^{Met}_{CAU}$. The AAU anticodon is modified to IAU, and the UAU anticodon is double modified to ΨAΨ. As is discussed in the section on wobble behavior of inosine below, the IAU does not efficiently read the codon AUA. The tRNA with the ΨAΨ anticodon is responsible for reading AUA. Key takeaways are that Ile and Met are distinguished and correctly transferred, and lysidine, agmatidine, aminovaleramide and the ΨAΨ anticodon were evolved to accomplish this task in different systems. In animal mitochondria a different solution was developed with AUA no longer meaning Ile. The very small genome size of animal mitochondrial DNA probably allowed for an evolutionary pathway for changing the meaning of the AUA codon [20].

Interestingly, there exist archaea which have a different solution for reading the codon AUA. In GtRNAdb [3,4] the presence of a $tRNA^{Ile}_{UAU}$ instead of a $tRNA^{Ile}_{CAU}$ can be seen, in the unusual archaeon *Nanoarchaeum equitans*. There are no indications that this organism uses the double modification to ΨAΨ. How *N. equitans* manages to not read the AUG codon with its supposedly unmodified UAU anticodon is a clear subject for future study. In any case, many different solutions for reading the codon AUA as Ile exist in nature, and inosine is not exactly one of them, as the ΨAΨ anticodon of eukaryotes is more important for this biochemical task. Even though slightly beyond the primary scope of this review, the focus on the AUN codon box in this section provides a comprehensive perspective on how complex decoding can be, how nature can find many different ways to solve the same problem, and how the traditional view of inosine recognizing three codons can obscure the role of inosine as it can be mistaken to be crucial in distinguishing between codon AUA and codon AUG.

One final twist to the complex situation of the AUN codon box is that despite the general absence of a tRNA with a G-starting anticodon when a tRNA with an I-starting anticodon is present, there is at least one exception to this rule. In human beings several copies of $tRNA^{Ile}_{GAU}$ are present in the genome in addition to $tRNA^{Ile}_{AAU}$ genes and $tRNA^{Ile}_{UAU}$ genes, as can be seen in GtRNAdb [3,4]. An obvious research question is in what circumstances these $tRNA^{Ile}_{GAU}$ genes are expressed, and what purpose they serve.

The discovery of major deviations from the so-called universality of inosine

When tRNA was first sequenced in 1965, the particular species which was selected (a *Saccharomyces cerevisiae* $tRNA^{Ala}$ isoacceptor) happened to be one with inosine at position 34 in the anticodon [21]. The result of this happenstance was that the presence of inosine in the anticodon was firmly imposed in the collective memory of the molecular biology community. Crick consolidated this piece of knowledge in his famous *Journal of Molecular Biology* article introducing the Wobble Rules [22]. This paper described the wobble behavior of G in position 34 (recognizing in addition to the cognate C-ending codon also the U-ending codon) and that of U in position 34 (recognizing in addition to the cognate A-ending codon also the G-ending codon). In addition, it included the wobble behavior of I in position 34 ("recognizing the U-, C-, and A-ending codons"). This information on wobble behavior became textbook knowledge. The impression was laid down that inosine is a universal aspect of living cells, and resulted in descriptions such as "*In all domains of life, A34 of the tRNA transcript is almost always deaminated to form inosine at the wobble position (I34)*" (from [23]). It was only later [24] (compared to the times of [21;22]) that the insight emerged that I34 is not present in one of the three domains of life: the Archaea (still called "Archaebacteria" in [24]). Experimental work demonstrating that in organisms as diverse as the bacterium *Escherichia coli*, the baker's yeast *S. cerevisiae*, and the human being, the A-to-I editing proteins are essential for cell viability reinforced the incorrect idea of universality of inosine: "... *I34 is a tRNA modification crucial for deciphering the*

genetic code" [25]. The modification is essential in *these* organisms (*E. coli, S. cerevisiae*, animals) but *not* in all organisms. Later, multiple large taxonomic lineages in the domain Bacteria [26] were found to be devoid of A-to-I editing at position 34 of tRNA. Thus it has been amply demonstrated that A-to-I editing at position 34 of tRNA is not needed for viability *in general.* An important aim of this review is to shed light on the distribution of inosine modifications across different domains of life and their functional implications. Inosine modification at anticodon position 34 is *not* universally present in all three domains of life (see above), and it has been suggested [27,28] that the most important functional implication of the inosine modification is improving translational efficiency.

These updated insights into the taxonomic distribution of A-to-I editing at tRNA position 34 can be combined with current insights into the evolution of the major branches in the tree of life [29–33]. A consistent interpretation is that the A-to-I modification at position 34 is a characteristic that was developed somewhere on the way between the Last Universal Common Ancestor (LUCA) and the Last Bacterial Common Ancestor (LBCA), (as indicated in Figure 3). An important takeaway from this perspective is that LUCA likely did not contain the A-to-I modification at position 34. Although this concept is not present in existing literature, the taxonomic distribution mentioned above implies this emerging understanding.

This placement of the evolutionary origin of inosine now allows its comparison with the origin of other key modifications, particularly pseudouridine, thiouridine and different methylations (namely of purine rings, pyrimidine rings and ribose sugars in tRNA). Interestingly, these latter tRNA modifications are universally present, indicating that they are much more ancient and most likely already present in LUCA. This insight is of prime importance for research on the origin of the genetic code because it implies that the wobble behavior of inosine did not contribute to the early stages of evolution.

Inosine in archaea

Strictly speaking, there is no inosine in archaeal tRNA. In this respect, care should be taken when considering the modification of A_{57}. The A_{57} nucleotide in the T-loop is first methylated, and only afterwards this nucleoside is modified to m^1I (see [34]). Inosine without methylation is therefore not a constituent of archaeal tRNA. The nucleoside at position 57 in archaea contributes to the stabiliza-

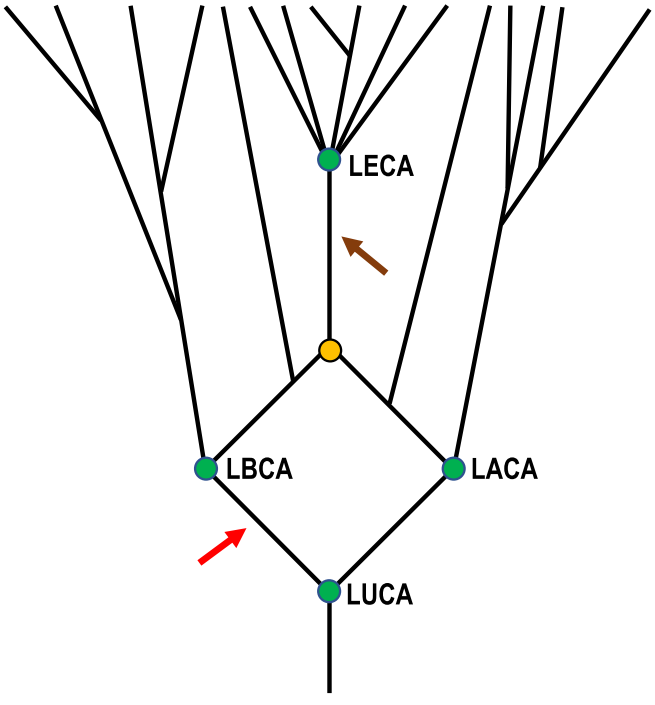

Figure 3. A highly schematized history of life. The red arrow indicates the emergence of A34-to-I34 editing in the anticodon ICG between the LUCA (Last Universal Common Ancestor; which did not have ICG) and the LCBA (Last Bacterial Common Ancestor; which did have ICG). LACA denotes the Last Archaeal Common Ancestor. The brown arrow indicates the emergence of I34 divergence between the merger at the basis of eukaryogenesis (yellow dot) and the LECA (Last Eukaryotic Common Ancestor). Please note that only branches of the Tree of Life with surviving descendants are shown. All the indicated ancestors must have had many contemporaries with all descendants going extinct: these are not depicted for the sake of clarity.

tion of the elbow of the three-dimensional structure of the tRNA, as explained in detail in [35]. The enzyme responsible for the m^1A to m^1I conversion has not yet been identified. This points to the fact that it probably is a totally unrelated enzyme in comparison to the well-known family of enzymes that is responsible for the A_{34} to I_{34} conversion in bacteria and eukaryotes. In other words: when speaking about "inosine" in archaea, we are referring to a different modification (methylinosine), executed by a different enzyme, in a different part of tRNA and with a different function. The m^1I nucleoside in the T-loop of archaea is entirely distinct from the I nucleosides in the anticodon-loop of bacteria and eukaryotes.

Inosine in bacteria

In bacteria, inosine is found at positon 34 of the anticodon in one of the $tRNA^{Arg}$ isoacceptors. However, this is not the case in all bacteria. There are large taxonomic groups in the bacteria (i.e. Spirochaetes, Chloroflexi and Thermotogae) which do not contain the inosine modification in tRNA at all [26]. As Diwan and Agashe [26] convincingly argue, the modification enzyme which changes the ACG anticodon to an ICG anticodon was most probably present in the LBCA, and the large taxonomic groups in the bacteria which do not have inosine have lost this property somewhere on the way from the LBCA to their own common ancestor. Bacteria lacking the I anticodon modification can be readily found using the information available in GtRNAdb [3,4]. Apart from the well-known high-level taxonomic groups missing inosine mentioned above (i.e. Spirochaetes, Chloroflexi and Thermotogae), a quick inspection of the GtRNAdb pages (in October 2024) yielded a number of additional high-level taxonomic bacterial groups missing inosine. In the names listed on GtRNAdb, these are: Armatimonadetes, C. Saccharibacteria, Elusimicrobia, *Thermobaculum*, C. division NC10, C. division SR1 and C. division WWE3.

Iyer and co-workers [36] have placed the bacterial A-to-I modification enzyme in a broader context of evolution of enzymes with "the deaminase fold". In the view that these scientists present, the ancestral deaminase domain was a C-to-U modification enzyme involved in nucleotide metabolism. The protein had single nucleotides as substrate, not RNA sequences. Having arisen early in bacterial evolution, the phylogenetic tree of enzymes with the deaminase fold then split in two large branches. One of these branches diverged into three subbranches, and one of these is the branch of A-to-I modification enzymes (but also see the Discussion in [37]). Seminal work resulted in the discovery of the enzymes performing the A-to-I modification: the discovery of Tad1, the I_{37} producing enzyme in eukaryotes [38] and the discovery of ADAT2 and ADAT3 which form the I_{34}-producing complex in eukaryotes [39]; the series of articles started with a milestone article in *Journal of Molecular Biology* which pointed the way forward [40]. As the last of the series of discoveries of A-to-I editing enzymes, the bacterial A-to-I modification enzyme, TadA, was discovered around the turn of the century [41]. Of note, the close relationship between A-to-I editing and C-to-U editing brought forward by Iyer and colleagues [36] also shines through with the dual activity of ADAT2/ADAT3 of *Trypanosoma* parasites in performing C_{32}-to-U_{32} editing in addition to A_{34}-to-I_{34} editing [42]. C-to-U editing has also been demonstrated for CDAT8, an enzyme of the archaeon *Methanopyrus kandleri*, which performs C_8-to-U_8 editing and belongs to the ADAT family according to phylogenetic analysis ([43] and references therein). The isolated occurrence of this enzyme in the archaea suggests horizontal gene transfer from eukaryotes to *Methanopyrus kandleri*, followed by acquiring a different function.

Genomics research has shown that some A-starting anticodons other than arginine are rarely but consistently present in certain groups of bacteria. Recently, thorough investigation into one of these non-arginine A-starting anticodons in a bacterial species was reported [44]. This work has demonstrated that TadA in the Firmicute species *Streptococcus pyogenes* performs A-to-I modification not only on position 34 of $tRNA^{Arg}_{ACG}$ but also on position 34 of $tRNA^{Leu}_{AAG}$. As mentioned in the introduction, $tRNA^{Leu}$ differs from $tRNA^{Arg}$ in having an extra stem instead of a short variable loop. However, this is not what is recognized by TadA. The anticodon-loop of the tRNA, which *is* the part of the molecule screened by TadA, is nearly identical for the two tRNAs studied in *S. pyogenes*, providing an explanation for the observed modifications. In addition to the expanded number of tRNA substrates (a limited expansion, from one to two), the TadA of this bacterium also acts on mRNAs, on stretches with a nucleotide sequence that is very similar to the anticodon-loops of *S. pyogenes* $tRNA^{Arg}_{ACG}$ and $tRNA^{Leu}_{AAG}$. This leads to differences in the proteins being produced compared to the situation when the mRNAs were not changed by TadA, because the I in the mRNA is read by the ribosome as a G instead of an A (leading to changes in the protein product such as having a Glu at a certain position instead of a Lys). These changes in mRNA by TadA are not limited to Firmicutes: they are also known from Proteobacteria ([45] and references therein). Being present in both Firmicutes and Proteobacteria, dual duty of TadA on both tRNA and mRNA probably is a general bacterial characteristic (please nota bene: for those clades which do not have lost TadA). It is important to realize that the conclusion "because the I in the mRNA is read by the ribosome as a G" is a simplification of the actual molecular biology in the bacterial cytosol [46]. In fact, the I is read as a G in most instances, but occasionally is read as an A, and sometimes even as a U. This more accurate

description of the situation was reported by Licht and co-workers [46]. Furthermore, the details differ for different codons, and in different contexts of codons. Also the amount of protein being produced changes, because of ribosomal stalling followed by release and subsequent breakdown of a truncated protein. We will also encounter the need to step back from simplification and bring nuance to our view in the section on wobble rules below.

In another Firmicute bacterium, *Oenococcus oeni*, in addition to the anticodons ACG and AAG two more A-starting anticodons are present (AGA and AGU), but these are not modified to I-starting anticodons [47]. In summary, until now, I_{34} in bacteria is only known from $tRNA^{Arg}_{ACG}$ (abundant) and $tRNA^{Leu}_{AAG}$ (very rare).

Inosine at position 34 in eukaryotes

In eukaryotes, inosine is found in the anticodon-loop at position 34 (*at least some* tRNAs always have I at this position in eukaryotes), and at position 37 (below a separate section is devoted to I_{37}). In most eukaryotic lineages, tRNAs with eight different anticodons are modified to contain I_{34} [48,49,5]. The modification of A_{34} to I_{34} is done in eukaryotes by a *heterodimeric* complex [39]. This is in contrast to the homodimeric structure of this enzyme in bacteria. Guy and Phizicky [50] have suggested that the ability of the complex to modify eight different tRNA species is *dependent on* the heterodimeric character of the complex. In this proposal, the homodimeric complex consisting of two TadA protein molecules known from bacteria would not be able to expand its repertoire extensively. This concept is further elaborated by Dixit et al. [51], while Roura Frigole and co-workers have focused on specific details of the substrate-recognition process [52]. Recent cryo-EM work beautifully illustrates the subtleties of the interaction between tRNA substrates and ADAT2/ADAT3 [53]. Please note that *S. cerevisiae* does not have the tRNA with anticodon AAG. In our opinion, this should be seen as secondary simplification of an organism with a reduced genome, which points to the limits of the utility of baker's yeast as a model organism.

The Standard Genetic Code has a very special, regular structure (see [54] and references therein). Eight of the 16 codon boxes contain four codons specifying the *same* amino acid; these are called fourfold-degenerate codon boxes. The distribution of the eight fourfold-degenerate codon boxes is not random [54]. The A-starting anticodons which are modified to I-starting anticodons in eukaryotes are in 7 of the 8 cases found in these fourfold-degenerate codon boxes. The exception (i.e. the single fourfold-degenerate codon box where the I-starting anticodon is never found) is the Gly codon box (i.e. the GGN codons). This codon box is thus *not* part of the expansion repertoire of the heterodimeric complex. This observation can be understood based on the particular sequence characteristics of the $C_{35}C_{36}$ anticodon-loop of $tRNA^{Gly}$, as shown in [55]. Contrary to the GGN codon box for Gly, the AUN split codon box shared by Ile and Met *is* part of the expansion repertoire. The evolution from a homodimeric complex as known in bacteria to a heterodimeric complex as known in eukaryotes (known as Tad2/Tad3 mainly in *S. cerevisiae* and as ADAT2/ADAT3 mainly in mammals) proceeded via the way of duplication, diversification and neofunctionalization of the single gene encoding the component from which a bacterial homodimeric complex is made. The enzymes performing the A-to-I modification *outside* the mitochondria in eukaryotic cells likely evolved from the bacterial partner in the eukaryogenesis process, specifically the mitochondrion. Gene duplication, which enabled the expansion of protein-coding genes in eukaryotes, is a key feature of eukaryotic evolution. This expansion is facilitated by the capacity of eukaryotes to support larger genomes, which might have led to neofunctionalization of many genes. Enhanced DNA repair mechanisms, required for maintaining larger genomes, distinguish eukaryotes from archaea and bacteria, in line with the error threshold theory (see [56] and references therein). One of the theories seeking an explanation for the enhanced DNA repair mechanisms of eukaryotes is the concept that the internal oxygen production of the bacterial-partner-in-eukaryogenesis *inside* the cell of the archaeal-partner-in-eukaryogenesis necessitated this higher DNA repair (in addition to necessitating the nucleus-cytoplasm separation) [57,58]. Figure 3 depicts the evolutionary development of the enzyme performing the A-to-I modification, with the innovation introducing the homodimeric complex occurring somewhere between the LUCA and the LBCA, and the innovation expanding the modification machinery to being a heterodimeric complex occurring somewhere between the FECA (the First Eukaryote Common Ancestor, i.e. the chimaera of archaeal and bacterial cooperation during eukaryogenesis) and the LECA (the Last Eukaryote Common Ancestor). It should be noted that LECA arose through a long way of fast evolution since the FECA. For the background in evolutionary biochemistry over deep time that led to this line of thought, see [17,59–64]. While it is ADAT2 which has the active catalytic site, the two components of the heterodimeric complex *both* are functional. ADAT3 has a *different* function from catalysis; it is *essential* for substrate recognition.

The observed taxonomic variation in the number of different anticodons containing the A-to-I modification has led to research on the evolutionary origin of this pattern. In [47], a progression of deaminated anticodons is described, starting with none in so-called deeply rooted, thermophilic bacteria, then one (ACG) in most bacteria, via six in an organism from the so-called primitive SAR supergroup of eukaryotes, to eight in

animals. The SAR supergroup is a major clade of eukaryotes in which the Stramenopiles, the Alveolates and the Rhizaria are united. Brown algae and diatoms are well-known stramenopile subgroups. Dinoflagellates, ciliates and apicomplexa (among which the malaria parasite) are well-known alveolate subgroups. Foraminiferans and radiolarians are well-known rhizarian subgroups. The above-presented "progression" is deceiving. The LBCA likely had the A-to-I modification [26], and so-called deeply rooted, thermophilic bacteria are descendants of an ancestor which lost the A-to-I modification. The SAR supergroup should not be seen as primitive eukaryotes because ciliate cells are exceedingly complex and kelp weed is the largest multicellular organism known. The state with only six I-starting anticodons mentioned is likely a secondary state of an organism which descended from an ancestor with eight I-starting anticodons. The proper way to see evolution on the large scale is that A-to-I modification is not present in the domain Archaea, A-to-I modification for the anticodon ACG is found in many, but not all, lineages of bacteria, and A-to-I modification for tRNAs of eight amino acids probably was the state of LECA. The simultaneous absence of the A-starting anticodon and the G-starting anticodon in codon boxes presented in [47] (see their Table 2) points to the possibility of the U-starting anticodon taking over the function (superwobble, see later section in this review). However, this interpretation would require thorough manual curation and bioinformatics analyses to ensure that tRNAscan-SE did not miss tRNA genes with unusual sequences (for a case showing the need for thorough manual curation when using tRNAscan-SE see [65]).

It should be noted that inosine plays a crucial role in antibody production in mammals, as shown by Guigère and colleagues [66]. Antibodies, produced in large quantities for immunoprotection, require translation systems adapted for this purpose. The constant regions of antibodies, which make up the majority of antibody production, are enriched in C-ending codons. These are read by I-starting anticodons rather than G-starting anticodons, which are absent in eukaryotes in the eight codon boxes where I-starting anticodons are used (but see the information about human $tRNA^{Ile}_{GAU}$ genes in the section on the AUN codon box above). Similarly, proteins involved in cell adhesion, particularly those with low-complexity domains, rely on I-starting anticodons. These domains, enriched with amino acids decoded by tRNAs with I-starting anticodons in mammals, slow down translation and can induce frameshifting and stalling [67]. Depletion of tRNAs with I_{34} impairs cell morphology and adhesion [67]. These tRNAs with I-starting anticodons, termed TAPSLIVR-tRNAs [68,47], are unique to eukaryotes and allow for the high density of certain amino acids in protein sequences, such as low-complexity domains, which are not found in archaea or bacteria. This feature emerged after the development of the heterodimeric complex.

Inosine at position 37 in eukaryotes

The presence of inosine at position 37 of tRNA was already found by Holley and co-workers [21]. As mentioned above, the first sequenced tRNA happened to be $tRNA^{Ala}$ of *S. cerevisiae*. In a striking coincidence, this also is the *only* tRNA with I_{37}. All 19 other amino acids do *not* show I_{37}. Therefore I_{37} is very rare among the tRNAs transferring all 20 amino acids, but it is generally present in $tRNA^{Ala}$ in all eukaryotes. Compared to other tRNA modifications, not much research has been dedicated to I_{37}. The nucleoside is further methylated to m^1I_{37} [24].

In 2013, research on the role of I_{37} in salt stress in embryophytes [69] was reported and was further studied in [70]. The molecular details of the function of I_{37} in these plants and in other eukaryotes, however, are still unclear, and so much so, that in a recent review specifically focusing on modifications of the anticodon-loop, I_{37} was not part of the scope [71]. The enzyme responsible for I_{37} modification, Tad1, has been identified long ago (in bakers yeast [38], in human beings [72], in mice [73] and in *Drosophila* [74]), but progress on the understanding of the precise functioning of I_{37} has proven difficult. Modification at position 37 (with other modifications than inosine) is known to optimize the dynamics of translation, e.g. preventing frameshifting (see e.g. [75,76]). Translation needs to proceed smoothly: hypomodification has been shown to disturb proteome integrity by way of ribosome stalling [77]. Possibly the lack of the I_{37} modification in eukaryotic $tRNA^{Ala}$ results in problems like these, but at the moment these are speculations without experimental evidence behind it. Clearly research on I_{37} is a neglected corner of tRNA modification biology.

The incomplete-saturated state of the I_{34} modification

Inosine modification has traditionally been considered as a stable tRNA modification. However, recent findings challenge this perspective. Research on human embryonic stem cells has shown that inosine modification levels can vary in response to cell state changes [78], suggesting that inosine may belong to the growing class of dynamically regulated tRNA modifications [79–81]. In circumstances of unusual tRNA modification state, mRNAs with special codon usage (termed Modification Tunable Transcripts, MoTTs [82]) can be preferentially read. In this way, dynamical regulation of tRNA modification is of the utmost importance in selective mRNA translation, proteome composition and cell fate [9]. Despite the findings in [78], it remains uncertain whether inosine in human cells qualifies as a fully dynamic tRNA

modification. A defining criterion for dynamic regulation is the presence of both a writer enzyme (introducing the modification) and an eraser enzyme (removing it). While Bornelöv and colleagues observed incomplete deamination with physiological relevance [78], an eraser enzyme is yet to be identified. Beyond human cells, incomplete I_{34} modification has also been reported in *S. cerevisiae* [83], suggesting it is not just a characteristic of animals, but should be considered to be present in all organisms of the opisthokont supergroup. Furthermore, it was also found in the malaria parasite *Plasmodium falciparum* [84] from the SAR supergroup, and therefore seems to be widespread in eukaryotes. However, dynamically regulated insosine modification may not be restricted to the eukaryotic domain of life, because it has also been reported in the bacterium *S. pyogenes* [44]. This suggests that when A_{34}-to-I_{34} modification is present in a system, dynamic regulation of the modification is also present.

Determining tRNA modifications in their exact position in the tRNA sequence and determining the relative abundances and modification states of all species of tRNA over time (e.g. before and after a stress condition has started) and place ((e.g. in different parts of an embryo) are formidable technical challenges. Recently great progress has been achieved in this field (see e.g. [85] and references therein). An important takeaway from this work is that default software settings remove the vast majority of tRNA reads. The sequence of a tRNA gene is in general (taking tRNA introns in consideration) less than 100 nt, and the default software settings discussed remove sequences shorter than 200 nt [85].

A special focus on the wobbling by inosine

It is important to realize that the standard textbook idea of "Inosine recognizes three codons: the U-ending codon, the C-ending codon and the A-ending codon" is a simplification of a complex situation. A number of important recent developments necessitate a more sophisticated view of wobbling by inosine (and wobbling in general). For decades, a strict division between anticodon loop modifications and tRNA body modifications has been textbook knowledge. Anticodon loop modifications are seen as governing reading behavior and body modifications are seen as governing tRNA stability. The recent article by Kompatscher and co-workers [86] introduces a change in this view. In this paper, the $tRNA^{Gly}_{UCC}$ molecules of *E. coli* and *Mycoplasma mycoides* are compared. The crucial difference between these two molecules is that the *E. coli* tRNA recognizes the codons GGA and GGG, whereas the *M. mycoides* molecule recognizes all 4 codons from the Gly codon box in the genetic code: GGU, GGC, GGA, and GGG. Such recognition of all 4 codons by a single tRNA is known under different names, but the most used name is: superwobble. A difference between the mature versions of the two molecules which immediately catches the eye is that the U_{34} of the *E. coli* molecule is modified, where the U_{34} of the *M. mycoides* molecule is not (see Figure 1 in [86]). One possibility was that the differences in decoding behavior were caused by this characteristic. However, by adding unmodified tRNA molecules to an *in vitro* translation system, Kompatscher and co-workers show that the *E. coli* molecule with U_{34} in unmodified state is not able to perform the superwobble. The *M. mycoides* molecule, remarkably, is able to superwobble even if the U_{34} is replaced by A_{34} or G_{34}. By transplantation of the different parts of the molecule into the alternative scaffold (the T-arm of the *M. mycoides* molecule transplanted into the scaffold of the *E. coli* molecule and so on), Kompatscher and co-workers show that the essential feature for superwobbling is a difference in the T-stem. The *E. coli* molecule has a T-stem consisting of 5 G-C pairs. The superwobbling *M. mycoides* molecule has only three G-C pairs in the T-stem: G_{49}-U_{65} and A_{50}-U_{64} are the base-pairs of the stem which are not G-C. The GC-content of the T-arm thus plays a key role in superwobbling. The view that the governing of reading behavior is exclusively with the anticodon loop modifications therefore is untenable. Importantly, T-stems are generally very GC-rich.

A quick inspection of the pages of GtRNAdb [3,4] for *E. coli* and *M. mycoides* (in September 2024) reveals that the discovery of Kompatscher and co-workers is not restricted to the Gly codon box. Relatively G-C poor T-arms are also present in tRNAs decoding the codon boxes for Ala, Pro and Val, and in the fourfold degenerate codon box for Leu; these four codon boxes show superwobbling behavior too as judged by the absence of other tRNAs decoding these boxes. The implication is that Kompatscher and co-workers have unraveled a fundamental aspect of tRNA decoding behavior.

The wobbling behavior of I-starting anticodons is traditionally considered as one recognizing three codons: the U-ending codon, the C-ending codon and the A-ending codon. This view goes back all the way to Crick [22]. However, it is important to note that the wobbling behavior of tRNAs containing I_{34} is not the same in the different codon boxes. One such case is the AUN codon box in eukaryotes, where the recognition of the A-ending codon (i.e. AUA) by the I-starting anticodon (i.e. IAU) is so poor that the presence (in addition to the presence of the $tRNA^{Ile}_{IAU}$) of a special tRNA with a UAU anticodon (with both U nucleosides modified) for AUA-recognition is necessary. Very poor reading of the CGA codon by the ICG anticodon in *S. cerevisiae* has also been known for many years (see [83] and references therein). Importantly, specific cases like these do not provide sufficient evidence to be able

to conclude that such a feature is universal for all organisms: the details of decoding power of a tRNA set often differ from system to system. Above, the variability in pairing behavior of inosine was already encountered [46], although in that case the pairing was between *inosines in the mRNA* and anticodons without inosine. The phenomenon at the heart of the matter, however, is the same: interaction of inosine with the other nucleosides. As stated above: The details differ for different codons, and in different contexts of codons (see [46]).

We have just seen that on the one hand the wobbling of inosine is sometimes more restricted than what is generally presumed. On the other hand, the wobbling behavior of inosine is sometimes presented in literature as more extended than the original three codons proposed by Crick: a superwobble by I_{34} (see e.g. [87]). In the light of the discussion on superwobble above, it seems entirely possible that unmodified A is performing the superwobble here, with sequence adaptations of the tRNA body. Until now, superwobbling by I_{34} has not been convincingly demonstrated.

An important lesson from these considerations regarding wobbling by inosine is to realize the simplifications that are inherent to summarizing the decoding behavior of tRNA sets in simple tables with decoding rules of the kind one prints out and tapes on the wall: "The" Wobble Rules. It should be noted that Curran [11] and Percudani [88] have made the same point long ago.

The inosine modification in human disease

A rapidly emerging theme in the field of tRNA biology, and particularly tRNA modifications, is their *disease relevance* [89–91]. This recently identified class of diseases is now referred to as *modopathies* ([92] and references therein). The inosine modification is no exception to the trend: the implications of mutation in the *ADAT3* gene in human beings can be far-reaching. In 2013 a G-to-A transition mutation was reported that changed a valine codon into a methionine codon [93] in *ADAT3*. Patients carrying this mutation in homozygous state were described as presenting severe intellectual disability with misalignment of the eyes (strabismus). Since then, more mutations in *ADAT3* have been described [94–97], all with consequences for intellectual ability. In [96] (and also in [97]), two different mutations are described, with the patients receiving one damaged allele from the father and the other damaged allele from the mother (this is called compound heterozygosity). The implications of both homozygous *ADAT3* mutations and compound heterozygous *ADAT3* mutations can be so severe that they show themselves early in childhood. In such cases children can present intellectual disability, hypotonia, short stature, feeding difficulties, strabismus, microcephaly, and absent or limited speech. These medical reports show that reduced inosine modification in humans has a severe impact on neurodevelopment. Defective synthesis of proteins with low-complexity sequence stretches rich in leucine, isoleucine, valine, serine, proline, threonine, alanine and arginine probably plays a role in this impact [67].

Recently, the ADAT2/ADAT3 complex of the mouse has been studied with and without the above valine-to-methionine mutation [98]. This study shows that the change in ADAT3 most likely hinders optimal presentation of the tRNA anticodon-arm to the catalytic site in ADAT2. The work on the murine ADAT2/ADAT3 was preceded by work on the yeast ADAT2/ADAT3, providing a broader view of this enzyme [99]. This area of research promises to ultimately uncover ways to alleviate the problems which these patients face. An example how basic tRNA research can improve the life of patients with modification mutations has recently emerged in Japan, for a modification different from the inosine modification. Oral administration of a high dose of taurine suppressed stroke recurrence in patients with the MELAS modopathy in Japan, which involves mutations in tRNA genes (see [25] and references therein). With respect to the inosine modification, it should be realized that children who are unable to speak due to compound heterozygosity for *ADAT3* may have profound capacity for *nonverbal* language development [95]. This is an aspect which should not be forgotten, as a special program for nonverbal language development may make these children and their parents much happier.

Recently, the inosine modification has been found to also play a role in certain kinds of cancer [9]. During the development of a cancer, the genome of cancer cells can change by way of deletion of genes which in normal growth are indispensable. Dedon and Begley [9] found a cluster of genes containing *ADAT3* which can be deleted during tumor growth. They arrived at this conclusion by mining a dataset based on 47,534 patient samples from 55 forms of cancer. According to their heat map, in approximately 20 forms of cancer, gene deletion of *ADAT3* is sometimes present. The forms of cancer with the highest frequency of *ADAT3* deletion are B-lymphoblastic leukemia/lymphoma, cervical squamous cell carcinoma, serous ovarian cancer and uterine serous carcinoma/uterine papillary serous carcinoma. In normal eukaryotic cells, life is impossible without the ADAT2/ADAT3 complex. The heat map of Dedon and Begley suggests that certain cancer cell lines are able to live without this complex. In future experimental research it would be important to verify for one of such cell lines that these eukaryotic cells are really living without I_{34}.

The latest chapter in the evolution of inosine research involves its application in biotechnology. TadA has been engineered to develop enzymes capable of inducing targeted DNA modifications [37,100,101], offering a potential approach for DNA repair in living organisms. This represents a promising advancement in medical science, though it also necessitates careful consideration of potential risks associated with such deep cellular interventions. Apart from medical aspects, this work [37] provides insights into the early evolution of inosine modification through the study of "resurrected proteins" (see [102] and references therein).

Perspectives and future directions

With the surge of recent enthusiasm for studying tRNA modifications (mainly motivated by the combined drive of the tRNA modification enzyme identity of many genes involved in human disease and fast technological progress) also our old acquaintance inosine is back at the forefront of scientific interest. In the genomics era, the fact that inosine at position 34 of tRNA is *not* a universal aspect of living cells has become abundantly clear. Archaea do not possess I_{34}, and neither do many bacteria. This is an evolutionary perspective which needs to be emphasized.

The dual duty of TadA as an enzyme both introducing inosine in tRNA and in mRNA [44,45] in many bacteria (i.e. those bacteria which *do* possess I_{34}) is something which has not yet been generally appreciated. The general differences in the functioning of epitranscriptomics between a bacterial clade with TadA compared to a bacterial clade without TadA are a subject of general interest in evolutionary bacteriology.

Rapid developments are occurring in our understanding of the eukaryotic A-to-I editing ADAT2/ADAT3 complex, and the cellular proteome heavily dependent on its activity [53,66,67]. Many exciting discoveries are expected to emerge in this field. An evolutionary implication is the realization that a single glance at the tRNA set of an organism allows one to know if the organism is a eukaryote or not (5 or more A-starting anticodons of different kind means: eukaryote; 3 or less A-starting anticodons of different kind means: *not* a eukaryote).

The inosine modification at position 37 of the anticodon-loop has been with us as long as the position 34 modification [21]; however, this aspect of tRNA biology has remained largely unstudied. What is I_{37} in e.g. human $tRNA^{Ala}_{AGC}$ doing? Why does specifically $tRNA^{Ala}$ need this modification? Is this modification indeed present in all eukaryotes?

The dynamic nature of tRNA modifications is "charging to the front", and regulated incomplete modification status is also found with respect to I_{34} [78,83,44]. Fortunately, rapid technological progress (e.g. [85]) promises that insight in the exact abundances and modification states of the total of tRNAs present in a cell at a certain moment will be in sight soon.

Nuance needs to be brought to the field concerning the wobble behavior of inosine. Too often it is assumed that inosine reads A-ending codons without difficulty. The reality is more complex, and is different for different codon boxes of the genetic code, for different organisms, and for different codon contexts. The recent report [86] of superwobbling of A-starting and U-starting anticodons, depending on G-C content of the T-stem should make us humble, and hesitant to make overly simplistic tables presenting wobble behavior.

Many of these new and exciting developments are of eminent interest for the field of human disease. The molecular basis for many phenomena of intellectual disability and speech impairments until recently remained totally unclear, but can now be attributed to hypomodification of tRNAs during embryological development. Also in the field of cancer research, tRNA modification has entered as one of the biological factors in the subject. The need for verification of cancer cells living despite the absence of I_{34} in these cells [9] has been indicated in the section on inosine in human disease.

Finally, the understanding of tRNA biology throws light upon main events in the evolutionary development of life in general: development of I_{34} for arginine on the way from LUCA to the bacteria, and explosive expansion of I_{34} all over the genetic code table at the emergence of eukaryotes, followed by the full exploitation of the possibilities this offered in multicellular animals.

CRediT authorship contribution statement

Peter T.S. van der Gulik: Writing – original draft. **Wouter D. Hoff:** Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are much indebted to Dave Speijer for making the figures, discussion with respect to tRNA modifications and general help with the process of producing this article, e.g. implementing the reference manager and proofreading. Without his guidance this review would not have happened.

We are also grateful to two anonymous reviewers for their comments that greatly improved the manuscript.

Received 13 January 2025;
Accepted 30 April 2025;
Available online 17 May 2025

Keywords:
Inosine;
Wobble;
Modopathies;
Eukaryogenesis;
Nanopore

Abbreviations:
ADAT, Adenosine deaminase acting on tRNA; CDAT, Cytosine deaminase acting on tRNA; FECA, First eukaryote common ancestor; I, Inosine; LACA, Last archaeal common ancestor; LBCA, Last bacterial common ancestor; LECA, Last eukaryote common ancestor; LUCA, Last universal common ancestor; Ψ, Pseudourine; TAD, tRNA-specific Adenosine Deaminase

References

[1]. Brennan, T., Sundaralingam, M., (1976). Structure of transfer RNA molecules containing the long variable loop. *Nucleic Acids Res.* **3**, 3235–3251.
[2]. Fan, J.-Y., Huang, Q., Ji, Q.-Q., Wang, E.-D., (2019). LeuRS can leucylate type I and type II tRNALeus in *Streptomyces coelicolor*. *Nucleic Acids Res.* **47**, 6369–6385. https://doi.org/10.1093/nar/gkz443.
[3]. Chan, P.P., Lowe, T.M., (2009). GtRNAdb: a database of transfer RNA genes detected in genomic sequence. *Nucleic Acids Res.* **37**, D93–D97. https://doi.org/10.1093/nar/gkn787.
[4]. Chan, P.P., Lowe, T.M., (2016). GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. *Nucleic Acids Res* **44**, D184–D189. https://doi.org/10.1093/nar/gkv1309.
[5]. Grosjean, H., De Crécy-Lagard, V., Marck, C., (2010). Deciphering synonymous codons in the three domains of life: co-evolution with specific tRNA modification enzymes. *FEBS Lett.* **584**, 252–264. https://doi.org/10.1016/j.febslet.2009.11.052.
[6]. Schultz, S.K., Kothe, U., (2024). RNA modifying enzymes shape tRNA biogenesis and function. *J. Biol. Chem.* **300**, 107488. https://doi.org/10.1016/j.jbc.2024.107488.
[7]. Phizicky, E.M., Hopper, A.K., (2010). tRNA biology charges to the front. *Genes Dev.* **24**, 1832–1860. https://doi.org/10.1101/gad.1956510.
[8]. Saleh, S., Farabaugh, P.J., (2024). Posttranscriptional modification to the core of tRNAs modulates translational misreading errors. *RNA* **30**, 37–51. https://doi.org/10.1261/rna.079797.123.
[9]. Dedon, P.C., Begley, T.J., (2022). Dysfunctional tRNA reprogramming and codon-biased translation in cancer. *Trends Mol. Med.* **28**, 964–978. https://doi.org/10.1016/j.molmed.2022.09.007.
[10]. Borén, T., Elias, P., Samuelsson, T., Claesson, C., Barciszewska, M., Gehrke, C.W., Kuo, K.C., Lustig, F., (1993). Undiscriminating codon reading with adenosine in the wobble position. *J. Mol. Biol.* **230**, 739–749. https://doi.org/10.1006/jmbi.1993.1196.
[11]. Curran, J.F., (1995). Decoding with the A:I wobble pair is inefficient. *Nucl. Acids Res.* **23**, 683–688. https://doi.org/10.1093/nar/23.4.683.
[12]. Murphy, F.V., Ramakrishnan, V., (2004). Structure of a purine-purine wobble base pair in the decoding center of the ribosome. *Nature Struct. Mol. Biol.* **11**, 1251–1252. https://doi.org/10.1038/nsmb866.
[13]. Lim, V.I., (1995). Analysis of action of the wobble adenine on codon reading within the ribosome. *J. Mol. Biol.* **252**, 277–282. https://doi.org/10.1006/jmbi.1995.0494.
[14]. Muramatsu, T., Nishikawa, K., Nemoto, F., Kuchino, Y., Nishimura, S., Miyazawa, T., Yokoyama, S., (1988). Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. *Nature* **336**, 179–181. https://doi.org/10.1038/336179a0.
[15]. Mandal, D., Köhrer, C., Su, D., Russell, S.P., Krivos, K., Castleberry, C.M., Blum, P., Limbach, P.A., Söll, D., RajBhandary, U.L., (2010). Agmatidine, a modified cytidine in the anticodon of archaeal $tRNA^{Ile}$, base pairs with adenosine but not with guanosine. *PNAS* **107**, 2872–2877. https://doi.org/10.1073/pnas.0914869107.
[16]. Ikeuchi, Y., Kimura, S., Numata, T., Nakamura, D., Yokogawa, T., Ogata, T., Wada, T., Suzuki, T., Suzuki, T., (2010). Agmatine-conjugated cytidine in a tRNA anticodon is essential for AUA decoding in archaea. *Nature Chem. Biol.* **6**, 277–282. https://doi.org/10.1038/nchembio.323.
[17]. van der Gulik, P.T.S., Hoff, W.D., (2011). Unassigned codons, nonsense suppression, and anticodon modifications in the evolution of the genetic code. *J. Mol. Evol.* **73**, 59–69. https://doi.org/10.1007/s00239-011-9470-3.
[18]. Miyauchi, K., Kimura, S., Akiyama, N., Inoue, K., Ishiguro, K., Vu, T.-S., Srisuknimit, V., Koyama, K., Hayashi, G., Soma, A., Nagao, A., Shirouzu, M., Okamoto, A., Waldor, M.K., Suzuki, T., (2024). A tRNA modification with aminovaleramide facilitates AUA decoding in protein synthesis. *Nature Chem. Biol.*. https://doi.org/10.1038/s41589-024-01726-x.
[19]. Takemoto, C., Spremulli, L.L., Benkowski, L.A., Ueda, T., Yokogawa, T., Watanabe, K., (2009). Unconventional decoding of the AUA codon as methionine by mitochondrial tRNA Met with the anticodon f 5 CAU as revealed with a mitochondrial in vitro translation system. *Nucleic Acids Res.* **37**, 1616–1627. https://doi.org/10.1093/nar/gkp001.
[20]. Massey, S.E., (2008). The proteomic constraint and its role in molecular evolution. *Mol. Biol. Evol.* **25**, 2557–2565. https://doi.org/10.1093/molbev/msn210.
[21]. Holley, R.W., Apgar, J., Everett, G.A., Madison, J.T., Marquisee, M., Merrill, S.H., Penswick, R.J., Zamir, A., (1965). Structure of a ribonucleic acid. *Science* **147**, 1462–1465.
[22]. Crick, F.H.C., (1966). Codon-anticodon pairing: the wobble hypothesis. *J. Mol. Biol.* **19**, 548–555.

[23]. Agris, P.F., Eruysal, E.R., Narendran, A., Väre, V.Y.P., Vangaveti, S., Ranganathan, S.V., (2018). Celebrating wobble decoding: half a century and still much is new. *RNA Biol.* **15**, 537–553. https://doi.org/10.1080/15476286.2017.1356562.

[24]. Grosjean, H., Auxilien, S., Constantinesco, F., Simon, C., Corda, Y., Becker, H.F., Foiret, D., Morin, A., Jin, Y. X., Fournier, M., Fourrey, J.L., (1996). Enzymatic conversion of adenosine to inosine and to N1-methylinosine in transfer RNAs: a review. *Biochimie* **78**, 488–501.

[25]. Suzuki, T., (2021). The expanding world of tRNA modifications and their disease relevance. *Nature Rev. Mol. Cell Biol.* **22**, 375–392. https://doi.org/10.1038/s41580-021-00342-0.

[26]. Diwan, G.D., Agashe, D., (2018). Wobbling forth and drifting back: the evolutionary history and impact of bacterial tRNA modifications. *Mol. Biol. Evol.* **35**, 2046–2059. https://doi.org/10.1093/molbev/msy110.

[27]. Novoa, E.M., Pavon-Eternod, M., Pan, T., Ribas de Pouplana, L., (2012). A role for tRNA modifications in genome structure and codon usage. *Cell* **149**, 202–213. https://doi.org/10.1016/j.cell.2012.01.050.

[28]. Novoa, E.M., Ribas De Pouplana, L., (2012). Speeding with control: codon usage, tRNAs, and ribosomes. *Trends Genet.* **28**, 574–581. https://doi.org/10.1016/j.tig.2012.07.006.

[29]. Imachi, H., Nobu, M.K., Nakahara, N., Morono, Y., Ogawara, M., Takaki, Y., Takano, Y., Uematsu, K., Ikuta, T., Ito, M., Matsui, Y., Miyazaki, M., Murata, K., Saito, Y., Sakai, S., Song, C., Tasumi, E., Yamanaka, Y., Yamaguchi, T., Kamagata, Y., Tamaki, H., Takai, K., (2020). Isolation of an archaeon at the prokaryote–eukaryote interface. *Nature* **577**, 519–525. https://doi.org/10.1038/s41586-019-1916-6.

[30]. Skejo, J., Garg, S.G., Gould, S.B., Hendriksen, M., Tria, F.D.K., Bremer, N., Franjević, D., Blackstone, N.W., Martin, W.F., (2021). Evidence for a syncytial origin of eukaryotes from ancestral state reconstruction. *Genome Biol. Evol.* **13**, evab096. https://doi.org/10.1093/gbe/evab096.

[31]. Moody, E.R., Mahendrarajah, T.A., Dombrowski, N., Clark, J.W., Petitjean, C., Offre, P., Szöllősi, G.J., Spang, A., Williams, T.A., (2022). An estimate of the deepest branches of the tree of life from ancient vertically evolving genes. *Elife* **11**, e66695. https://doi.org/10.7554/eLife.66695.

[32]. López-García, P., Moreira, D., (2023). The symbiotic origin of the eukaryotic cell. *C. R. Biol.* **346**, 55–73. https://doi.org/10.5802/crbiol.118.

[33]. Moody, E.R.R., Álvarez-Carretero, S., Mahendrarajah, T.A., Clark, J.W., Betts, H.C., Dombrowski, N., Szánthó, L.L., Boyle, R.A., Daines, S., Chen, X., Lane, N., Yang, Z., Shields, G.A., Szöllősi, G.J., Spang, A., Pisani, D., Williams, T.A., Lenton, T.M., Donoghue, P.C.J., (2024). The nature of the last universal common ancestor and its impact on the early Earth system. *Nature Ecol. Evol.* **8**, 1654–1666. https://doi.org/10.1038/s41559-024-02461-1.

[34]. Torres, A.G., Piñeyro, D., Filonava, L., Stracker, T.H., Batlle, E., Ribas De Pouplana, L., (2014). A-to-I editing on tRNAs: biochemical, biological and evolutionary implications. *FEBS Lett.* **588**, 4279–4286. https://doi.org/10.1016/j.febslet.2014.09.025.

[35]. Roovers, M., Droogmans, L., Grosjean, H., (2021). Post-transcriptional modifications of conserved nucleotides in the T-loop of tRNA: a tale of functional convergent evolution. *Genes* **12**, 140. https://doi.org/10.3390/genes12020140.

[36]. Iyer, L.M., Zhang, D., Rogozin, I.B., Aravind, L., (2011). Evolution of the deaminase fold and multiple origins of eukaryotic editing and mutagenic nucleic acid deaminases from bacterial toxin systems. *Nucleic Acids Res.* **39**, 9473–9497. https://doi.org/10.1093/nar/gkr691.

[37]. Zhang, S., Yuan, B., Cao, J., Song, L., Chen, J., Qiu, J., Qiu, Z., Zhao, X.-M., Chen, J., Cheng, T.-L., (2023). TadA orthologs enable both cytosine and adenine editing of base editors. *Nature Commun.* **14**, 414. https://doi.org/10.1038/s41467-023-36003-3.

[38]. Gerber, A., Grosjean, H., Melcher, T., Keller, W., (1998). Tad1p, a yeast tRNA-specific adenosine deaminase, is related to the mammalian pre-mRNA editing enzymes ADAR1 and ADAR2. *EMBO J.* **17**, 4780–4789. https://doi.org/10.1093/emboj/17.16.4780.

[39]. Gerber, A.P., Keller, W., (1999). An adenosine deaminase that generates inosine at the wobble position of tRNAs. *Science* **286**, 1146–1149. https://doi.org/10.1126/science.286.5442.1146.

[40]. Auxilien, S., Crain, P.F., Trewyn, R.W., Grosjean, H., (1996). Mechanism, specificity and general properties of the yeast enzyme catalysing the formation of inosine 34 in the anticodon of transfer RNA. *J. Mol. Biol.* **262**, 437–458.

[41]. Wolf, J., Gerber, A.P., Keller, W., (2002). tadA, an essential tRNA-specific adenosine deaminase from Escherichia coli. *EMBO J.* **21**, 3841–3851. https://doi.org/10.1093/emboj/cdf362.

[42]. Rubio, M.A.T., Gaston, K.W., McKenney, K.M., Fleming, I.M.C., Paris, Z., Limbach, P.A., Alfonzo, J.D., (2017). Editing and methylation at a single site by functionally interdependent activities. *Nature* **542**, 494–497. https://doi.org/10.1038/nature21396.

[43]. Mao, X.-L., Eriani, G., Zhou, X.-L., (2024). ADATs: roles in tRNA editing and relevance to disease. *ABBS*. https://doi.org/10.3724/abbs.2024125.

[44]. Wulff, T.F., Hahnke, K., Lécrivain, A.-L., Schmidt, K., Ahmed-Begrich, R., Finstermeier, K., Charpentier, E., (2024). Dynamics of diversified A-to-I editing in *Streptococcus pyogenes* is governed by changes in mRNA stability. *Nucleic Acids Res.*gkae629. https://doi.org/10.1093/nar/gkae629.

[45]. Yang, X., Sun, T., Jia, P., Li, S., Li, X., Shi, Y., Li, X., Gao, H., Yin, H., Jia, X., Yang, Q., (2023). A-to-I RNA editing in *Klebsiella pneumoniae* regulates quorum sensing and affects cell growth and virulence. *Adv. Sci.* **10**, 2206056. https://doi.org/10.1002/advs.202206056.

[46]. Licht, K., Hartl, M., Amman, F., Anrather, D., Janisiw, M. P., Jantsch, M.F., (2019). Inosine induces context-dependent recoding and translational stalling. *Nucleic Acids Res.* **47**, 3–14. https://doi.org/10.1093/nar/gky1163.

[47]. Rafels-Ybern, À., Torres, A.G., Camacho, N., Herencia-Ropero, A., Roura Frigolé, H., Wulff, T.F., Raboteg, M., Bordons, A., Grau-Bove, X., Ruiz-Trillo, I., Ribas De Pouplana, L., (2019). The expansion of inosine at the wobble position of tRNAs, and its role in the evolution of

proteomes. *Mol. Biol. Evol.* **36**, 650–662. https://doi.org/10.1093/molbev/msy245.
[48]. Gerber, A.P., Keller, W., (2001). RNA editing by base deamination: more enzymes, more targets, new mysteries. *Trends Biochem. Sci.* **26**, 376–384.
[49]. Marck, C., Grosjean, H., (2002). tRNomics: analysis of tRNA genes from 50 genomes of eukarya, archaea, and bacteria reveals anticodon-sparing strategies and domain-specific features. *RNA* **8**, 1189–1232. https://doi.org/10.1017/S1355838202022021.
[50]. Guy, M.P., Phizicky, E.M., (2014). Two-subunit enzymes involved in eukaryotic post-transcriptional tRNA modification. *RNA Biol.* **11**, 1608–1618. https://doi.org/10.1080/15476286.2015.1008360.
[51]. Dixit, S., Henderson, J.C., Alfonzo, J.D., (2019). Multi-substrate specificity and the evolutionary basis for interdependence in tRNA editing and methylation enzymes. *Front. Genet.* **10**, 104. https://doi.org/10.3389/fgene.2019.00104.
[52]. Roura Frigolé, H., Camacho, N., Castellví Coma, M., Fernández-Lozano, C., García-Lema, J., Rafels-Ybern, À., Canals, A., Coll, M., Ribas De Pouplana, L., (2019). tRNA deamination by ADAT requires substrate-specific recognition mechanisms and can be inhibited by tRFs. *RNA* **25**, 607–619. https://doi.org/10.1261/rna.068189.118.
[53]. Dolce, L.G., Zimmer, A.A., Tengo, L., Weis, F., Rubio, M. A.T., Alfonzo, J.D., Kowalinski, E., (2022). Structural basis for sequence-independent substrate selection by eukaryotic wobble base tRNA deaminase ADAT2/3. *Nature Commun.* **13**, 6737. https://doi.org/10.1038/s41467-022-34441-z.
[54]. Lehmann, J., Libchaber, A., (2008). Degeneracy of the genetic code and stability of the base pair at the second position of the anticodon. *RNA* **14**, 1264–1269.
[55]. Saint-Léger, A., Bello, C., Dans, P.D., Torres, A.G., Novoa, E.M., Camacho, N., Orozco, M., Kondrashov, F. A., Ribas De Pouplana, L., (2016). Saturation of recognition elements blocks evolution of new tRNA identities. *Sci. Adv.* **2**, e1501860. https://doi.org/10.1126/sciadv.1501860.
[56]. Eigen, M., (2002). Error catastrophe and antiviral strategy. *PNAS* **99**, 13374–13376. https://doi.org/10.1073/pnas.212514799.
[57]. Speijer, D., (2015). Birth of the eukaryotes by a set of reactive innovations: new insights force us to relinquish gradual models. *Bioessays* **37**, 1268–1276. https://doi.org/10.1002/bies.201500107.
[58]. Speijer, D., Hammond, M., Lukeš, J., (2020). Comparing early eukaryotic integration of mitochondria and chloroplasts in the light of internal ROS challenges: timing is of the essence. *mBio* **11**, e00955-20. https://doi.org/10.1128/mBio.00955-20.
[59]. Woese, C.R., Kandler, O., Wheelis, M.L., (1990). Towards a natural system of organisms: proposal for the domains archaea, bacteria, and eucarya. *PNAS* **87**, 4576–4579.
[60]. de Duve, C., (2005). The onset of selection: natural selection started to drive the evolution as soon as molecular replication became possible. *Nature* **433**, 581–582.
[61]. van der Gulik, P.T.S., Hoff, W.D., (2016). Anticodon modifications in the tRNA set of LUCA and the fundamental regularity in the standard genetic code. *PLoS One* **11**, e0158342. https://doi.org/10.1371/journal.pone.0158342.
[62]. van der Gulik, P.T.S., Hoff, W.D., Speijer, D., (2017). In defence of the three-domains of life paradigm. *BMC Evol. Biol.* **17**, 218. https://doi.org/10.1186/s12862-017-1059-z.
[63]. van der Gulik, P.T.S., Hoff, W.D., Speijer, D., (2023). Renewing Linnaean taxonomy: a proposal to restructure the highest levels of the natural system. *Biol. Rev.* **98**, 584–602. https://doi.org/10.1111/brv.12920.
[64]. van der Gulik, P.T.S., Hoff, W.D., Speijer, D., (2024). The contours of evolution: In defence of Darwin's tree of life paradigm. *Bioessays* **46**, 2400012. https://doi.org/10.1002/bies.202400012.
[65]. van der Gulik, P.T.S., Egas, M., Kraaijeveld, K., Dombrowski, N., Groot, A.T., Spang, A., Hoff, W.D., Gallie, J., (2023). On distinguishing between canonical tRNA genes and tRNA gene fragments in prokaryotes. *RNA Biol.* **20**, 48–58. https://doi.org/10.1080/15476286.2023.2172370.
[66]. Giguère, S., Wang, X., Huber, S., Xu, L., Warner, J., Weldon, S.R., Hu, J., Phan, Q.A., Tumang, K., Prum, T., Ma, D., Kirsch, K.H., Nair, U., Dedon, P., Batista, F.D., (2024). Antibody production relies on the tRNA inosine wobble modification to meet biased codon demand. *Science* **383**, 205–211. https://doi.org/10.1126/science.adi1763.
[67]. Torres, A.G., Rodríguez-Escribà, M., Marcet-Houben, M., Santos Vieira, H.G., Camacho, N., Catena, H., Murillo Recio, M., Rafels-Ybern, À., Reina, O., Torres, F. M., Pardo-Saganta, A., Gabaldón, T., Novoa, E.M., Ribas de Pouplana, L., (2021). Human tRNAs with inosine 34 are essential to efficiently translate eukarya-specific low-complexity proteins. *Nucleic Acids Res.* **49**, 7011–7034. https://doi.org/10.1093/nar/gkab461.
[68]. Rafels-Ybern, À., Torres, A.G., Grau-Bove, X., Ruiz-Trillo, I., Ribas De Pouplana, L., (2018). Codon adaptation to tRNAs with Inosine modification at position 34 is widespread among Eukaryotes and present in two Bacterial phyla. *RNA Biol.* **15**, 500–507. https://doi.org/10.1080/15476286.2017.1358348.
[69]. Zhou, W., Karcher, D., Bock, R., (2013). Importance of adenosine-to-inosine editing adjacent to the anticodon in an Arabidopsis alanine tRNA under environmental stress. *Nucleic Acids Res.* **41**, 3362–3372. https://doi.org/10.1093/nar/gkt013.
[70]. Dannfald, A., Favory, J.-J., Deragon, J.-M., (2021). Variations in transfer and ribosomal RNA epitranscriptomic status can adapt eukaryote translation to changing physiological and environmental conditions. *RNA Biol.* **18**, 4–18. https://doi.org/10.1080/15476286.2021.1931756.
[71]. Smith, T.J., Giles, R.N., Koutmou, K.S., (2024). Anticodon stem-loop tRNA modifications influence codon decoding and frame maintenance during translation. *Semin. Cell Dev. Biol.* **154**, 105–113. https://doi.org/10.1016/j.semcdb.2023.06.003.
[72]. Maas, S., Gerber, A.P., Rich, A., (1999). Identification and characterization of a human tRNA-specific adenosine deaminase related to the ADAR family of Pre-mRNA editing enzymes. *PNAS* **96**, 8895–8900.

[73]. Maas, S., Kim, Y.-G., Rich, A., (2000). Sequence, genomic organization and functional expression of the murine tRNA-specific adenosine deaminase ADAT1. *Gene* **243**, 59–66.

[74]. Keegan, L.P., Gerber, A.P., Brindle, J., Leemans, R., Gallo, A., Keller, W., O'Connell, M.A., (2000). The properties of a tRNA-specific adenosine deaminase from *Drosophila melanogaster* support an evolutionary link between pre-mRNA editing and tRNA modification. *Mol. Cell. Biol.* **20**, 825–833. https://doi.org/10.1128/MCB.20.3.825-833.2000.

[75]. Urbonavicius, J., Qian, Q., Durand, J.M.B., Hagervall, T. G., Bjork, G.R., (2001). Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO J.* **20**, 4863–4873. https://doi.org/10.1093/emboj/20.17.4863.

[76]. Bjork, G.R., Jacobsson, K., Nilsson, K., Johansson, M.J. O., Bystrom, A.S., Persson, O.P., (2001). A primordial tRNA modification required for the evolution of life? *EMBO J.* **20**, 231–239. https://doi.org/10.1093/emboj/20.1.231.

[77]. Nedialkova, D.D., Leidel, S.A., (2015). Optimization of codon translation rates via tRNA modifications maintains proteome integrity. *Cell* **161**, 1606–1618. https://doi.org/10.1016/j.cell.2015.05.022.

[78]. Bornelöv, S., Selmi, T., Flad, S., Dietmann, S., Frye, M., (2019). Codon usage optimization in pluripotent embryonic stem cells. *Genome Biol.* **20**, 119. https://doi.org/10.1186/s13059-019-1726-z.

[79]. Jonkhout, N., Tran, J., Smith, M.A., Schonrock, N., Mattick, J.S., Novoa, E.M., (2017). The RNA modification landscape in human disease. *RNA* **23**, 1754–1769. https://doi.org/10.1261/rna.063503.117.

[80]. Valadon, C., Namy, O., (2021). The importance of the epi-transcriptome in translation fidelity. *Non-Coding RNA* **7**, 51. https://doi.org/10.3390/ncrna7030051.

[81]. Mitchener, M.M., Begley, T.J., Dedon, P.C., (2023). Molecular coping mechanisms: reprogramming tRNAs to regulate codon-biased translation of stress response proteins. *Acc. Chem. Res.* **56**, 3504–3514. https://doi.org/10.1021/acs.accounts.3c00572.

[82]. Dedon, P.C., Begley, T.J., (2014). A system of RNA modifications and biased codon use controls cellular stress response at the level of translation. *Chem. Res. Toxicol.* **27**, 330–337. https://doi.org/10.1021/tx400438d.

[83]. Wada, M., Ito, K., (2023). The CGA codon decoding through tRNA Arg (ICG) supply governed by Tad2/Tad3 in Saccharomyces cerevisiae. *FEBS J.* **290**, 3480–3489. https://doi.org/10.1111/febs.16760.

[84]. Ng, C.S., Sinha, A., Aniweh, Y., Nah, Q., Babu, I.R., Gu, C., Chionh, Y.H., Dedon, P.C., Preiser, P.R., (2018). tRNA epitranscriptomics and biased codon are linked to proteome expression in *Plasmodium falciparum*. *Mol. Syst. Biol.* **14**, e8009.

[85]. Lucas, M.C., Pryszcz, L.P., Medina, R., Milenkovic, I., Camacho, N., Marchand, V., Motorin, Y., Ribas De Pouplana, L., Novoa, E.M., (2024). Quantitative analysis of tRNA abundance and modifications by nanopore RNA sequencing. *Nature Biotechnol.* **42**, 72–86. https://doi.org/10.1038/s41587-023-01743-6.

[86]. Kompatscher, M., Bartosik, K., Erharter, K., Plangger, R., Juen, F.S., Kreutz, C., Micura, R., Westhof, E., Erlacher, M.D., (2024). Contribution of tRNA sequence and modifications to the decoding preferences of *E. coli* and *M. mycoides* tRNAGlyUCC for synonymous glycine codons. *Nucleic Acids Res.* **52**, 1374–1386. https://doi.org/10.1093/nar/gkad1136.

[87]. Žihala, D., Eliáš, M., (2019). Evolution and unprecedented variants of the mitochondrial genetic code in a lineage of green algae. *Genome Biol. Evol.* **11**, 2992–3007. https://doi.org/10.1093/gbe/evz210.

[88]. Percudani, R., (2001). Restricted wobble rules for eukaryotic genomes. *Trends Genet.* **17**, 133–135.

[89]. Suzuki, T., Nagao, A., Suzuki, T., (2011). Human mitochondrial tRNAs: biogenesis, function, structural aspects, and diseases. *Annu. Rev. Genet.* **45**, 299–329. https://doi.org/10.1146/annurev-genet-110410-132531.

[90]. Burgess, R.W., Storkebaum, E., (2023). tRNA dysregulation in neurodevelopmental and neurodegenerative diseases. *Annu. Rev. Cell Dev. Biol.* **39**, 223–252. https://doi.org/10.1146/annurev-cellbio-021623-124009.

[91]. Pinzaru, A.M., Tavazoie, S.F., (2023). Transfer RNAs as dynamic and critical regulators of cancer progression. *Nature Rev. Cancer* **23**, 746–761. https://doi.org/10.1038/s41568-023-00611-4.

[92]. Chujo, T., Tomizawa, K., (2021). Human transfer RNA modopathies: diseases caused by aberrations in transfer RNA modifications. *FEBS J.* **288**, 7096–7122. https://doi.org/10.1111/febs.15736.

[93]. Alazami, A.M., Hijazi, H., Al-Dosari, M.S., Shaheen, R., Hashem, A., Aldahmesh, M.A., Mohamed, J.Y., Kentab, A., Salih, M.A., Awaji, A., Masoodi, T.A., Alkuraya, F.S., (2013). Mutation in *ADAT3* , encoding adenosine deaminase acting on transfer RNA, causes intellectual disability and strabismus. *J. Med. Genet.* **50**, 425–430. https://doi.org/10.1136/jmedgenet-2012-101378.

[94]. Salehi Chaleshtori, A.R., Miyake, N., Ahmadvand, M., Bashti, O., Matsumoto, N., Noruzinia, M., (2018). A novel 8-bp duplication in ADAT3 causes mild intellectual disability. *Hum. Genome Var.* **5**, 7. https://doi.org/10.1038/s41439-018-0007-9.

[95]. Thomas, E., Lewis, A.M., Yang, Y., Chanprasert, S., Potocki, L., Scott, D.A., (2019). Novel missense variants in ADAT3 as a cause of syndromic intellectual disability. *J. Pediatr. Genet.* **08**, 244–251. https://doi.org/10.1055/s-0039-1693151.

[96]. Ramos, J., Proven, M., Halvardson, J., Hagelskamp, F., Kuchinskaya, E., Phelan, B., Bell, R., Kellner, S.M., Feuk, L., Thuresson, A.-C., Fu, D., (2020). Identification and rescue of a tRNA wobble inosine deficiency causing intellectual disability disorder. *RNA* **26**, 1654–1666. https://doi.org/10.1261/rna.076380.120.

[97]. Del-Pozo-Rodriguez, J., Tilly, P., Lecat, R., Vaca, H.R., Mosser, L., Balla, T., Gomes, M.V., Ramos-Morales, E., Brivio, E., Salinas-Giégé, T., VanNoy, G., England, E.M., Lovgren, A.K., O'Leary, M., Chopra, M., Gable, D., Alnuzha, A., Kamel, M., Almenabawy, N., O'Donnell-Luria, A., Neil, J.E., Gleeson, J.G., Walsh, C.A., Elkhateeb, N., Selim, L., Srivastava, S., Nedialkova, D. D., Drouard, L., Romier, C., Bayam, E., Godin, J.D., (2024). Neurodevelopmental disorders associated variants in *ADAT3* disrupt the activity of the ADAT2/ADAT3 tRNA deaminase complex and impair neuronal migration. *MedRxiv*. https://doi.org/10.1101/2024.03.01.24303485.

[98]. Ramos-Morales, E., Bayam, E., Del-Pozo-Rodríguez, J., Salinas-Giegé, T., Marek, M., Tilly, P., Wolff, P.,

Troesch, E., Ennifar, E., Drouard, L., Godin, J.D., Romier, C., (2021). The structure of the mouse ADAT2/ADAT3 complex reveals the molecular basis for mammalian tRNA wobble adenosine-to-inosine deamination. *Nucleic Acids Res.* **49**, 6529–6548. https://doi.org/10.1093/nar/gkab436.

[99]. Liu, X., Chen, R., Sun, Y., Chen, R., Zhou, J., Tian, Q., Tao, X., Zhang, Z., Luo, G., Xie, W., (2020). Crystal structure of the yeast heterodimeric ADAT2/3 deaminase. *BMC Biol.* **18**, 189. https://doi.org/10.1186/s12915-020-00920-2.

[100]. Neugebauer, M.E., Hsu, A., Arbab, M., Krasnow, N.A., McElroy, A.N., Pandey, S., Doman, J.L., Huang, T.P., Raguram, A., Banskota, S., Newby, G.A., Tolar, J., Osborn, M.J., Liu, D.R., (2023). Evolution of an adenine base editor into a small, efficient cytosine base editor with low off-target activity. *Nature Biotechnol.* **41**, 673–685. https://doi.org/10.1038/s41587-022-01533-6.

[101]. Gao, Z., Jiang, W., Zhang, Y., Zhang, L., Yi, M., Wang, H., Ma, Z., Qu, B., Ji, X., Long, H., Zhang, S., (2023). Amphioxus adenosine-to-inosine tRNA-editing enzyme that can perform C-to-U and A-to-I deamination of DNA. *Commun. Biol.* **6**, 744. https://doi.org/10.1038/s42003-023-05134-0.

[102]. Mascotti, M.L., (2022). Resurrecting enzymes by ancestral sequence reconstruction. In: Magnani, F., Marabelli, C., Paradisi, F. (Eds.), *Enzyme Engineering*. Springer US, New York, NY, pp. 111–136. https://doi.org/10.1007/978-1-0716-1826-4_7.