Noncanonical inputs and outputs of tRNA aminoacylation

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Abstract

The aminoacylation reaction is one of most extensively studied cellular processes. The so-called "*canonical*" reaction is carried out by direct charging of an amino acid (aa) onto its corresponding transfer RNA (tRNA) by the cognate aminoacyl-tRNA synthetase (aaRS), and the *canonical* usage of the aminoacylated tRNA (aa-tRNA) is to translate a messenger RNA codon in a translating ribosome. However, four out of the 22 genetically-encoded aa are made "*noncanonically*" through a two-step or indirect route that usually compensate for a missing aaRS. Additionally, from the 22 proteinogenic aa, 13 are *noncanonically* used, by serving as substrates for the tRNA- or aa-tRNA-dependent synthesis of other cellular components. These nontranslational processes range from lipid aminoacylation, and heme, aa, antibiotic and peptidoglycan synthesis

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to protein degradation. This chapter focuses on these noncanonical usages of aa-tRNAs and the ways of generating them, and also highlights the strategies that cells have evolved to balance the use of aa-tRNAs between protein synthesis and synthesis of other cellular components.

1. Introduction

Translation relies on the constant synthesis and delivery to the decoding ribosomes of a complete set of accurately aminoacylated transfer RNAs (aa-tRNAs). aa-tRNAs are the products of an enzymatic reaction termed tRNA aminoacylation, that is catalyzed by a family of ubiquitous enzymes called aminoacyl-tRNA synthetases (aaRSs) [1]. Elucidation of the aminoacylation reaction was concomitant with the characterization of tRNA and aaRSs [2–7]. In the "*canonical*" aminoacylation reaction, using a specific amino acid (aa), aaRSs catalyze the ATP-dependent formation of an aminoacyl-adenylate (aa∼AMP), which enables this activated aa to be transferred directly onto the accepting 3′- or 2′OH of the terminal adenosine of the cognate tRNA (Fig. 1A). "*Canonical*" tRNA aminoacylation is often also termed "*direct*" tRNA aminoacylation and signifies that the aa and tRNA that are linked are cognate pairs and that, upon release, the aa-tRNA can be used directly in protein synthesis. Contrarily, the "*non-canonical*" tRNA aminoacylation, also termed "*alternate*" or "*indirect pathway,*" starts with an aa that is first charged onto a non-cognate tRNA by an aaRS, followed by its conversion into the cognate aa by a second enzymatic activity. The first description of a non-canonical pathway for tRNA aminoacylation occurred as early as 1968 by M. Wilcox and M. Nirenberg, who demonstrated that direct formation of glutaminyl-$tRNA^{Glutamine}$ (Gln-$tRNA^{Gln}$) by a glutaminyl-tRNA synthetase (GlnRS) was absent in three *Bacilli* species; the evidence indicated that they all required first the aminoacylation of $tRNA^{Gln}$ with glutamate (Glu), followed by the tRNA-dependent conversion of Glu into Gln, thereby demonstrating that tRNA can be a cofactor in aa biosynthesis [8]. The description of the first non-canonical aminoacylation reaction almost coincided with the first report of the non-canonical utilization of an aa-tRNA, *e.g.,* not for protein synthesis, and was that of lysyl-$tRNA^{Lysine}$ in the synthesis of *O*-lysyl phosphatidylglycerol in *Staphylococcus aureus* [9].

From the 22 genetically-encoded aa that have been identified to date, 18 are exclusively aminoacylated onto their cognate tRNAs through the

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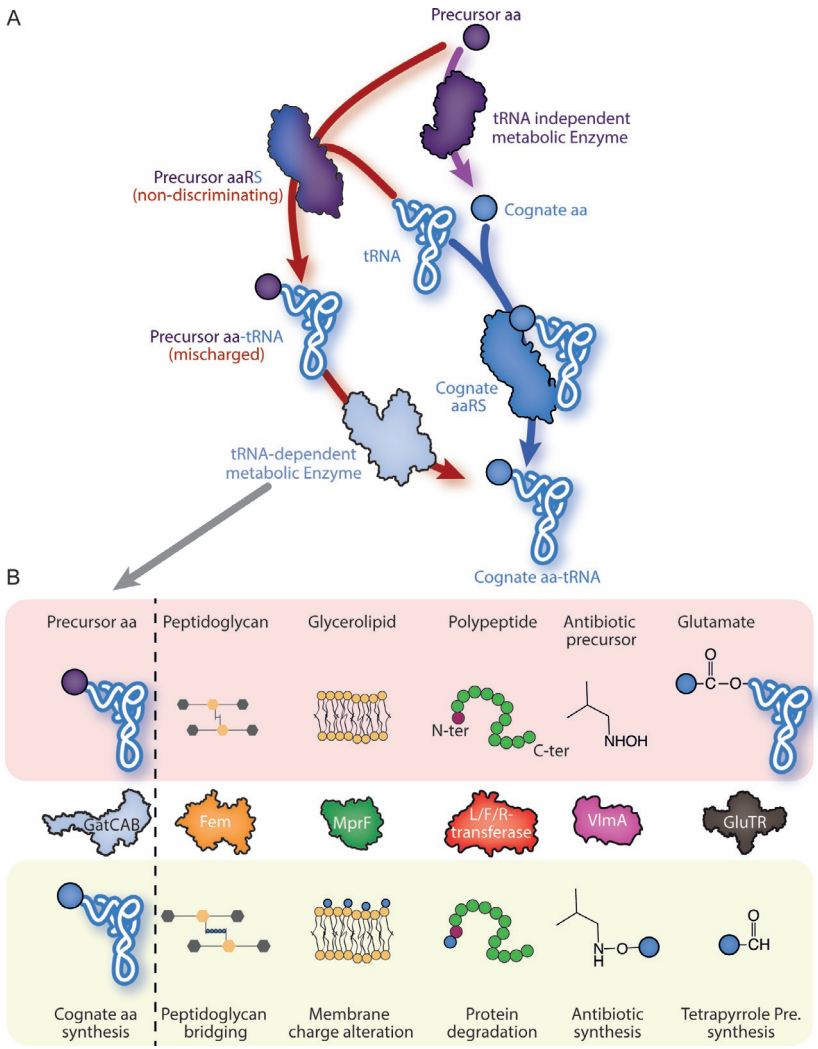

Fig. 1 Canonical and noncanonical tRNA aminoacylation reaction and nontranslational aa-tRNA usages. (A) Overview of the canonical tRNA aminoacylation (right side, blue arrows) and of the noncanonical 2-step reaction (left side, red arrows). For details see Fig. 2. (B) Schematic representations of the nontranslational usages of aa-tRNAs described in this chapter. The molecule that will be tRNA-dependently or aa-tRNA-dependently modified is indicated in the top row, the enzyme responsible for the tRNA-dependent modification in the middle and the product of the reaction or the fate of the compound tRNA-dependently modified is indicated in the bottom row.

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canonical reaction (Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Pyrrolysine (Pyl), Ser, Thr, Trp, Tyr and Val), one is strictly aminoacylated *via* a non-canonical aminoacylation reaction (Selenocysteine, Sec) and three are charged onto their corresponding tRNA either canonically or non-canonically (Asn, Cys and Gln). All four indirect pathways of tRNA aminoacylation have three main common features. They proceed *via* two consecutive steps (an exception is Sec-tRNA^{Sec} formation in archaea and eukaryotes that requires three reactions; see Fig. 2). The first step is the mis-charging of a tRNA with a non-cognate aa by a so-called non-discriminating aaRS (ND-aaRS) [10,11]. The second step is the conversion of the misacylated aa into the cognate one, while it is attached to the tRNA by a tRNA-dependent modifying enzyme [12]. Sometimes the ND-aaRS and the tRNA-dependent modifying enzyme form a complex that catalyzes both reactions without the release of the mischarged aa-tRNA intermediate [13]. The last common feature is that the noncognate aa of the misacylated aa-tRNA intermediate is always a metabolic precursor of the cognate final aa, in the tRNA-independent and regular metabolic pathway (*e.g.,* Asp for Asn, Glu for Gln, O-phosphoserine (Sep) for Sec; Fig. 1A). Among the 22 proteinogenic aa, 13 (Ala, Arg, Asp, Glu, Gly, Leu, Lys, Met, Phe, Thr, Trp, Val and Ser) can serve as substrates for the tRNA-dependent synthesis of other cellular components. Noteworthy, when the aa of an aa-tRNA is used for a non-canonical pathway, the tRNA carrier is generally not specific to the non-canonical usage but diverted from protein synthesis. This often requires adaptation of other components of the translation machinery that limit the negative impact that rerouting of aa-tRNAs could have on the efficiency or accuracy of protein synthesis [14]. The purpose of this chapter is to present the fascinating diversity of the non-canonical usages made by organisms of aa-tRNAs (Fig. 1B).

2. Non canonical aminoacylation of tRNAs and tRNA-dependent synthesis of amino acids

2.1 The two non-standard amino acids

In addition to the common set of 20 proteinogenic aa, two additional and non-standard aa are used for protein synthesis in some organisms: selenocysteine (Sec) and pyrrolysine (Pyl), respectively, known as the 21st and 22st genetically-encoded aa.

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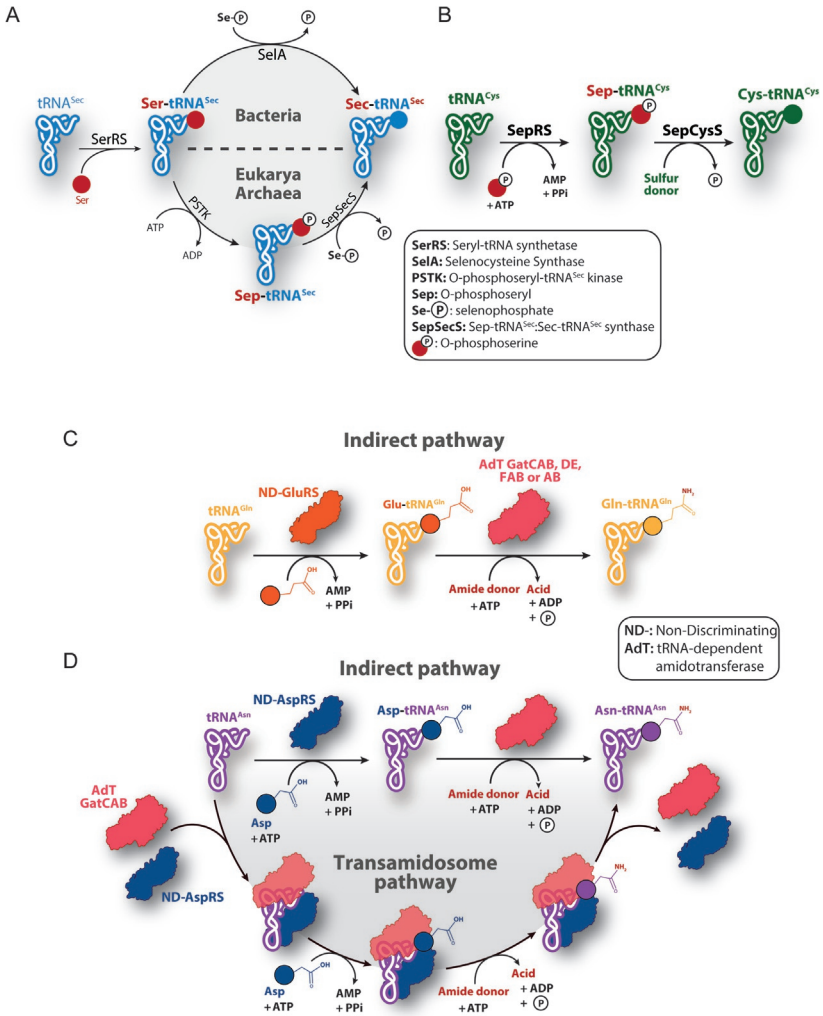

Fig. 2 Non-canonical pathways of aa-tRNA biosynthesis. (A): Biosynthesis of Sec-tRNA^{Sec} using a post-charging conversion of Sec by a two-step pathway in bacteria (upper panel) and a three-step pathway in eukaryotes and archaea (lower panel); **(**B) Two-step biosynthesis of Cys-tRNA^{Cys} in archaea lacking CysRS. (C) Two-step transamidation pathway of Gln-tRNA^{Gln} synthesis used by bacteria, archaea, mitochondria and chloroplasts. GatCAB AdT: Glutamyl-tRNA^{Gln} amidotransferase used by bacteria, most eukaryotic organelles; GatDE: Glutamyl-tRNA^{Gln} amidotransferase used by archaea; GatFAB AdT: Glutamyl-tRNA^{Gln} amidotransferase of yeast mitochondria; GatAB: Glutamyl-tRNA^{Gln} amidotransferase used by apicoplast. (D) Two-step transamidation pathway or transamidosome-mediated route of Asn-tRNA^{Asn} synthesis used by bacteria, archaea deprived of AsnRS or of asparagine synthetase. The amide donor is either Gln or Asn and is deaminated to form Glu or Asp (acid) during transamidation, respectively. *Panel B: adapted from J. Yuan, S. Palioura, J.C. Salazar, D. Su, P. O'Donoghue, M.J. Hohn, A.M. Cardoso, W.B. Whitman, D. Söll, RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 18923–18927.*

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2.1.1 The 21st proteinogenic aa: Selenocysteine (Sec)

Selenocysteine incorporation has been discovered in 1986 both in mammals and bacteria [15,16]. Its incorporation into proteins depends on an in-frame UGA "opal" STOP codon, which is read through by ribosomes. Sec residues are found in the active sites of selenoproteins (25 genes in mammals, but none in fungi and higher plants). This non-ubiquitous proteinogenic aa has a higher nucleophilic reactivity than cysteine (Cys), thus facilitating selenoproteins in performing enhanced redox reactions and maintain redox homeostasis [17]. Sec is generated *via* two consecutive steps [18] (Fig. 2A) by tRNA-dependent modification of a Ser precursor attached to the opal suppressor $tRNA^{Sec}$ [19]. Indeed, because $tRNA^{Sec}$ and $tRNA^{Ser}$ share the same tRNA identity elements, $tRNA^{Sec}$ can be recognized by the seryl-tRNA synthetase (SerRS) and charged with Ser to form Ser-$tRNA^{Sec}$ [20]. However, an additional base pair in the acceptor stem of $tRNA^{Sec}$—in comparison to all known tRNAs—prevents the recognition of the misacylated Ser-$tRNA^{Sec}$ by the elongation factor thermo-unstable (EF-Tu), thus precluding misincorporation of Ser into proteins [21–23]. After serylation of $tRNA^{Sec}$ by SerRS, the Ser residue is either (i) directly selenylated by SelA to form Sec-$tRNA^{Sec}$ in bacteria [19], or (ii) phosphorylated by *O*-phosphoseryl-tRNA-kinase (PSTK) to form *O*-phosphoseryl-$tRNA^{Sec}$ (Sep-$tRNA^{Sec}$), the latter activated aa being subsequently selenylated in a tRNA-dependent manner by the SepSecS enzyme in eukaryotes and archaea [24]. Both SelA and SepSecS are pyridoxal-5′-phosphate (PLP)-dependent enzymes and use selenophosphate as a selenium donor (Fig. 2A). Selenocysteinyl-$tRNA^{Sec}$ is then transported to translating ribosomes by specific elongation factors: SelB in bacteria [25] and the eEFSec•SBP2 complex in eukaryotes [26,27]. Finally, incorporation of Sec requires pausing of the ribosomes at UGA "Sec" codons, which requires a *cis*-acting stem loop structure, called SElenoCysteine Insertion Sequence or SECIS, located downstream of the "Sec" UGA codons of selenoprotein mRNAs. Note that SECIS elements differ in length and structure between bacteria, archaea and eukaryotes [28–32].

2.1.2 The 22nd proteinogenic aa: Pyrrolysine (Pyl)

Pyrrolysine is a modified lysine with a 4-methylpyrroline-5-carboxylate group linked by an amide to the ε-amino group (ε-N). It was first discovered in 2002 in methanogenic archaea and later in several bacteria [33,34]. In methanogenic archaea, all genes encoding methylamine methyltransferases

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(methane-generating enzymes) contain an in-frame UAG "amber" STOP codon that is translated into a pyrrolysyl residue. As for Sec, Pyl active site residues appear to be crucial for the activity of enzymes involved in methanogenesis [35]. Pyrrolysyl-tRNA^{Pyl} is synthesized by direct pyrrolysylation of an amber-suppressor tRNA^{Pyl} by the class IIc PylRS [36]. Pyl incorporation has become the first known example to date of direct aminoacylation of a tRNA with a non-standard proteinogenic aa. The structure of tRNA^{Pyl} differs from the classical tRNA structure because it displays for example a D- and TΨC-loop without their conserved representative residues [37]. Contrary to Sec-tRNA^{Sec} and its *trans*-specific factors, Pyl-tRNA^{Pyl} is transported to the A-site of the ribosome by the standard elongation factor EF-Tu [38]. In addition, in some cases the mRNA sequence context seems important for Pyl insertion, and a specific stem loop called PYrroLysyl Insertion Sequence or PYLIS has sometimes, but not always, been found directly next to the "amber" codon on mRNAs encoding for methylamine methyltransferases [39].

2.2 tRNA-dependent pathways of amino acid biosynthesis

Selenocysteine is not the only aa that requires multiple steps to be incorporated into proteins. In numerous organisms, analogous indirect pathways are also needed for the incorporation of standard aa into proteins. In those cases, the biosynthesis of the proteinogenic aa is tRNA-dependent and linked to the production of the corresponding cognate aa-tRNA.

For the majority of eukaryotes and very few bacteria, the 20 different types of standard aa-tRNAs are produced by a full and unique set of 20 different aaRSs. But in the majority of bacteria and all archaea, one or more aaRS genes is missing, suggesting that the aminoacylation of corresponding orphan tRNAs is performed by a non-canonical route. The organism that best illustrates missing aaRSs is the hyperthermophilic methanogenic archaea *Methanocaldococcus jannaschii*, whose genome was sequenced in 1996 and that lacks four expected aaRS genes: LysRS, CysRS, AsnRS and GlnRS [40]. It was shown in this archaeon that the lack of CysRS is compensated by a tRNA^{Cys}-dependent two-step pathway (Fig. 2B) similar to that of Sec-tRNA^{Sec} formation in bacteria. In this pathway, tRNA^{Cys} is first mischarged by a dedicated *O*-phosphoseryl-tRNA synthetase (SepRS) with Sep, before tRNA-dependent conversion of the Sep moiety into Cys by the PLP-dependent SepCysS enzyme that ressembles SepSecS [41].

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AsnRS and especially GlnRS are also often missing in prokaryotes and in organelles [42]. All archaea and the majority of bacteria lack GlnRS, whereas AsnRS can be found in most bacteria and half of archaea. While all organelles do possess a dedicated AsnRS, an organellar GlnRS is never found, except in some protozoans [43]. When AsnRS and/or GlnRS is/are absent, direct tRNA charging with the two respective cognate aa—Asn and Gln—is compensated for by an indirect route called the transamidation pathway. It is a two-step pathway in which the metabolic precursors of Gln and Asn, respectively, Glu and Asp, are first attached onto the non-cognate $tRNA^{Gln}$ and $tRNA^{Asn}$ by ND-GluRS or ND-AspRS [10,44] (Fig. 2C). Once released from ND-aaRSs, the mischarged Glu- or Asp-tRNAs are transferred to tRNA-dependent amidotransferases (AdTs). AdTs first ATP-dependently activate the γ- or β-carboxyl group of the misacyled Glu- and Asp-tRNAs by phosphorylation, the activated side chains then being amidated, using an amido group donor, to form Gln-$tRNA^{Gln}$ or Asn-$tRNA^{Asn}$ [45]. All AdTs, regardless of their origins or types, usually use the γ-amide of a free Gln, with the release of Glu, to produce this amido group [46–48]. AdTs are complexes that always possess an amidase subunit to generate the amido group necessary for the amidation reaction, and a subunit that binds the misacylated aa-tRNA substrates and catalyzes the activation of the carboxyl group of the mischarged aa before amidation. The amido donor travels from one subunit to the other, enabling amidation of the misacylated tRNA, a reaction termed "transamidation." Accessory proteins also participate in the formation of the complex.

To date, four types of tRNA-dependent AdTs are known: the heterotrimeric GatCAB and GatFAB and the heterodimeric GatDE and GatAB ("Gat" for glutamyl-$tRNA^{Gln}$ amidotransferase). The GatCAB AdT (A: amidase, B: aa-tRNA binding, COOH activation and transamidation subunit, C: accessory chaperone) usually is a bispecific Glu/Asp-AdT that can generate Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$, such as in bacteria. In archaea GatCAB is a specific Asp-AdT [49]. The heterodimeric GatDE (E: amidase subunit, D: aa-tRNA binding, COOH activation and transamidation subunit) is an archaea-specific Glu-AdT [48] that only produces Gln-$tRNA^{Gln}$. In most eukaryotic organelles, such as mitochondria and chloroplasts, an organellar AdT is also required, and it is of the GatCAB-type. It functions as a Glu-AdT that synthesizes organellar Gln-$tRNA^{Gln}$ *in vivo* [50,51]. Note that in plants, there is only a single organellar GatCAB AdT that is dually-targeted to mitochondria and chloroplasts. Of note, in mitochondria of the yeast *Saccharomyces cerevisiae*, formation of

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the mitochondrial Gln-tRNA^{Gln} pool relies on a heterotrimeric GatFAB that differs from the homologous bacterial GatCAB by the presence of a fungi-specific GatF subunit that replaces structurally GatC. Recently, a heterodimeric GatAB AdT lacking a "C" subunit was found in the apicoplast of the human parasite *Plasmodium falciparum* to function as a Glu-AdT [52].

All organisms using an AdT to generate Gln-tRNA^{Gln} or Asn-tRNA^{Asn} or both also possess ND-GluRS and/or ND-AspRS that, in addition to regular Glu-tRNA^{Glu} and Asp-tRNA^{Asp}, respectively, provide the misacylated Glu-tRNA^{Gln} and Asp-tRNA^{Asn} [10,44]. Some organisms such as *Helicobacter pylori*, possess in addition to regular "discriminating" GluRS, a dedicated supernumerary tRNA^{Gln}-mischarging GluRS2 [11,53]. In organelles, the mitochondrial GluRS usually is the ND-GluRS that participates in the organellar transamidation pathway [51]. In plants, there is a dual-targeted ND-GluRS that participates in the chloroplast and mitochondrial transamidation pathways [50]. The yeast *S. cerevisiae* is however an exception, because it is the cytosolic GluRS that canonically produces the cytoplasmic Glu-tRNA^{Glu} pool which is imported into mitochondria, to specifically mischarge the mitochondrial tRNA^{Gln}. The *bona fide* mitochondrial GluRS only aminoacylates the mitochondrial tRNA^{Glu} [43,54].

Organisms that use the transamidation pathway to generate amidated aa-tRNAs have an adapted EF-Tu that has been shown to lack significant binding capacity for the misacylated Glu-tRNA^{Gln} and Asp-tRNA^{Asn} intermediates, thereby preventing misincorporation of Glu and Asp at Gln and Asn codons [13,54,55]. Another way to generate amidated aa-tRNAs without challenging the genetic code with the mischarged intermediates is the formation of so-called transamidosomes. These complexes are formed by binding of the ND-aaRS to the AdT in a tRNA-dependent manner. In the transamidosomes that have been described to date, the misacylated Asp-tRNA^{Asn} or Glu-tRNA^{Gln} generated by the complexed ND-AspRS or -GluRS are channeled to the AdT active site, without being released, and transamidated to release only the final amidated forms from the particle [56]. Bacterial (ND-AspRS•tRNA^{Asn}•GatCAB, Fig. 2D) and archaeal (ND-GluRS•tRNA^{Gln}•GatDE; [55]) transamidosomes have been characterized. The stoichiometry of their components (tRNA, ND-aaRS and AdT) varies as well as their stability and catalytic mechanism. The transamidosome of *Thermus thermophilus* is composed of 2 dimeric ND-AspRSs (four enzymes), four tRNA^{Asn} and 2 GatCAB AdTs (two enzymes); 2 tRNA^{Asn} molecules are substrates and two others are scaffolding

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components that ensure the stability of the ribonucleoprotein particle throughout catalysis [14]. In *H. pylori*, only transient Glu- or Asp-transamidosomes have been identified [57,58].

While these four indirect pathways are compensatory routes for the synthesis of a given aa-tRNA when the cognate aaRS is absent, for two of them, Sec and Asn, they are (Sec) or can sometimes be (Asn) the sole metabolic pathway for the synthesis of the aa, which is therefore strictly tRNA-dependent [44].

3. aa-tRNA-dependent aminoacylation of lipids and cell-wall synthesis

3.1 Multiple peptide resistance factor (MprF)

In the early 60s, Macfarlane reported the presence of *O*-L-lysyl and *O*-L-alanyl esters of phosphatidylglycerol (PG) in *Clostridium welchii* and in *Staphylococcus aureus* [59]. However, that the enzymatic synthesis of lysyl-PG in *S. aureus* involves the transfer of the lysyl moiety from Lys-tRNA^{Lys} onto PG was not demonstrated until 1966 [9]. Over the last decades, a large number of studies detected aminoacylated lipids in several bacterial species, and showed that Lys-tRNA^{Lys} and Ala-tRNA^{Ala} are usually the aa donors for lipid aminoacylation and that PG, diacylglycerol or cardiolipin are the aa acceptors. Arg-PG and could also be detected but to a lesser extent [60]. In 2001, Peschel et al. demonstrated in *S. aureus* that the *mprF* gene encodes the lysyl-phosphatydyl glycerol synthase (LysPGS) responsible for LysPG synthesis [61]. They also showed that the cytoplasmic domain of LysPGS is responsible for the transfer of L-Lys from Lys-tRNA^{Lys} onto PG at the inner leaflet of the plasma membrane, whereas the N-terminal integral membrane domain flips the newly synthesized LysPG to the outer membrane leaflet [62]. The addition of positively- charged or neutral aa to PG by aminoacyl-phosphatidyl glycerol synthases (aaPGSs) reverses or neutralizes the net negative charge of the cell envelope [63], thereby decreasing the susceptibility of bacteria to positively-charged antimicrobial agents. Furthermore, aaPGS-dependent membrane remodeling participates in the adaptation of bacteria to environmental changes (pH, osmolarity, temperature), increases immune escape [64,65] and enhances virulence of *S. aureus* and *Listeria monocytogenes* upon infection of epithelial cells and macrophages in mice [66,67].

Some aaPGSs are specific for a single aa-tRNA (*e.g.,* Lys- or Ala-tRNA), and others exhibit broader substrate specificity and can utilize up to three

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different aa-tRNAs as aa donors (*e.g.,* Lys-, Ala- or Arg-tRNA) [68,69]. It has been shown that the acceptor-stem of the tRNA and in particular its fifth base pair and the Cα of the corresponding aa are essential elements for the recognition of Ala-$tRNA^{Ala}$ by the AlaPGS [70,71]. Structural analysis showed that the catalytic domain of AlaPGS from *Pseudomonas aeruginosa* and LysPGS from *Bacillus licheniformis* harbor a so-called dupli-GNAT (GCN5 *N*-acetyltransferase) domain, where the two GNATs are separated by a positively-charged alpha helix [72]. Such similar structures have also been described for other aminoacyl-tRNA transferases (AAT), including the Fem and L/F transferases described below. Site-directed mutagenesis based on the structures of aaPGSs, demonstrated that this positively- charged helix is essential for aa-tRNA recruitment. However, it is still not understood how bacteria regulate the balance between the usage of the aa-tRNA for protein synthesis *versus* lipid aminoacylation. Interestingly, *Mycobacterium* spp. encode a LysPGS (LysX) that is fused directly to a C-terminal lysyl-tRNA synthetase (LysRS) that is essential for the LysPG synthesis activity. This suggests that, in *Mycobacterium* spp., a dedicated pool of Lys-$tRNA^{Lys}$ is synthesized and used by LysPGS, to produce LysPGs and that this pool escapes protein synthesis [73].

4. FemXAB

The peptidoglycan sacculus (murein) is a unique and essential structural element of bacterial cell walls found outside of the plasma membrane. It contributes to the maintenance of cell shape to preserve the cell integrity, and serves as a scaffold for the anchoring of other cell envelope components such as proteins and teichoic acids in Gram-positive bacteria.

Peptidoglycan synthesis is achieved by a succession of enzymatic events, that start with the formation of the well-known lipid II. This lipid harbors a so-called stem-peptide composed of the L-Ala_1-D-Glu_2-X_3-D-Ala_4-D-Ala_5 pentapeptide where X_3 is variable among species (L-Lys, δ-L-ornithine or ω-L, L-diaminopimelic acid) (Fig. 3A). At this stage, Fem transferases, which are non-ribosomal peptidyl transferases, use aa-tRNA as aa donors to form an "interpeptide bridge" that, in a later step, serves to cross-link the X_3 of the newly-synthesized peptidoglycan subunit (Lipid II released from its undecaprenyl-diphosphate) to the D-Ala_4 of an adjacent peptidoglycan stem-peptide [74,75]. The formation of the stem-peptide is tRNA-independent, whereas Fem ligase-mediated formation of the interpeptide bridge was shown to be tRNA-dependent.

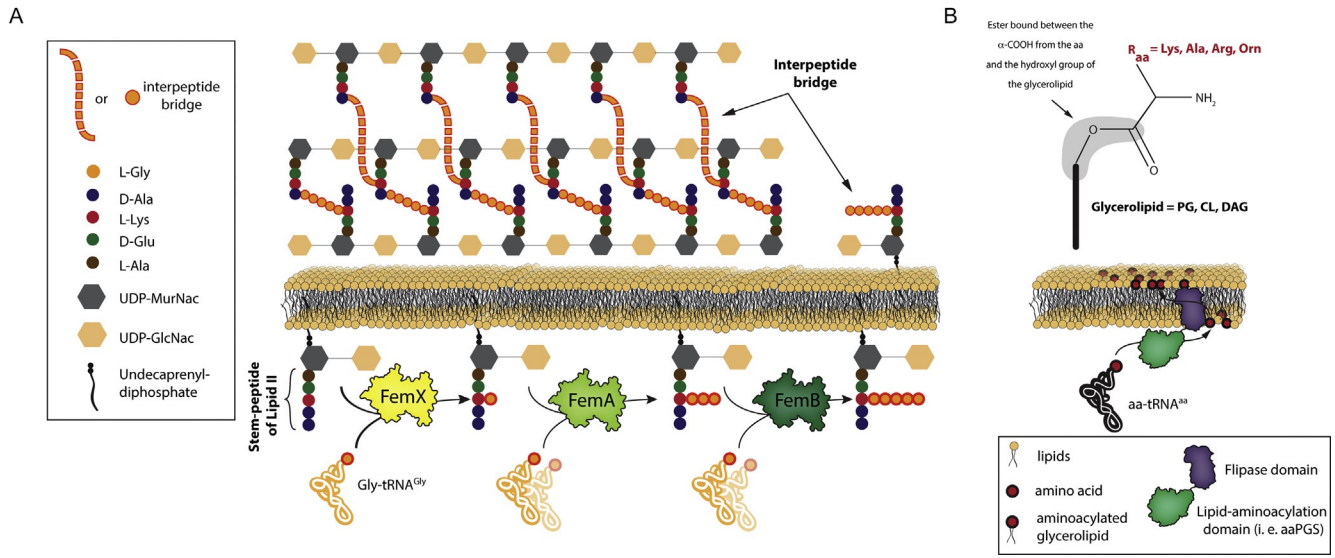

Fig. 3 Aminoacyl-tRNA-dependent cell wall remodeling in prokaryotes. (A) In *S. aureus*, the stem peptide composed of L-Ala$_1$-D-Glu$_2$-Lys$_3$-D-Ala$_4$-D-Ala$_5$ is added onto Lipid I to form the well-characterized Lipid II. Aminoacyl-tRNA transferases (ATT) belonging to the Fem ligases family then successively add a variable number of aa to form the interpeptide bridge. For example, in *S. aureus* FemX adds a single glycine, FemA adds Gly2 and Gly3 and finally, FemB adds Gly4 and Gly5 onto the L-Lys of the stem-peptide. The resulting peptidoglycan subunit is then flipped out to the outer cytoplasmic-membrane leaflet, and the link to the growing peptidoglycan saccule is made by PBP transpeptidases. MurNac: N-Acetylmuramic acid, GlcNac: N-Acetylglucosamine. (B) MprF and MprF-like proteins contain a C-terminal ATT domain (green) that reroutes aa-tRNA^{aa} from the translational machinery and transfers the aa moiety onto glycerolipids, and a N-terminal flipase domain (purple) that exposes the aminoacylated glycerolipid to the outer membrane leaflet. As schematized in the upper panel, different aa/ lipid combinations were documented in prokaryotes as reviewed by Slavetinsky et al. [60]. PG: phosphatidylglycerol, DAG: diacylglycerol, CL: cardiolipin, R_{aa}: lateral chain of the indicated aa.

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In *S. aureus*, 3 Fem ligases are required. FemX adds the first glycine residue (Gly_1) onto the stem-peptide at the ε-NH_2 group of L-Lys_3 [76,77], while FemA and FemB are essential for the addition of $Gly_{2,3}$ and $Gly_{4,5}$, respectively. These proteins are highly specific with respect to the position of the Gly residues that they attach [78]. Among the five $tRNA^{Gly}$ isoacceptors encoded by *S. aureus*, three of them have a weak binding capacity to EF-Tu, due to the replacement of G_{49}-U_{65} and G_{51}-C_{63} by A_{49}-U_{65} and A_{51}-U_{63} base pairs in the T-loop [79,80]. Furthermore, Rohrer et al. showed that the three $tRNA^{Gly}$ capable of escaping the translational machinery have G_{18} and G_{19} replaced by UU or CU [81]. The Fem factors are known to be essential for methicillin resistance, and are found in both resistant and susceptible *S. aureus* strains. The phenotypes of the FemAB null mutants of *S. aureus* showed a reduction of cell wall Gly content and strong morphological aberrations during cell division and separation [76]. The inactivation of the *femAB* operon reduces the interpeptide to a monoglycine, inducing a poorly crosslinked peptidoglycan. Decreased growth rate and hypersusceptibility to antibiotics such as methicillin were observed in *femAB* deleted strains, making FemAB a potential target to restore β-lactam susceptibility in methicillin-resistant *S. aureus* (MRSA) [82].

Staphylococcus simulans, *S. epidermidis* and *S. capitis* incorporate a variable ratio of L-Ser/Gly residues (but also L-Ala for *S. epidermidis*) into the interpeptide bridges to escape pentaglycine endopeptidase-mediated degradation. To our knowledge, $tRNA^{Ser}$ modifications are not involved in the distribution of Ser-$tRNA^{Ser}$ between peptidoglycan cross-linking and translation. Thus, bacteria may adapt the ratio of Ser-$tRNA^{Ser}$ used for each mechanism according to environmental conditions. *S. epidermis* encodes at least two $tRNA^{Gly}$ isoacceptors employed exclusively for interpeptide-chain formation [83]. Both contain CC or UU instead of the universal G_{18} and G_{19} di-nucleotide. Furthermore, the supplementary C_{32}-G_{38} base pair in the anticodon loop and, to a lesser extent, the impaired U_{10}-U_{25} base pairs as well as the weak post-transcriptional modification ratio in the D-loop are also considered as crucial to determine the fate of Gly-$tRNA^{Gly}$ isoacceptors.

In *S. pneumoniae*, MurM (homologue of the *S. aureus* FemA) adds L-Ser or L-Ala as the first residue to the L-Lys of the stem peptide [78,84], and MurN then adds L-Ala to form an interpeptide chain composed only of two residues [84].

Finally, *Weissella viridescens* harbors either a L-Ala-L-Ser or a L-Ala-L-Ser-L-Ala interpeptide chain [85]. Villet et al. showed that the C_{71} and C_{72}

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from the $tRNA^{Ala}$ are critical nucleotides for recognition by FemX, whereas the G_3-U_{70}, base pair considered as a strong identity determinant for recognition by AlaRS is dispensable [86]. Because MurM and FemX of *W. viridescens* recognize only two aa-tRNAs [87,88], it has been suggested that these two proteins may be involved in the complex discrimination between protein and peptidoglycan synthesis [89].

In summary, Gly-$tRNA^{Gly}$, Ala-$tRNA^{Ala}$, Ser-$tRNA^{Ser}$ but also Thr-$tRNA^{Thr}$ participate in peptidoglycan interpeptide chain formation. For a more detailed review of the interpeptide composition across bacterial species, refer to reports of Dare & Ibba or Schleifer & Kandler [83,90].

5. aa-tRNA-dependent synthesis of hemes

Only a small subset of the aa produced by the cell are used in protein synthesis, and we have seen that from this small pool some can be deviated to build molecules other than proteins. There are also several other pathways that require non-proteogenic aa and among them, the synthesis of heme necessitates δ-aminolevulinic acid (ALA), a pathway that is tRNA-dependent. ALA can be synthesized either from free glycine and succinyl-CoA (eukaryotes) or glutamate (bacteria and green plants), when the latter is bound to its cognate $tRNA^{Glu}$ (reviewed in [91], Fig. 4).

ALA is the first molecule in the tetrapyrrole synthesis pathway that leads to heme biosynthesis in mammals and chlorophyll in plants. ALA biogenesis in plants and bacteria has been studied for more than four decades, and employs a simple three-step pathway. First, Glu is charged onto $tRNA^{Glu}$ by GluRS [92–94]. Second, $tRNA^{Glu}$-bound Glu is reduced to glutamate

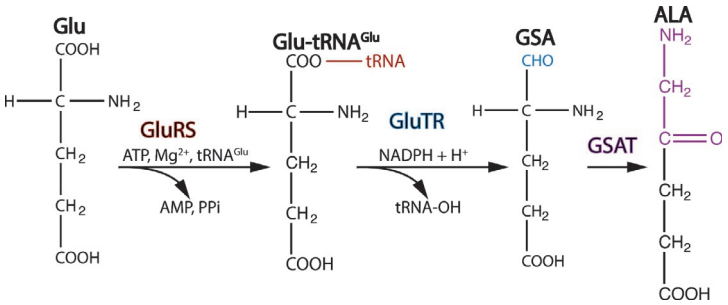

Fig. 4 C5 pathway of ALA formation. Glutamate (Glu) is attached directly onto $tRNA^{Glu}$ by GluRS. Glu is then reduced to glutamate-1 semi-aldehyde (GSA) by glutamyl-tRNA reductase (GluTR) and further converted by glutamate aminomutase (GSAT) to δ-aminolevulinic acid (ALA).

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semi-aldehyde (GSA) and detached from $tRNA^{Glu}$ by glutamyl-tRNA reductase (GluTR) [95,96]. Finally, GSA is converted to the final product ALA by GSA aminomutase (GSAT) [97].

The finding that Glu is used to produce ALA in green plants [98,99] preceded the discovery that $tRNA^{Glu}$ is required for ALA production [92–94]. Thus, Glu-$tRNA^{Glu}$ formation is an absolute intermediate in the GSA formation by GluTR, an enzyme that has been shown to form a complex with GluRS [100] and together, both proteins have strict requirements for $tRNA^{Glu}$ [101,102].

In bacteria, GluTR is encoded by the *hemA* gene that is part of the *hemAXCDBL* operon in which *hemX* codes for an integral membrane protein capable of negatively affecting the cellular concentration of *hemA* [103]. GluTR activity is rate-limiting in ALA formation [104] and in plants, it represents an ideal target for designing herbicides [105]. Its activity has been shown to be regulated by acting on the protein stability that is increased in heme limiting conditions in *S. typhimurium* [106]. Later, FLU, another negative regulator of GluTR was identified. Flu is a membrane-bound protein involved in chlorophyll biosynthesis that interacts directly with GluTR but not with GSAT [107,108]. It must be noted that although the levels of GluTR and GSAT control ALA production, they do not control directly chlorophyll synthesis in *Chlamydomonas reinhardtii*. A complex between GSAT and GluTR has been identified and proposed to protect the highly- reactive GSA intermediate [109].

ALA is the universal precursor of tetrapyrroles like hemes, and the latter have been shown to downregulate not only the activity of GluTR but also of GluRS, which is more surprising [110]. In addition, hemes are also capable of inhibiting aminoacylation activity of a Zn^{2+}-deprived form of human tryptophanyl-tRNA synthetase [111]. Moreover, holocytochrome *c* (*e.g.,* heme bound) can interact with tRNA, and this interaction was shown to mediate caspase-mediated apoptosis [112].

An essential GluTR-interacting protein named GluTRB that is localized in the thylakoid membrane, participates in the sub-compartmentalization of GluTR, to separate ALA pools in order to balance production of heme and chlorophyll [113]. The interaction of GluTRBP with GluTR stimulates its activity, but the latter is still inhibited in a heme-dependent concentration [114]. Binding of GluTRBP to GluTR also prevents FLU from inhibiting GluTR, but a ternary complex consisting of GluTR, GluTRBP and FLU can be formed, suggesting a biological role for the ternary complex in the regulation of the plant GluTR [115]. The complexity of this regulation

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of GluTR expression/activity is reinforced by the finding that GluTR can be degraded from its N-terminus by the Clp protease, but not when protected by GluTRBP [116].

ALA utilization by bacteria leads to heme, an essential cofactor for *S. aureus* growth and colonization that becomes toxic at high concentrations, thereby underlining the necessity for these organisms to control tightly heme homeostasis [117]. In plants, subplastidial allocation of GluTR in the stroma and in membrane fractions reveals that when membrane-bound, GluTR is less active, and this is the result of the concerted actions of GluTR interacting proteins (FLU, GluTRBP, Clp protease and cpSRP43) [118,119]. Finally, in higher plants there are two isoforms for GluTR: GluTR1 that is expressed predominantly in green tissue, and GluTR2 that is expressed constitutively in all organs. It was recently shown that heme binding to GluTRBP inhibits its interaction with GluTR, thereby making it accessible to Clp protease, which participates in GluTR regulation [120].

Overall, the utilization of Glu-tRNA^{Glu} outside translation represents an intriguing example of how various metabolic pathways are interconnected. Glu-tRNA^{Glu} utilization for ALA production controls heme and chlorophyll biosynthesis, two molecules that are crucial for other cellular events that contribute to the life cycle of various organisms, and whose production seems to be subjected to tight and complex control.

6. aa-tRNA-dependent formation of antibiotics

Peptide-based antibiotics can, at least in part, be synthesized tRNA-dependently, and are an important class of molecules because they provide organisms with defenses to fight against invading microbes, or to help them survive in a given ecological niche [89,121]. There is a group of antibiotics whose composition is based on peptides. There are two ways of synthesizing peptide-based antibiotics that both require tRNA: ribosomally- or non-ribosomally-synthesized peptidic antibiotics [122]. Those that are ribosomally-synthesized include defensins (30–40 aa in length; disrupt membrane integrity) that are a large class of Cys-rich peptide antibiotics whose Cys residues are regionally-specifically oxidized to stabilize the molecule and provide protease resistance. Similarly, lantibiotics are low molecular weight lanthionine-containing cyclic peptides that, like defensins, perturb membrane integrity. This class of molecules belongs to the superfamily of RiPP (ribosomally synthesized peptides that undergo extensive post-translational modifications). They are synthesized as precursors that

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consist of an N-terminal leader peptide fused to a C-terminal core peptide containing modifications. Interestingly for some of them, a Glu-tRNA^{Glu} is used by a dehydratase to modify aa residues of the ribosomally-made peptide parts (reviewed in [123]). Aromatic heterocyclic peptide scaffolds derived from Cys, Ser and Thr residues [124] are another large family of ribosomally-derived antibiotics like microcin B17, patellamides and streptolysin S. It is noteworthy that microcins have a broad diversity of chemical structures and modifications, and are widely used as antitumoral and/or antimicrobial drugs [125]. The synthesis of these antibiotic compounds is rather complex and there are tens of genes that are required, and tRNAs are either involved as regulators of this process [122] or as carrier molecules for additional modifications with aa. tRNA-based regulation of antibiotic production is, for example, used by *Streptomyces coelicolor*. In this filamentous bacterium there is a single gene (*bldA*) that encodes the only tRNA^{Leu} capable of decoding the UUA rare Leu codon. The expression of this specific tRNA^{Leu} is very low in fresh cultures, but increases to reach its maximum as cultures get older. The tRNA^{Leu} expression pattern correlates with morphological differentiation and changes in antibiotic production [126]. This suggests a means of limiting ribosomal-mediated antibiotic synthesis in the host to physiologically appropriate circumstances by modulating tRNA availability. A similar mode of regulation has also been proposed for biofilm production, in which Hha proteins decrease biofilm production by repressing transcription of tRNAs decoding rare codons (argU and proL) normally involved in fimbriae production [127].

The second tRNA-dependent mode of synthesizing peptide-based antibiotics are the non-ribosomal antibiotic pathways. These rely on a series of enzymatic reactions that involve at one step an aa-tRNA. For example, valanimycin production involves a gene cluster containing 14 genes in *Streptomyces viridifaciens*, in which the VlmA protein catalyzes the transfer of a seryl residue from Ser-tRNA^{Ser} to the hydroxyl group of isobutylhydroxylamine to produce the O-seryl-isobutylhydroxylamine ester [128]. An intriguing fact concerning valanimycin production is that the Ser-tRNA^{Ser} used in the reaction is produced by a dedicated SerRS, named VlmL, encoded by the *vlmL* gene [129]. Similarly, production of a streptothricin-related antibiotic requires an intermediate, in which an amino sugar is aminoacylated with Gly by a Fem-like enzyme in a tRNA-dependent manner [130]. Overall, the enzymes involved in these types of modifications belong to the class of the aa-tRNA-dependent transferases that were identified over 50 years ago [131–133].

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Although aa-tRNAs can be used to remodel several compounds, they can also be substrates for the synthesis of cyclic dipeptide-based antibiotics. They were long thought to be products released prematurely from the ribosome until the discovery of cyclodipeptides synthases [134]. Cyclodipeptides are further subjected to various modifications until the final product is obtained [135]. A strategy employing various enzymes to modify a core scaffold to produce differential tailoring is often used in nature to generate structural diversity. Such small molecule diversity enables easy evolution of new biological functions, and allows fine-tuning of existing ones [135,136]. Cyclodipeptide synthases genes, are often clustered with tailoring enzyme genes, most likely to facilitate the overall regulation of the cyclodipeptide-based compound production (for a review see [123]). Cyclic dipeptides are an interesting class of molecules because they constitute a rather convincing alternative over conventional antibiotics, as exemplified in their role in preventing quorum sensing pathogenesis of uropathogens [137].

7. aa-tRNA-dependent degradation of proteins

In 1963, the participation of aa-tRNAs in protein degradation was first reported in prokaryotes, in which it was shown that ribosome-deprived extracts were still able to catalyze tRNA-dependent aminoacylation of proteins [138]. In eukaryotes, it was noted 23 years later that specific aa residues found in the N-terminus (N-t) of proteins could decrease dramatically the half-life of the corresponding protein in *S. cerevisiae* [139]. An enzymatic activity able to transfer the aa moiety of an aa-tRNA to the N-t of proteins leading to protein degradation was also identified in eukaryotes [140], and is presently termed the N-end rule pathway [141].

In bacteria, the N-end rule pathway includes three components: an aa-tRNA transferase responsible for the transfer of the aa moiety onto the protein that will be degraded, a N-recognin that binds the aminoacylated protein signaling it to the degradation machinery, the Clp protease complex. In eukaryotes, an additional preliminary step involving deamination of amide aa residues in N-t of the protein is catalyzed by a deamination enzyme. This enables the subsequent recognition by the aa-tRNA transferase, followed by signaling of the protein for degradation by the proteasome [142].

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7.1 N-end rule pathway in prokaryotes

In 1963, Kaji et al. identified a protein fraction in *E. coli* that was able to transfer radioactively-labeled Leu onto proteins in the absence of ribosomes [138], thereby describing for the first time the activity of a leucyl-tRNA protein transferase. In 1973, Soffer demonstrated that Leu, Phe, and to a lower extent Met and Trp, can also be transferred onto the N-t residue of α-casein [143]. Later, it was discovered that the nature of the N-t residue of the targeted protein modulates the aa conjugation efficiency [139]. The residues onto which aa are efficiently transferred efficiently include the basic residues Arg, Gln, Lys and Pro, which were thereafter termed destabilizing residues. However, the mechanism linking addition of aa to the N-t of a protein to its degradation was not identified until Tobias et al. showed in 1991that a bacterial strain lacking the Clp ATP-dependent protease degraded much less efficiently a protein carrying destabilizing residues [144]. This proved that Leu (or Phe) conjugation mediates degradation by recruiting the Clp protease [144]. The gene coding the leucyl−/phenylalanyl-tRNA transferase (L/F transferase) was identified 2 years later [145]. Notably, this gene is located near the gene encoding ClpA, a component of the Clp protease complex, which participates in the N-end rule pathway. In 1995, the gene encoding the protein was cloned, overexpressed and purified, allowing the development of an *in vitro* assay that lead to the characterization of its catalytic mechanism [146]. Since then, the structure of the aa-tRNA transferase was determined in complex with an aa-tRNA analog [147], and the mechanism of the peptide bond formation between the N-t residue and the aa transferred by the aa-tRNA transferase has been elucidated [148].

In sum, protein aminoacylation in prokaryotes is mediated by L/F transferases that modify a residue in N-t (preferentially recognizing a basic residue), which enables the recognition of the protein by ClpS, which in turn signals delivery of the proteins to the Clp protease complex for degradation. Note that ClpS belongs to the family of N-recognins, which are proteins that bind to aminoacylated proteins and enable their delivery to the degradation machinery (Fig. 5C).

7.2 N-end rule pathway in eukaryotes

In *S. cerevisiae* and higher eukaryotes, proteins are not aminoacylated directly to induce their degradation (Fig. 5B). Instead, a first step of deamidation converts the amide aa Asn and Gln to Asp and Glu. These deamination

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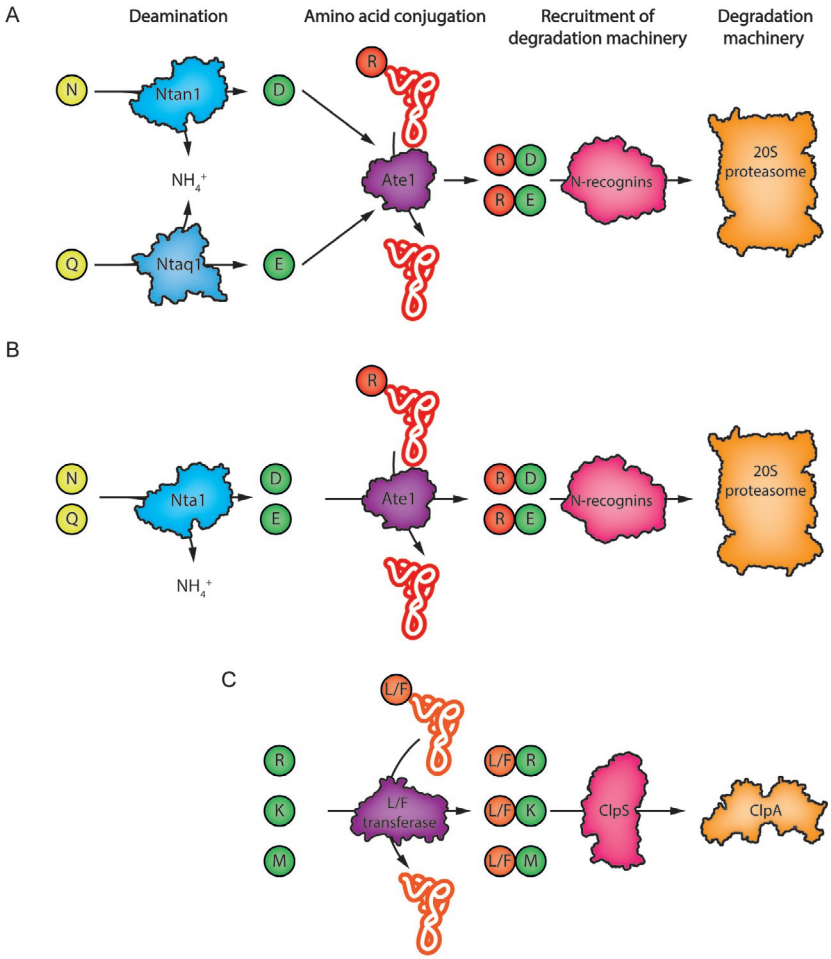

Fig. 5 Overview of the N-end rule pathway leading to protein degradation. (A) In mammals, protein degradation starts by the deamination of Gln (Q) or Asn (N) in first position at the N-ter of the protein. This leads to the arginylation of the N-ter of by Ate1, and subsequently to the binding of the protein by a N-recognin, which delivers it to the proteasome. (B) In fungi, the pathway is very similar, the sole difference being that the deamination step is performed by one enzyme both for Gln and Asn. (C) In prokaryotes there is no deamination step: Arg (R), Lys (K) and Met (M) are recognized by a leucyl-phenylalanine-tRNA transferase (L/F transferase) which transfers either Leu (L) or Phe (P) on the first amino acid in the N-ter of the protein. This so-called destabilizing residue is recognized by ClpS, which delivers the protein to the ClpA protease for degradation. *Panel C: adapted from T. Tasaki, S.M. Sriram, K.S. Park, Y.T. Kwon, The N-end rule pathway, Annu. Rev. Biochem. 81 (2012) 261–289.*

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reactions are performed by Nta1 in fungi [149]. In mammals, deamination of Gln and Asn is performed by Ntaq1 and Ntan1, respectively [141,150] (Fig. 5A). After deamination, these residues can be aminoacylated by a unique arginyl-tRNA transferase, Ate1, both in *S. cerevisiae* and mammals [151,152]. Upon arginylation, the protein can be ubiquitinylated, a step mediated by a N-recognin. This leads finally to the degradation of the protein by the 26S proteasome.

This N-end rule mechanism is the only noncanonical pathway in which proteinogenic aa-tRNAs are used for protein degradation rather than for their synthesis. In this case, aa-tRNAs are diverted not only from their anabolic use, but become essential components of the protein catabolic pathway.

8. Concluding remarks

This chapter has been dedicated. Mainly to the fascinating diversity of the nontranslational mechanisms and processes that employ the aa moiety of an aa-tRNA. The first descriptions of these noncanonical usages of aa-tRNAs (Leu-tRNA^{Leu} to modify proteins and of Lys-tRNA^{Lys} in *O*-lysyl-phosphatidylglycerol synthesis) coincided with the characterization of the conventional aminoacylation reaction for protein synthesis [5–7,9]. Yet, these nontranslational usages are generally less well known than the primary historical role of aa-tRNA in protein synthesis, despite being equally essential for the cell. There is no doubt that new processes and functions will be unraveled in the future, and will enrich the existing functional repertoire of aa-tRNAs. As an example, the arginyltransferase Ate1, which transfers the arginyl moiety from Arg-tRNA^{Arg} to the N-terminus of proteins targeted for degradation by the N-end rule pathway, has recently also been shown in mammalian cells and in *Dictyostelium discoideum* to be implicated in the aminoacylation of internal residues of β-actin [153,154]. Interestingly the steady-state level of arginylated β-actin is relatively low but tends to increase in migratory cells, demonstrating that this post-translational aminoacylation is actively regulated in cells [153]. Arginylation of protein is not restricted to β-actin, because an increasing number of eukaryotic proteins appear to undergo Ate1-mediated arginylation of internal Asp and Glu residues; these arginylated proteins participate in myriad cellular processes ranging from embryogenesis, to cell migration and protein homeostasis [155].

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There are however many unanswered issues when considering the non-translational processes that rely on the use of aa-tRNAs. One of the most important is how aa-tRNAs escape the translational machinery, and how cells achieve a balance between the use of the same aa-tRNA species for protein synthesis and/or for other cellular processes? In fact, though the various non-canonical usages of aa-tRNAs we describe in this chapter have been studied actively, it remains largely unknown how aa-tRNAs are diverted from their primary and canonical usage. In some cases, organisms have found a strategy that eliminates the competition between translation and nontranslational usages of an aa-tRNA by producing a dedicated pool of aa-tRNAs for a given non-canonical process. For example, *S. epidermis* encodes two $tRNA^{Gly}$ isoacceptors dedicated exclusively to interpeptide-chain formation. Moreover, sometimes organisms have even acquired or evolved a supernumerary aaRS dedicated to the synthesis of specific aa-tRNAs (*e.g.,* LysRS fused to LysPGS in *Mycobacterium* for LysPG synthesis). Dedicating a given pool of tRNAs to nontranslational processes could also be accomplished by post-transcriptionally modifying tRNA nucleotides. Indeed, tRNAs undergo a series of post-transcriptional modifications, and some of them could potentially also serve to assign a given tRNA pool to a given nontranslational function. Finally, it was recently shown that, in eukaryotes, a single aaRSs can be localized in two or more subcellular compartments, and that these spatially-distinguished isoforms (called echoforms) can serve different roles, including the noncanonical roles that rely on the synthesis of a compartment-specific aa-tRNA [156].

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