Transfer RNA: from pioneering crystallographic studies to contemporary tRNA biology

Pablo Fernández-Millán, Cédric Schelcher, Joseph Chihade, Benoît Masquida, Philippe Giegé and Claude Sauter*

Highlights

- tRNA was the first class of large RNA to be crystallized and understood structurally
- tRNA, as the adaptor molecule, is a key player in translation
- The intrinsic structural flexibility of tRNA is a pivotal feature for its diverse functions
- Our understanding of tRNA biology has expanded widely in the -omics era

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Transfer RNA: from pioneering crystallographic studies to contemporary tRNA biology

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Abstract

Transfer RNAs (tRNAs) play a key role in protein synthesis as adaptor molecules between messenger RNA and protein sequences on the ribosome. Their discovery in the early sixties provoked a worldwide infatuation with the study of their architecture and their function in the decoding of genetic information. tRNAs are also emblematic molecules in crystallography: the determination of the first tRNA crystal structures represented a milestone in structural biology and tRNAs were for a long period the sole source of information on RNA folding, architecture, and post-transcriptional modifications. Crystallographic data on tRNAs in complex with aminoacyl-tRNA synthetases (aaRSs) also provided the first insight into protein:RNA interactions. Beyond the translation process and the history of structural investigations on tRNA, this review also illustrates the renewal of tRNA biology with the discovery of a growing number of tRNA partners in the cell, the involvement of tRNAs in a variety of regulatory and metabolic pathways, and emerging applications in biotechnology and synthetic biology.

Keywords

transfer RNA; crystallography; protein synthesis; genetic code; translation; RNA:protein recognition

Introduction

Since the mid-1950s crystallography has accompanied the birth and the development of molecular biology, providing biologists with pictures of their favorite biomolecules and helping them describe biological processes at an atomic scale [1]. The determination of the iconic three-dimensional structure of the DNA double helix by Watson and Crick [2] revolutionized the understanding of life. It led Crick to formulate his central dogma explaining the propagation of genetic information by DNA replication, transcription into RNA and translation into proteins, following a genetic code that associates every base triplet or codon in the DNA/RNA sequence with an amino acid in the polypeptide chain [3,4]. Yet an essential piece of the puzzle was missing and Crick proposed the 'adaptor hypothesis' in 1955, postulating the existence of a dedicated class of molecules making the link between nucleic acid and protein sequences [3]. Soon after a family of small RNAs, first called soluble RNAs (sRNAs) and quickly renamed transfer RNAs (tRNAs), were isolated along with an essential family of enzymes, aminoacyl-tRNA synthetases (aaRSs), which catalyze the specific coupling of amino acids to their tRNA carriers [5].

At this point, it became crucial to gain an insight into the structural properties of these molecules in order to understand the specificity of substrate recognition and of catalytic mechanisms ensuring the fidelity of tRNA aminoacylation reactions and, by extension, of protein synthesis. This triggered a worldwide effort to decipher the crystal structure of tRNAs and related enzymes. In 1966 the sequencing of $tRNA^{Ala}$ from yeast revealed complementary stretches of nucleotides suggesting a 2D folding resembling a cloverleaf [6]. The next three sequences of $tRNA^{Tyr}$, $tRNA^{Phe}$, and $tRNA^{Ser}$ [7–9] reinforced the idea of a four-arm organization. They also led to the concept of anticodon base triplet complementary to the codon of the carried amino acid, establishing a physical link between nucleic acid and protein alphabets/sequences. Hence, it became clear that all tRNAs should share a common scaffold to ensure their recognition by the ribosome and by elongation factors, while also expressing a specific identity with regard to their cognate aaRSs. The quest for structural data led to a first important breakthrough in the mid-seventies with the determination of the crystal structure of yeast $tRNA^{Phe}$ by two groups in the US and one in the UK [10–12], followed by that of yeast $tRNA^{Asp}$ in France [13]. These crystallographic studies revealed the characteristic L-shape of adaptor molecules. The story continued with the determination of the first crystal structures of tRNA:aaRS complexes in the early nineties [14] and culminated in the 2000s with the description of the adaptors bound to the A, P and E sites of the ribosome [15].

In the context of the celebration of a century of X-ray crystallography, the aim of this review is to illustrate how early studies of the key protagonists in the translational machinery such as tRNAs have contributed to many aspects to structural biology and how vital this tRNA research continues to be. Solving the initial structures represented a considerable challenge and required methodological developments in sample preparation, crystallization and crystallography. For almost 15 years, tRNAs were the sole source of structural information about RNA and provided the basis for the understanding of RNA architecture and function, as well as information about RNA:protein recognition and the role of RNA modifications. Finally, while tRNAs entered textbooks in the nineties as central actors in translation [16], a number of functional questions have recently emerged from the genome-wide examination of tRNA diversity. These include the discovery of new tRNA partners and related pathways and the observation of tRNA mutations related to human pathologies, stimulating novel research directions on tRNA function, structure, dynamics, and biotechnology.

From first crystal structures to the -omics era

A first step towards the crystallographic analysis of tRNAs was the separation of different species from natural sources like baker's yeast. RNA is distinguished from DNA by the presence of an hydroxyl group in 2' position of the sugar which confers both flexibility and reactivity, making RNA molecules much more labile and difficult to work with than DNA. In the sixties the method of countercurrent distribution, based on partitioning tRNAs according to their hydrophobicity in aqueous and organic solvents, played a key role. This technique allowed the preparation of pure samples on the hundreds of milligram scale required for biochemical characterization, sequencing and eventually crystallization [17]. The introduction of the vapor diffusion method was also crucial for the success of tRNA crystallization [18]. Other micromethods, such as dialysis or free-interface diffusion, were developed to crystallize aaRSs and aaRS:tRNA complexes because they were better adapted to sensitive

biological samples and have since been widely adopted. A new discipline called biocrystallogenesis, aiming at better understanding and mastering the process of biological crystallization, directly emerged from the difficulties encountered in the preparation of crystals suitable for diffraction studies [19]. These efforts eventually yielded tRNA crystals diffracting at resolution of ~3 Å at the turn of the seventies.

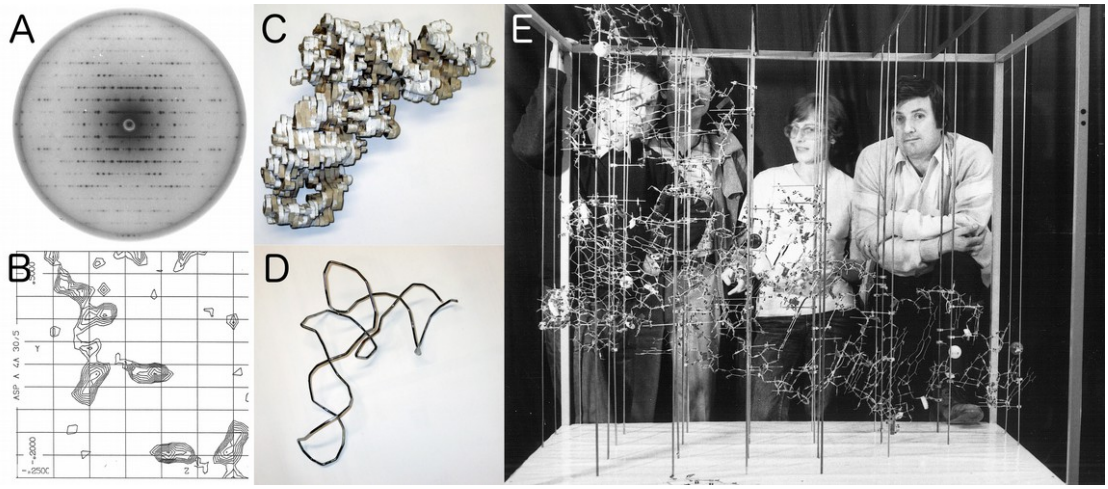

Figure 1: Determination of yeast tRNA^{Asp} crystal structure [13]. Structural investigations on yeast tRNA^{Asp} started in the seventies and illustrate well how crystallographic studies were performed at the time. (A) Diffraction patterns were collected on photographic films from which reflection intensities were quantified. Multiple isomorphous replacement with heavy atoms was used to determine sets of phases and to compute slices of electron density maps (B). A low resolution model in balsa wood was first assembled (C), then the tRNA backbone was built in metal wire by connecting the positions of phosphates, which were easily identified in the electron density map (D). Using an optical device called Richards box (see https://en.wikipedia.org/wiki/Frederic_M._Richards) and electron density maps manually transferred on glass windows, the French research team at IBMC, Strasbourg (E) could build a wire frame atomic model (scale: 2 cm = 1Å) showing the characteristic L-shape of yeast tRNA^{Asp}. From the left to the right: J.-C. Thierry, R. Giegé, M.-B. Comarmond, D. Moras (courtesy of R. Giegé).

Today, when it has become possible to determine X-ray structures on a simple laptop computer, it is difficult to imagine how solving a tRNA structure once represented long and tedious work, collecting diffraction pattern on photographic films and building a model manually in hand-drawn electron density maps (**Figure 1**). These pioneering crystallographic studies led to the well-known L-shape model. The building of full atomic models revealed how helical domains of the cloverleaf get stacked pairwise (acceptor and T helices, D and anticodon helices; **Figure 2A**) to form the two arms of the L, held together by a network of tertiary interactions predicted from sequence conservations by Levitt in 1969 [20]. Although the angle between the arms in tRNA^{Asp} is more open than in tRNA^{Phe} (100° instead of 90°), a sign of the intrinsic flexibility of tRNA backbone, both tRNAs adopt a similar fold, in agreement with the boomerang-shape proposed based on early small angle X-ray scattering (SAXS) measurements [21]. This canonical 2D cloverleaf and 3D L structure were later confirmed in a dozen crystal structures of free tRNAs, as well as by tens of thousands of tRNA sequences derived from a growing number of genomes and collected in dedicated databases such as the tRNAdb (http://trna.bioinf.uni-leipzig.de) [22]. Exceptions to this general pattern have been found in animal mitochondria, where tRNAs can display size reductions due to shorter loops in the D or T arms, and in some cases, the complete absence of one or two of arms (**Figure 2B**). The most extreme case is a functional tRNA consisting of only 42 nucleotides (to be compared with usual sizes of 70-95 nts) recently described in the mitochondria of the worm *Romanomermis culicivorax* [23]. This raises questions about the folding and the stability of such tRNAs and their recognition by cellular factors including the ribosome.

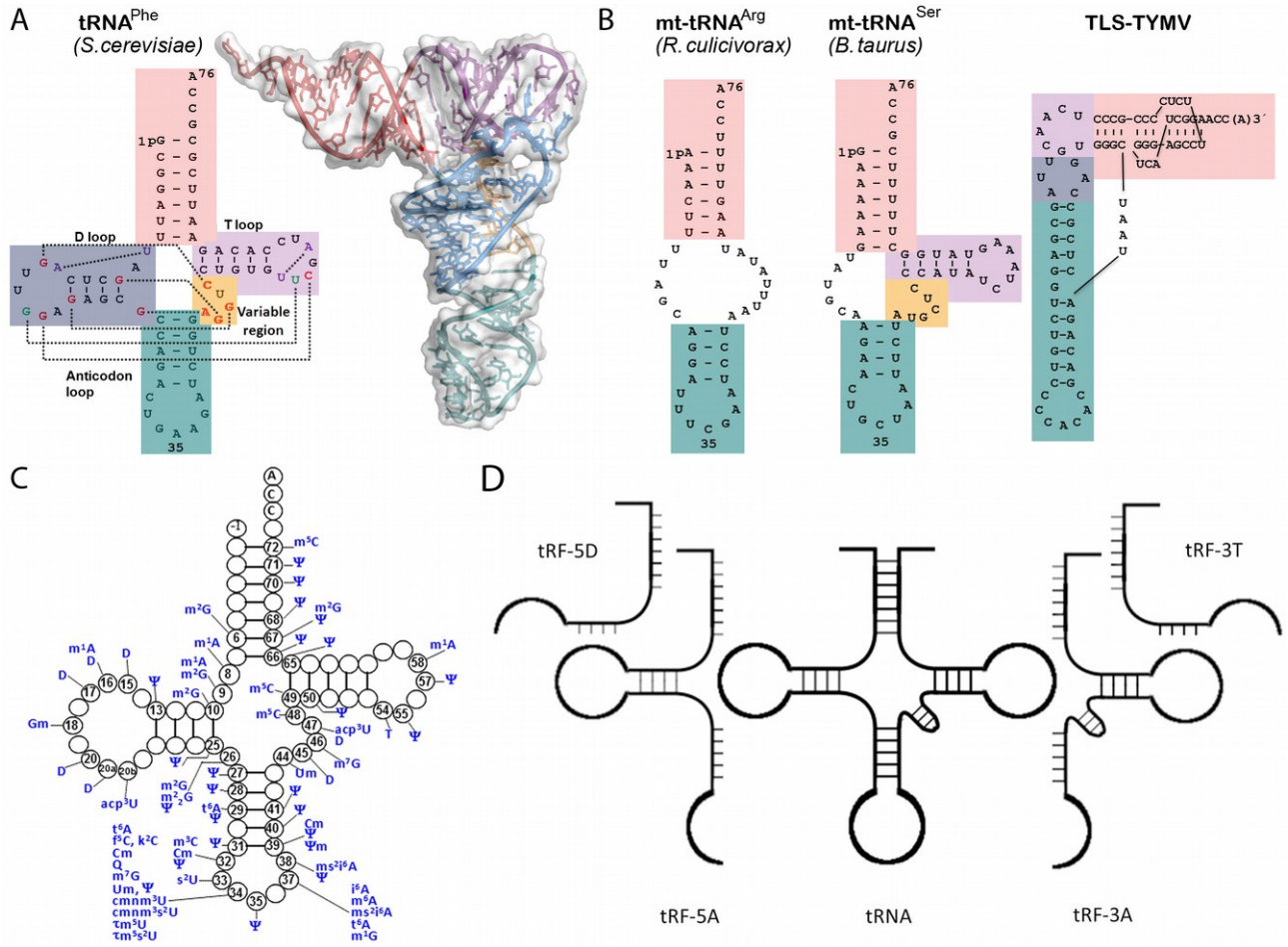

Figure 2: tRNA structural organization. (A) 2D and 3D structures of tRNA^{Phe} (PDBid 1TN1). Tertiary interactions that stabilize the L-shape are depicted on the cloverleaf as broken lines (more details in Figure 4). The arms of the cloverleaf are depicted with the same colors throughout the figure. (B) Examples of variations on the classical 2D organization with typical armless mitochondrial tRNAs and the tRNA-like structure (TLS) found in 3' of the genomic RNA of Turnip Yellow Mosaic Virus (TYMV). (C) Distribution of post-transcriptional modifications on a tRNA cloverleaf with conventional nucleotide numbering. (D) General nomenclature of tRNA-derived fragments (tRFs). Mature tRNAs and precursors can be cleaved at different positions to produce various tRFs. The atomic model of tRNA^{Phe} was rendered using PyMOL (Schrödinger), as well as atomic models in Figures 4 and 5.

Another structural characteristic of tRNAs is the presence of numerous post-transcriptionally modified nucleosides (**Figure 2C**). Besides methylated bases, the first sequence of yeast tRNA^{Ala} also revealed the presence of dihydrouridines in one loop, as well as a ribo-thymidine (T) and a pseudouridine (Ψ) in another, leading to the current names of D- and T- (or TΨC-) loops/stems as these modifications are almost universally conserved. Inosine was also found at position 34, soon identified as the first position of the anticodon, which base pairs with the third position of the codon in the messenger RNA. Following the 'wobble hypothesis' of Crick [3], an inosine at this position in the tRNA extends its decoding capacity to the synonymous codons ending by A, C or U (GCA, GCC and GCU in the case of tRNA^{Ala} with an IGC anticodon). For this reason, the number of tRNA species in a cell is always lower than the 61 codons, and can be as low as 22 in mammalian mitochondria, due to the presence of specific modifications at positions 34 and 37 of the anticodon loop [24]. Modified nucleosides can serve as recognition signals for cognate synthetases (see below) and are known to increase the stability of the tRNA scaffold, as reviewed in [25]. A classic example of modification that affects tRNA structure is the m1A at position 9 in human mitochondrial tRNA^{Lys} which drives the formation of the functional cloverleaf over an alternative hairpin structure [26]. More than a hundred such RNA modifications have been identified (**Figure 2C**) and their structures and positions in tRNAs, together with information on their biosynthetic pathways, have been cataloged in the RNA modification (http://rna-mdb.cas.albany.edu) and the Modomics (http://modomics.genesilico.pl) databases [27,28].

Exploring the mechanisms of tRNA:synthetase recognition

The first structural information on tRNA:synthetase interactions came more than a decade after the solution of $tRNA^{Phe}$ structure. The delay was mainly due to the difficulties in the preparation of homogeneous complexes for crystallization and crystal quality optimization. The first structures of tRNA:aaRS complexes specific for glutamine and aspartate [29,30] highlighted that, although synthetases catalyzed the same type of reaction, they diverge in terms of sequence and structure. The aaRS family was divided into two classes based on the topology of their catalytic domain [31]: a Rossmann fold, with its characteristic parallel β-sheet, is found in monomeric Class I aaRSs, while Class II enzymes are generally dimeric and are built around an antiparallel β-sheet. The determination of a number of crystal structures of tRNA:aaRS complexes has revealed the specificities and dynamics of class I and class II aaRS binding to tRNA.

The observation of close contacts between the partners, together with mutagenesis experiments on both partners, reinforced the concept of a second genetic code. This operational code involves tRNA determinants and tRNA anti-determinants, which are chemical signals defining the identity of a family of tRNAs with respect to their cognate aaRS or preventing the interaction and misacylation by a non cognate enzyme, respectively [32]. As might be expected, the anticodon region often constitutes a major identity element. In several complexes, anticodon nucleotides are distorted so as to be bound in specific enzymatic pockets (see, for example, the structure of the GlnRS-$tRNA^{Gln}$ complex [33]).

Identity elements are also frequently found in the acceptor stem, but the interpretation of specific protein contacts with this region of the tRNA is more difficult, since interactions with both the ribose-phosphate backbone and nucleotide bases may play a role. The clearest example may be provided by two recent structures of *A. fulgidus* alanyl-tRNA synthetase [34]. In one, the enzyme is complexed with a cognate tRNA, containing the G3:U70 wobble pair, which is an identity element, while the second is a complex with an A3:U70 tRNA mutant. The structures reveal a multifaceted set of protein-RNA interactions which direct the A76 nucleotide of the cognate tRNA into the aminoacylation active site, but distort the backbone of the mutant tRNA and prevent A76 from attaining the proper configuration for aminoacylation. Other tRNA:aaRS structures have demonstrated idiosyncratic modes of tRNA recognition that are difficult to describe within the framework of identity elements, such as *T. thermophilus* SerRS recognition of the long variable arm of $tRNA^{Ser}$ [35] and “shape-selective” contacts between *E.coli* CysRS and $tRNA^{Cys}$ [36].

These structural studies also shed light on the high degree of tRNA plasticity in tRNA:aaRS interactions [37]. For example, in the case of yeast aspartylation system, the comparison of $tRNA^{Asp}$ structure with the structure of its complex with AspRS revealed important conformational changes altering its structure upon enzyme binding, i.e. with the two arms of the overall L shape of $tRNA^{Asp}$ presenting a reduced angle [30]. In class I aaRSs including an editing domain, the 3' end of the tRNA can swing from the aminaocylation site to the editing site to allow hydrolysis of an incorrect amino acyl ester [38]. Despite the rapidly growing number of crystal structures of tRNA:aaRS complexes, the existing data remain highly biased in favor of bacterial systems, with few archaeal and eukaryotic structures available. Knowledge of organelle aminoacylation systems is even more scarce, with only a few isolated aaRS structures but no structures of complexes [37]. Recently, mitochondrial systems have drawn substantial interest due to the discovery of mutations in tRNAs and aaRSs associated with a variety of human pathologies [39,40]. Future investigations will be necessary to examine how these mutations affect expression, folding, binding, catalysis or other functions of tRNAs aside from aminoacylation and translation.

tRNA as template for RNA modeling

As mentioned above, the first tRNA structures were, for a long period, the only examples of complex RNA architecture (**Figure 3**). As such, they were instrumental in elaborating how double-stranded helices can be connected by structured loops in which all nucleotide edges (Watson-Crick, Hoogsteen, sugar) are used in base-pairing. Important roles of the 2' hydroxyl group of nucleotides were noted, such as its capacities to constrain the ribose pucker, forcing A-form RNA helices, and to simultaneously act as a donor and acceptor of hydrogen bond. Despite their relatively limited size and number, as the only available templates for RNA modeling, the tRNA crystal structures have long been a source of inspiration.

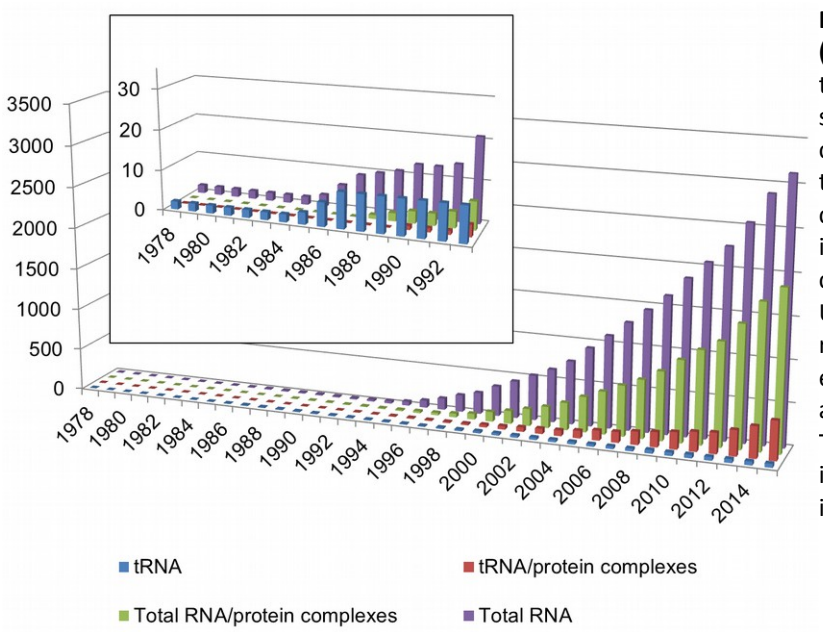

Figure 3: tRNA and RNA entries in the PDB (as of December 2014). The plot bar shows the number of PDB entries per year for structures of tRNA alone (red), tRNA:protein complexes (blue, this group includes tRNA:ribosome complexes), all RNA:protein complexes (green) and all isolated RNAs including tRNAs (violet). The inset gives a close-up view for the years 1978 to 1993. Until 1994 and the determination of first ribozyme structures, tRNAs were the exclusive source of information on the folding and architecture of complex RNA molecules. Then the study of the ribosome led to a burst in RNA and tRNA/RNA:protein complex data in the PDB.

The observation of an overall L-shape architecture stabilized by pairwise helix stacking led to the concept of RNA domain assembly. This assembly is further stabilized by loop-loop interactions on one side and base triples on the other (**Figure 4**). The stunning beauty of the structures was due to the harmony of the backbone curves resulting from interactions between bases in a layout so natural that the whole appeared to be unconstrained. As masterpieces resulting from millions of years of molecular adaptation, the tRNA crystal structures inspired structural biologists. The observation that the single strands joining the helical segments adopted precise architectures led to the idea that they could constitute individual structural motifs.

This essential RNA structural repertoire (See **Figure 4**) could in principle be used as a building set to generate theoretical RNA structural models by pasting together individual building blocks. However, in the eighties the organization of the secondary structures of ribosomal RNAs (rRNA) already indicated that the RNA structural diversity was not exhaustively contained in the tRNA structures. The number of loop residues well illustrates this idea. The tRNAs display seven- or eight-membered loops, while rRNAs contain less or more nucleotides in the loops closing hairpins. Moreover, rRNAs present internal loops or complex junctions linking two to five helical segments impossible to map onto tRNAs subdomains. At this stage, it was difficult to anticipate how the RNA structural repertoire would expand. However structural biologists could understand that the diversity of base-pairs beyond the Watson-Crick classics would be at play. Additional likely interactions were observed by analysis of tRNA crystal packing. Thus, building unknown motifs from interactions between nucleotides became a field of research. All atom modeling independently added stereochemical restraints on top of the secondary structure constraints, conferring significant predictive power to the method. Structural biologists could use RNA helices, base triples and kissing loops from the tRNA structures to build complex architectures like group I introns [41].

The number of available RNA crystal structures increased gradually (**Figure 3**), each time revealing new structural features that helped structural biologists to better understand RNA architecture and folding principle [42–44]. However the mist really cleared upon the appearance of the crystal structures of the ribosomal subunits [45–47], which multiplied the size of the structural repertoire by ~7 fold and revealed a wealth of magnificent and daring RNA folds, RNA-RNA and RNA-protein interaction motifs. Strikingly, most of these folds are recurrent, like the sarcin or loop E motif, the GNRA and UNCG tetraloops, kink-turns [48,49], C-loops [50], UA handles [51], and A-minor [52,53]. For example, the A-minor motif is very frequent due to the versatility of the O2' group interactions, although it cannot be deduced from comparative sequence analysis [54]. Some of these motifs are even reminiscent of tRNA: one can find a fair representation of anticodon loops, U-turns, as in the structure of the lariat-capping ribozyme [55], and T-loops, the latter being crucial for tRNA recognition by RNase P [56], T box regulators [57] and ribosome [15,58].

RNA modeling has never been as necessary as today since massive amounts of RNA sequences are being identified by new generation sequencing (NGS) methods at a rate far faster than conventional structural

methods such as crystallography can follow up. The continued efforts of structural biologists to increase the number of RNA crystal structures are thus mandatory to approach completion of the RNA structural repertoire which makes up the modeling building set. The identification of both secondary structures and nucleotides in proximity in RNAs by high-throughput chemical probing methods can then be coupled to automatic modeling methods based on the adjunction of RNA fragments. This type of approach, exemplified by MOHCA-seq coupled to modeling with Rosetta [59] have proven to reach subhelical resolution (10 Å).

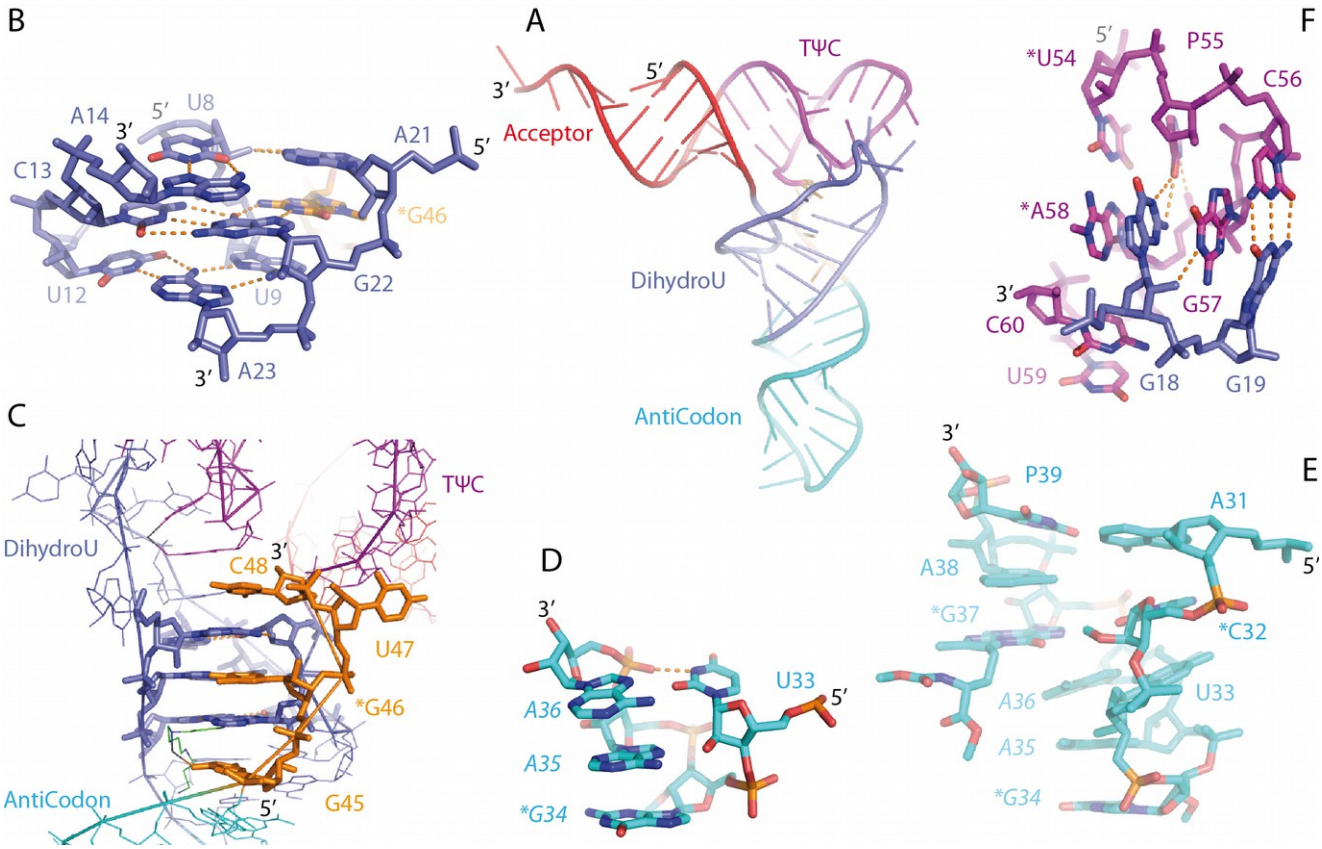

Figure 4: The early observation of tRNA structures shed light on RNA folding and architecture. (A) Overall ribbon view of the $tRNA^{Phe}$ crystal structure (PDB ID 1TN1). The different domains are indicated and colored individually. The variable loop lies behind the dihydrouridine stem loop (dim orange). (B) The core of the tRNA is stabilized by base triple interactions. In the foreground, the last two base pairs of the D stem are represented, U_{12}-A_{23}, and C_{13}-G_{22}. In the deep groove of the stem the Hoogsteen edge of U_9 and the Watson-Crick edge of $m7G_{46}$ interact with the Hoogsteen edge of A_{23} and of G_{22}, respectively. The first nucleotide of the D loop, A_{14}, is stabilized by U_8, which directly connects the Acceptor stem to the D domain. The resulting *trans* Watson-Crick/Hoogsteen interaction is further stabilized by A_{21}, the terminal residue of the D loop. $m7G_{46}$ stacks in between A_{21} and G_{22}. As seen in (C), stacking interactions extend farther than the base triple ensemble, which is wrapped by C_{48} and G_{45}. The latter interacts with the first base pair of the D stem (not shown), while the former stacks onto U_{59}, the unpaired penultimate nucleotide in the 3' side of the T loop. (D) The U-turn motif consists in the interaction between the N3 imino proton of U_{33} with the proximal phosphate oxygen atom of A_{36}. This motif is observed recurrently in RNA structures, for example in the T loop of the tRNA (P_{55} and $M1A_{58}$ in panel F). (E) A view of the overall anticodon loop shows the arrangement of the three nucleotides 34-36 (italic) involved in mRNA recognition. Hyper modification of nucleotide 37 prevents its interaction with a fourth nucleotide on the mRNA, which would result in mistranslation. (F) A view of the nucleotides involved in the interaction between the T and D loops depicts how stacking interactions promote close contact between nucleotides which result in exquisite hydrogen bonds. Notably, packing contacts in tRNA crystals between anticodon loops of adjacent tRNA molecules dictate the codon-anticodon interaction observed on the ribosome. Hydrogen bonds are indicated by solid dashed lines. '*' indicates modified nucleotides. *G_{46}: 7-methylguanosine; *G_{34}: 2'-O-methylguanosine; *C_{32}: 2'-O-methylcytidine; *U_{54}: 5-methyluridine; P_{55}: pseudouridine; *A58: 1-methyladenosine.

tRNA partners in the cell

As a central actor of the translation machinery, many of tRNA's partners are involved in the process of protein synthesis, including aaRSs, initiation and elongation factors that deliver aminoacyl-tRNAs to the ribosome. A growing number of functional complexes not directly related to protein synthesis have also been structurally characterized over the years, including maturation enzymes involved in tRNA biogenesis, regulatory T-boxes in mRNA and tRNA-like structures in viral RNAs as shown in **Figure 5**, illustrating the diversity of cellular functions associated with tRNAs.

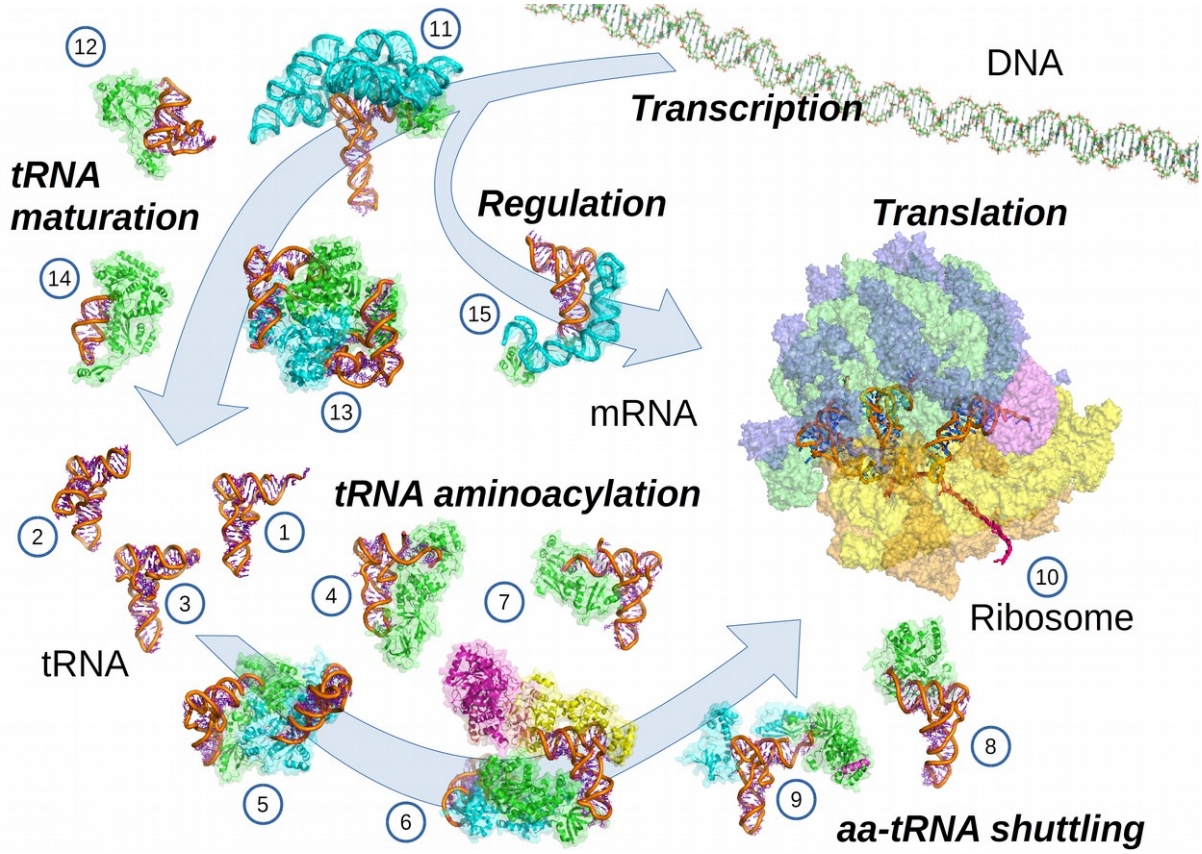

Figure 5: Structural gallery of tRNAs and their cellular partners. This selection of representative structures shows the diversity of tRNA partners and includes free tRNAs and tRNA-like molecules with (1) the yeast phenylalanyl-tRNA [10,11], (2) the murine selenocysteinyl-tRNA characterized by a large variable region and an acceptor stem with 8 base-pairs [60], (3) the tRNA-like structure present at the 3' of TYMV genomic RNA [61]; enzymatic complexes active in tRNA aminoacylation and protein synthesis, involving (4) a monomeric class I glutaminyl-tRNA synthetase [29], (5) a dimeric class II aspartyl-tRNA synthetase [30], (6) the transamidosome [62], (7) the methionyl-tRNA^{fMet}-formyltransferase [78]; bacterial and archeal elongation factors (8) EF-Tu [63] and (9) aIF2 [64], and (10) the bacterial ribosome bound to EF-Tu:tRNA [65]; tRNA maturation enzymes with (11) the ribonucleoproteic RNase P [56] (12) the ribonuclease Z [66], (13) tRNA-guanine transglycosylase associated with tRNA in so-called lambda open conformation [67] and (14) CCA adding-enzyme [68]; regulatory elements represented by (15) the T-box riboswitch [57]. All tRNAs are depicted with an orange backbone and pink bases, free tRNAs are represented, tRNA partners are shown with a transparent surface and different colors are used to distinguish their subunits.

tRNA biogenesis

Like many other RNA molecules, tRNAs are expressed as precursor transcripts and need to undergo several maturation steps in order to become functional. tRNA precursors are first cleaved by two endonucleases called RNase P and RNase Z, which remove 5' leader and 3' trailer sequences of tRNA precursors respectively. The RNase P function was believed to be ubiquitously performed by ribonucleoproteins containing a ribozyme (P RNA) until the recent discovery of protein-only RNase P enzymes in eukaryotes reviewed in [69]. The determination of the structure of the bacterial *Thermotoga maritima* RNase P in complex with tRNA has revealed that tRNA-P RNA recognition occurs through shape complementarity, specific intermolecular contacts and base-pairing interactions [56]. Interestingly, although a structure of protein-only RNase P in complex with tRNA has not yet been solved, initial biochemical and biophysical data suggest that protein-only and ribonucleoprotein RNase P share similar processes for specific tRNA recognition [70]. Atfer the initial end-trimming, tRNAs undergo several additional modification and editing events [71]. A number of structures of tRNA modifying enzymes in complex with complete tRNA have exposed several stages of the tRNA modification process, including initial RNA binding, the formation of covalent intermediates and the release of the modified RNA [72]. The structures of enzyme-tRNA complexes allowed tRNA modifying enzymes to be categorized in distinct classes depending on their requirement for the L-shape structure of tRNA for RNA recognition. Group I enzymes bind either nucleotides in the anticodon loop or residues embedded inside the tRNA structure, whereas group II enzymes require the three dimensional structure of tRNA for recognition [73]. The last step in tRNA maturation is the addition of a CCA 3' group by CCA adding enzymes (CCases). Structural analyses have revealed that CCases use a template independent RNA polymerization mechanism [68]. More recently, the determination of structures of archaeal CCA adding enzymes in complex with tRNA has revealed how the enzymes discriminate between the incorporation of C or A at position 76. The discrimination against incorporation of C at this position arises because protein flexibility results in the improper geometry of CTP in the active site [74].

tRNAs in the ribosome

Aminoacylated tRNA in complex with initiation and elongation factors reaches the ribosome where the incorporation of amino acids into the emerging protein chain through peptide bond formation is achieved. A rapidly increasing number of high-resolution structures of tRNA in complex with ribosome, ribosomal subunits and / or elongation factors at different stages of translation have been obtained by both X-ray crystallography and cryo-electron microscopy (cryoEM) [75]. These structures together with biochemical and computational approaches have revealed the plasticity of tRNA molecules during their interaction with the ribosome. It thus becomes increasingly evident that the intrinsic structural flexibility of tRNA is pivotal for protein synthesis [76]. In the ribosome, tRNAs bind the primary aminoacyl (A) site, travel to the peptidyl (P) site and leave the ribosome at the level of the exit (E) site. In this process, tRNA conformational changes are required for the concerted action of the ribosome's small and large subunits during translation initiation, for the decoding steps where aminoacylated tRNA are incorporated and for ribosome translocation [77].

Translation is initiated with the binding of the initiator $tRNA_i^{Met}$ to the P site of the small ribosomal subunit, where its anticodon basepairs with the mRNA start codon. Initiator tRNAs possess structural features that distinguish them from elongator tRNAs, inlcuding the elongator $tRNA^{Met}$, and enable their specific recognition and shuttling by initiation factors IF2/aIF2/eIF2. In *E. coli*, the initiator tRNA bears a C1-A72 mismatch (instead of a G1-C72 pair in the elongator) which is essential for the formylation of the methionine. This formyl group ensuring a specific binding to IF2, instead of EF1A (or EF-Tu), the elongation factor [78]. In Eukaryotes and Archaea, a weak base A1-U72 is a major identity element for binding to e/aIF2 whereas Ψ54, a major recognition signal for eEF1A, is absent. Met-$tRNA_i^{Met}$ is shuttled by IF2 or e/aIF2 to the P site of the ribosome. In all kingdoms, its anticodon stem contains 3 consecutive G-C pairs followed by an additional C32-A38 wooble pair [64,79]. This unique sequence leads to a specific anticodon loop conformation which is proposed to facilitate the accommodation of Met-$tRNA_i^{Met}$ in the P site .

In prokaryotes, the small subunit of ribosomes binds the initiator tRNA, initiation factors and mRNA to form the 30S pre-initiation complex in a sequential process. After the positioning of the mRNA, the anticodon of the initiator tRNA is base-paired with the initiation codon and the sequential release of initiation factors induces association of the large subunit to establish the 70S initiation complex [80]. In contrast, in eukaryotes, the initiation of translation is a more complex and tightly regulated process engaging over ten initiation factors, in

which eukaryotic ribosomes use a scanning mechanism resulting in the formation of the initiation complex with the initiator tRNA in its ultimate location [81,82]. In both eukaryotes and prokaryotes, the variety of conformations observed for initiator tRNAs reveals the plasticity of the acceptor arm and suggests that both the assembly of ribosome subunits and the integration of initiator tRNA into the P site might be regulated by the distinct conformations adopted by tRNA during the initial steps of translation [83].

When the ribosome is assembled, decoding of the mRNA can start and the delivery of elongator tRNAs is ensured by the elongation factors. The determination of the structure of ternary complex made of EF-Tu, Phe-tRNA^{Phe} and an analog of GTP represented an important breakthrough in the mid 90s and shed light on the recognition aminoacylated tRNAs [63]. Recent progress in ribosome crystallography enabled the observation of this ternary complex docked onto the ribosome (Figure 5) and revealed the structural plasticity of tRNAs [65]. Then, during the decoding process, the ribosome has to discriminate between cognate aminoacylated tRNA-elongation factor complexes and non-cognate complexes. This is achieved through a kinetic discrimination mechanism that allows incorrect tRNA species to be rejected at different stages. Decoding relies on Watson-Crick pairing between mRNA codons and tRNA anticodons together with 30S conformational changes [84]. The ribosome plays an active role in the accuracy of this process as it uses an ensemble of local and global conformational changes to control the fidelity of tRNA selection (e.g. [85,86]. Here again the structural flexibility of tRNA plays a fundamental role to allow the decoding process in both prokaryotic and eukaryotic systems [87].

For the translocation of ribosomes, peptide transfer takes place after the entry of tRNA in the A site guiding the formation of the pre-translocation complex poised to translocate tRNA-mRNA duplexes by one codon e.g. [87,88]. In this motion, the acceptor stems of tRNA in the A and P sites move 5’ to 3’, resulting in the hybrid A/P and P/E configurations [89]. This tRNA reorganisation is associated with the rotation of the 30S relative to the 50S subunit along with the stalk rearrangement as shown by cryoEM studies of prokaryote and eukaryote complexes, which revealed several tRNA intermediate configurations [76,90,91].

Interestingly, in some cases, tRNAs interact with ribosomes in ways that go beyond the primary association of tRNA as part of the mRNA decoding process. A tRNA was also found associated to the large subunit of the mitochondrial ribosome in animals. This tRNA, most likely mitochondrial tRNA^{Phe}, is referred to as CP tRNA. It is an integral component of the central protuberance (CP) of the 39S subunit, where it structurally replaces the 5S ribosomal RNA, which is not encoded in animal mitochondrial genomes, despite being ubiquitous in cytoplasmic ribosomes [92,93].

tRNA beyond translation

In addition to their canonical function, tRNA molecules participate in a variety of other processes [37], such as cell wall synthesis in bacteria (tRNA^{Ser}) or the porphyrin synthesis pathway (tRNA^{Glu}). Viruses frequently take over some feature of tRNA biology. In particular, retroviruses recruit particular host tRNAs for the priming of reverse transcription of their genomes [94]. Likewise, large DNA viruses such as bacteriophages, phycodnaviruses, and mimiviruses express their own tRNAs, most probably to regulate translation during viral infection [95]. In bacteria, the T-box riboswitch differs from all other identified riboswitches by using a tRNA as its effector. This riboswitch is believed to monitor the aminoacylation state of tRNAs and to control the expression of genes involved in a number of functions related to aminoacylation and the metabolism of amino acids [96]. The crystal structure of the highly conserved T-box riboswitch in complex with tRNA has revealed how the distal stem I region of the riboswitch interacts with the tRNA corner to anchor it to the riboswitch [57].

Beyond their canonical fold, the three-dimensional architecture of tRNAs is also found in a variety of other RNA molecules. These structures, called tRNA-like (TLS), are often found in 5’ and 3’ untranslated regions of plant mitochondrial mRNAs. They appear to serve as RNA processing signals for the maturation of mRNAs as they are recognized and cleaved by RNase P and RNase Z enzymes [97,98]. Similarly, a number of positive strand RNA plant viral genomes also possess 3'-tRNA-like structures. Although differing considerably from canonical tRNAs in terms of secondary structure, these TLS exhibit a three-dimensional fold that accurately mimics tRNAs and tRNA properties, including the capacity to be amino-acylated. These viral TLS often appear to be essential for viral replication [95]. For instance, the turnip yellow mosaic virus (TYMV) TLS (i) is recognized by ValRS like a true tRNA to amino-acylate the viral genomic RNA and (ii) binds the promoter region in the 3' untranslated region of the TYMV RNA for negative-strand synthesis. The determination of the TYMV

TLS three-dimensional structure has revealed its global tRNA mimicry and has also shown how it exploits a completely different array of intramolecular interactions to resemble tRNA. These specific interactions enable the TLS to switch conformations as required for its function [61].

Perspectives and new challenges of tRNA structural biology

Understanding the conformational dynamics of tRNAs

The gallery of tRNA structures, either as isolated molecules or in complex with cellular partners (**Figure 5**), highlights the flexibility of the L-shape scaffold. However, the cloverleaf behind this L-shape is not always the only 2D conformation possible, nor the most stable one. Defects in tRNA modification or mutations in mitochondrial tRNAs related to human pathologies were for instance shown to destabilize the cloverleaf and favor alternate 2D folds for which 3D models still need to be established [26,99]. Also, atypical tRNAs found in animal mitochondria, with sequence alterations ranging from a shortening of D/T loops, to the absence of D-arm, T-arm or both arms, deserve further investigation to understand their folding and their stability and to understand how translation factors can cope with such unusual and presumably flexible substrates. The example of the human mitochondrial aspartyl-tRNA synthetase suggests that the enzyme has relaxed its specificity and shows a higher plasticity than its *E. coli* homolog, an evolutionary response to the structural alteration of its cognate tRNA [100].

Understanding tRNA trafficking in the cell

In eukaryotes, gene expression is not restricted to the nucleus. It also takes place in organelles, in particular in mitochondria. The pool of tRNAs required for mitochondrial translation can be composed of both tRNAs encoded in mitochondria and in the nucleus, with the latter being expressed in the cytosol and imported to mitochondria by precise import systems. tRNA import is ubiquitous in eukaryotes, although very diverse. In yeast and in some animals, a full set of tRNA is encoded in mitochondria and tRNA import is not required a priori but nevertheless takes place [101]. In contrast, in trypanosomes, not a single tRNA is encoded in the mitochondrial genome and the full set of tRNA is imported. An intermediate situation is found in plants where a number of tRNAs are not encoded in mitochondria and have to be imported [102]. The precise nature of the factors involved in this process and their diversity throughout eukaryotes is only beginning to be unravelled. In yeast, the $tRNA^{Lys}_{1}$ is shuttled to the mitochondrial surface by the glycolytic enzyme enolase 2 and taken over by the precursor of the mitochondrial lysyl-tRNA synthetase to be co-imported in mitochondria through the protein import machinery [103]. In trypanosomes, tRNAs interact with the cytosolic elongation factor eEF1a to reach the mitochondrial surface. Then, two mitochondrial membrane proteins, Tb11.01.4590 and Tb09.v1.0420, together with Tim17 and Hsp70, are part of subsequent tRNA import machinery in the inner membrane showing that tRNA and protein import mechanisms share components [104]. Finally, in plants some TOM proteins are believed to act as tRNA receptors on the mitochondrial surface, while the voltage dependent anion channel (VDAC) may be the main translocation channel through the outer mitochondrial membrane [105]. In all of these systems tRNAs interact with β-barrel forming proteins such as VDAC, TOM40 or other components of the protein import machinery, presumably allowing them to cross mitochondrial membranes. In all cases, structural modeling predicts that mature tRNA have to undergo either complete or partial unfolding, or at least major structural rearrangements in order to cross mitochondrial membranes. The determination of structures of these factors in complex with tRNA will reveal the mechanism of tRNA import at atomic resolution and, more generally, will help scientists to understand how tRNA import can be fine-tuned to attain the pool of tRNA required for mitochondrial translation.

Understanding the biogenesis and functions of tRNA fragments

Transfer RNAs are key players in a variety of molecular processes, including, but not limited to, translation. For these crucial functions, tRNAs have to be quality-controlled and their turn-over regulated. Examples of this regulation include two tRNA surveillance pathways identified in yeast that degrade hypo-modified or mis-processed pre-tRNAs, as well as mature tRNAs lacking modifications. A nuclear tRNA surveillance pathway involving polyadenylation by the TRAMP complex and degradation by the exosome deals primarily with hypo-modified tRNAs, while another pathway termed the “rapid tRNA decay” pathway involves a CCAse and

degradation by Xrn1 [106].
Beyond tRNA degradation pathways, a number of other cellular activities generate tRNA-derived fragments (tRFs). The wide accumulation of numerous tRFs suggests that these tRNA cleavage products potentially represent a new class of small non-coding RNAs that may have important regulatory functions. tRFs are evolutionarily widespread and created by cleavage at a number of specific positions most probably by different endonuclease activities (**Figure 2D**). The discovery of small non-coding RNAs such as microRNA (miRNA) and small interfering RNA (siRNA) and their functions as major regulators of gene expression has been a major breakthrough [107]. Recent analysis of transcriptomes in all three domains of life has suggested that tRFs constitute another class of small regulatory RNAs with biologically relevant functions because (i) in most cases tRFs are not generated from abundant tRNAs, and the amount of tRFs does not correlate with initial tRNA abundance or with the corresponding tRNA gene copy number. (ii) The cleavage patterns of tRFs seem to be dependent on tRNA anticodons. (iii) Cleavage patterns of tRNAs often vary according to the cell status and / or developmental stages. (iv) Some tRFs have been found in association with key components of the RNA silencing machineries such as Argonaute or Piwi. The functions of tRFs are related to a rapidly growing number of molecular processes, including translation, translation initiation, RNA degradation, reverse transcription, gene silencing, vacuolar ribophagy and cell survival [108]. The determination of three-dimensional structures of tRFs alone and in complex with protein partners will aid in understanding how tRFs are generated as well as the mode of action and functional diversity of this novel class of non coding RNAs [108,109].

From tRNA to systems biology

The early observation of tRNAs capable of decoding nonsense UGA, UAA and UAG codons in *E. coli* [110], the reattribution of UGA to Phe in yeast mitochondria [111], and more recently the discovery of the 21st and 22nd amino acids, selenocysteine (Sec) and pyrolysine (Pyl), coded by of UGA and UAG [112,113], have contributed to the idea of utilizing nonesense codons to incorporate non-canonical amino acids (ncAAs) into proteins. The expansion of genetic code in vivo requires the introduction of an orthogonal pair consisting of a suppressor tRNA decoding a nonsense codon and a modified cognate synthetase able to transfer the desired ncAA without interfering with tRNAs of the host organism. This has been achieved in a variety of ways, including the selection of variants of archeal TyrRS [114,115] or PylRS [116,117] activating ncAAs. For optimal incorporation further engineering of host organism factors such as the elongation and release factors, or the peptidyl-transferase site of the ribosome, may be necessary. More than one hundred such compounds have already been successfully incorporated into proteins, introducing new functional groups for cross-linking or fluorescent labeling purposes or to mimic epigenetic modifications [118,119]. In an alternative approach, RNA aptamers known as flexizymes have been designed to aminoacylate any tRNA with ncAAs in cell free translation systems [120] by reassigning rare codons to ncAAs open up vast perspectives for the production of artificial proteins or more generally for the design of new organisms in the field of synthetic biology [121,122]

Concluding remarks

Forty years after the determination of the first tRNA crystal structure, the adaptor molecule has not yet revealed all its secrets. While at the end of the 90s, investigations carried out with model organisms may have given the feeling that the major aspects of tRNA:synthetase interactions had been revealed, the systematic exploration of genomes since the early 2000 and an increasing interest in pathology-related mutations, both in tRNAs and associated proteins, have revitalized the field and led to the discovery of many new aspects of tRNA biology. With the latest progress in cryoEM, one can easily anticipate a burst of new data describing how tRNAs move on the ribosome and how tRNA dynamics contributes to the decoding of mRNA from bacteria to higher eukaryotes, including cytosolic, mitochondrial and plastidal translation machineries. The integration of structural data from high resolution crystallography, cryoEM and correlative microscopy in the cell will provide a much deeper insight into their distribution and functions with applications in human medicine, drug design and synthetic biology, making the field of tRNA structural biology more active and open than ever.

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