Blastocrithidia—A Genetic Alien from the Planet Earth

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The standard genetic code, which applies almost without exception, is the key to our understanding of molecular biological processes. Although it is close to impossible to imagine that sparse code changes occur naturally given proteomic constraints, specific cases of codon usage alterations have been documented, mostly in unicellular eukaryotes. Here, we summarize what we have learned about *Blastocrithidia*, a little-known parasitic flagellate with all three stop codons reassigned to sense codons, which uses UAA as the only universal stop codon. We first describe its origin, life cycle, morphology, cultivation, and transformation, the combination of which predisposes it to become the first tractable eukaryote with a noncanonical genetic code. Next, we present our across-the-genome analysis revealing uneven distribution of in-frame stops and discuss the features distinguishing in-frame and genuine stop codons that allow for so-called position-specific termination. Finally, given what is known about stop codon readthrough by near-cognate transfer RNAs (tRNAs) and the fidelity of stop codon recognition by eukaryotic release factor 1 (eRF1), we propose a model illuminating how unique properties of *Blastocrithidia* tRNAs, combined with specific alterations of its eRF1, enable this massive deviation from the standard genetic code.

The genetic code is a universal feature of all extant life. It is almost invariably represented by its canonical version with only a very small fraction of organisms deviating from it in various ways (Crick 1968). These noncanonical genetic codes differ by codon reassignments and usually occur in the organelles and nuclei of eukaryotes, and, with lower frequency, in bacteria and viruses (J Lukeš, M Eliáš, A Kachale et al., unpubl.). While scattered across the tree of life, departures from the standard genetic code tend to appear in clusters, with most of them being associated with the nuclei of unicellular eukaryotes (protists) and mitochondria.

The ever-growing sequencing efforts, progressively covering the diversity of viruses, prokaryotes, and eukaryotes, unearthed almost 60 alternative genetic codes so far (J Lukeš, M Eliáš,

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A Kachale et al., unpubl.). Several mutually nonexclusive and partially overlapping theories explaining the deviations from the canonical genetic code have been proposed, namely, the ambiguous intermediate theory (Schultz and Yarus 1994c), the codon capture theory (Osawa and Jukes 1989), and the unassigned codon theory (Sengupta and Higgs 2005). Regrettably, so far, the noncanonical codes have been almost exclusively encountered either in organelles or in organisms for which methods of functional dissection are unavailable. Furthermore, carriers of these genetic codes are also generally rare, and the lack of (closely) related lineages with the standard genetic code precludes comparative studies that would make any validation of the above-listed theories possible.

The concept of proteomic constraint postulates that any change of the genetic code is inevitably associated with reduced fitness (Massey 2008). However, when genetic code alterations occur via the steps postulated by the codon capture theory, namely, the disappearance of a given codon from the genome and its reappearance with a new meaning and corresponding new decoding mechanism (Osawa and Jukes 1989), minimal to no fitness burden is expected. Moreover, the relatively high frequency of the alternative genetic codes in mitochondria can be well explained by nucleotide bias and the fact that just a handful of protein-coding genes are encoded by the mitochondrial DNA allowing, under certain conditions, reassignment(s) with low or even no fitness cost (J Lukeš, M Eliáš, A Kachale et al., unpubl.). In any case, the underlying reasons behind the emergence of each and every noncanonical genetic code are likely different, and it is reasonable to assume that many intermediates were lost in the evolutionary history of their bearers and will remain intractable.

Indeed, we can only speculate on what triggered the unique explosion of genetic code reassignments that occurred, for example, in ciliates. It could have been caused by the tendency of their translation apparatus to read through in-frame stop codons (Swart et al. 2016) or by its capacity to deal with widespread ribosomal frameshifting (Gaydukova et al. 2023). Alternatively, it might have been initiated by changes in their release factors or other component(s) of the translation machinery (Lozupone et al. 2001). Unless their common ancestor can be investigated (which is very unlikely because it is most probably extinct) and the extant ciliates can be genetically manipulated, we may never know. In any case, it is plausible to suggest that different preadaptations in different lineages made the "inner sanctum," as the canonical genetic code may well be called, conducive to changes (J Lukeš, M Eliáš, A Kachale et al., unpubl.).

The main limitation to our understanding of altered genetic codes known so far is that organisms, in which they evolved, cannot be axenically cultivated and are not amenable to genetic modifications. As a result, the possibilities to explore reasons why an organism would alter a code that supports all forms of life and is probably as old as Earth (Eigen et al. 1989) are very limited.

This unfavorable situation has changed with the discovery of a flagellated parasitic protist *Blastocrithidia nonstop* that has extensively deviated from the canonical code (Záhonová et al. 2016; Kachale et al. 2023). The same applies to three other recently sequenced and annotated members of the genus *Blastocrithidia* that share the same alteration of the genetic code and possess exactly the same set of unique features associated with it (see below) (Záhonová et al. 2025). *Blastocrithidia* spp. that belong to the class Kinetoplastea (supergroup Euglenozoa) (Fig. 1) qualify as promising models for numerous reasons: (1) phylogenetic relatedness with the functionally well-studied human pathogens *Trypanosoma* and *Leishmania* (Kostygov et al. 2024); (2) availability of several sequenced and well-annotated genomes (Záhonová et al. 2016, 2025; Kachale et al. 2023); (3) existence of a closely related sister genus *Obscuromonas* with a canonical genetic code allowing very useful comparative analyses (Fig. 1); (4) all the above-listed species can be axenically cultivated at high cell densities and for reasonable costs (Lukeš et al. 2021); (5) *B. nonstop* can be genetically modified using the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system

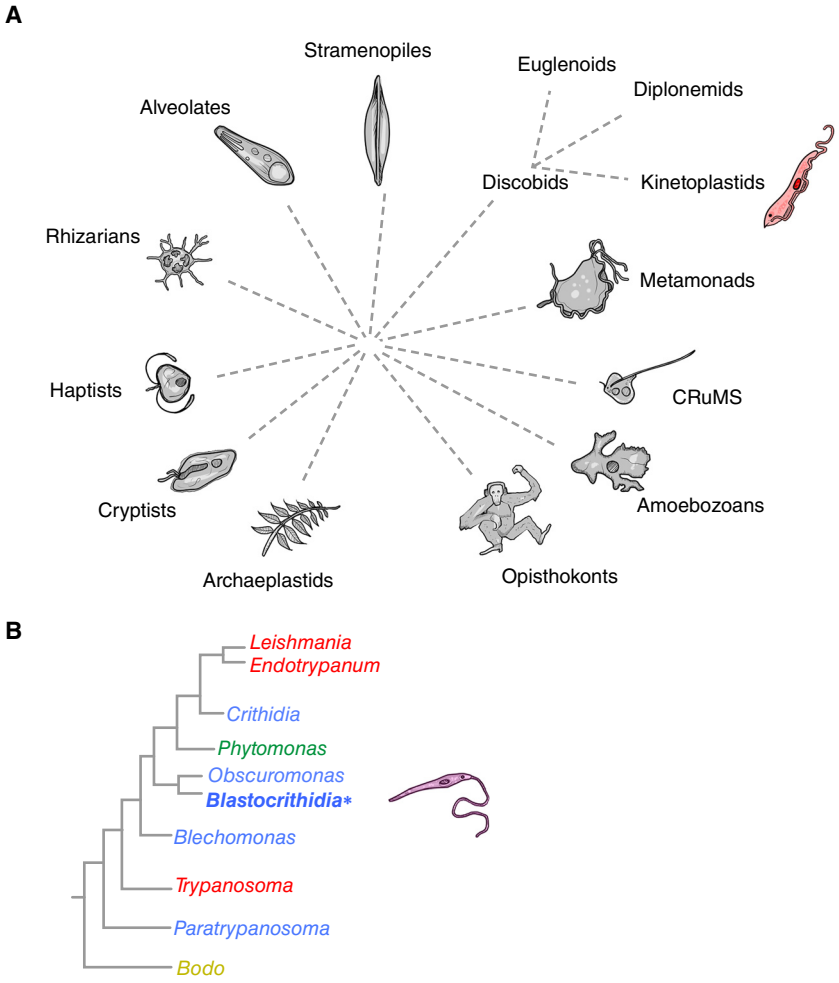

Figure 1. Phylogenetic position of *Blastocrithidia nonstop*. (*A*) Schematized phylogenetic tree of eukaryotes showing the main subgroups and thumbnail diagrams of their representatives. The diagram showing kinetoplastid protists is colored. (*B*) Simplified phylogenetic tree of kinetoplastid flagellates, featuring their most important members. The free-living outgroup, represented by the genus *Bodo*, is shown in yellow. Color-coding shows insect-only infecting genera *Paratrypanosoma*, *Blechomonas*, *Blastocrithidia*, *Obscuromonas*, and *Crithidia* in blue, plant-infecting *Phytomonas* in green, and the genera *Trypanosoma*, *Leishmania*, and *Endotrypanum*, which are responsible for serious human diseases, in red.

and gene tagging (see below); and (6) several *Blastocrithidia* spp. can infect insect hosts and thus complete the whole life cycle under laboratory conditions.

Hence, *Blastocrithidia* spp. may allow the dissection of their noncanonical code, interrogations of its potential advantages and limitations, and, eventually, its possible malleability. Understanding the code's plasticity will be uniquely informative for the extensive efforts to generate synthetic genomes. As has been recently experimentally confirmed in *Escherichia coli*, artificially expanded or contracted genetic codes provide extremely attractive features, namely, biocontainability, incorporation of nonnatural amino acids, as well as the resistance to viruses and horizontal gene transfers, to their synthetic bearers (de la Torre and Chin

2021; Zürcher et al. 2022; Nyerges et al. 2023). The proverb "Normals teach us rules, outliers teach us laws" fits here very well as it underlies the outstanding role of outliers for our understanding of a given, generally highly conserved, yet mutable, feature.

Below, we summarize what we have learned about these intriguing trypanosomatids so far. We commence with their inconspicuous morphology and life cycle, proceed to their unique genetic code, and conclude with what is known about the intricacies of their translation machinery that allows this "genetic alien from the planet Earth" to live on it. We believe that these obscure flagellated parasites hold even more secrets with outsized implications that need to be unearthed.

NONCANONICAL CODE HIDDEN BEHIND THE FACADE OF MUNDANE MORPHOLOGY AND LIFE CYCLE

All known members of the genus *Blastocrithidia* are morphologically very similar, represented here by the best studied *B. nonstop*. It has ~15-µm-long and 2–4-µm-thick promastigote-type cells with a single flagellum of variable length, which is supported by a prominent paraflagellar rod (Fig. 2). As shown by 4′,6-diamidino-2-phenylindole (DAPI) staining, the round nucleus is in the center of the cell, while the typical disk-shaped kinetoplast (=mitochondrial DNA) is at the base of the flagellum (Fig. 2A,C). In fact, all these features are characteristic for most members of the diverse and species-rich trypanosomatid flagellates (Kostygov et al. 2021). Under unfavorable conditions, the motile stages transform into the cystic stage termed the straphanger (Fig. 2B,C). Straphangers often remain associated with a flagellum of the promastigote cell but can also be isolated and investigated separately (Reduth and Schaub 1988).

Blastocrithidia spp. have a cosmopolitan distribution in various true bugs (Heteroptera) (Cerisola et al. 1971; Frolov et al. 2017; Záhonová et al. 2025). The genus was formally described by Laird (1959) from water striders of the genus *Gerris* (family Gerridae) in Canada. Morphologically similar trypanosomatids were first observed and reported in Indian bugs of the genera *Gerris* and *Microvelia* more than a century ago (Patton 1908). The prefix *Blasto-* is derived from the Greek "blastos" (="sprouting") referring to the appearance of the straphanger cysts associated with the flagellar apparatus. Together with its sister clade formed by the genus *Obscuromonas*, the members of which have a standard genetic code, *Blastocrithidia* spp. form a subfamily Blastocrithidiinae (Lukeš et al. 2021).

Their inconspicuous morphology is accompanied by a similarly inconspicuous life cycle, in general reminiscent of that of many other trypanosomatids (Frolov et al. 2021). It has been investigated (mostly microscopically) in several *Blastocrithidia* spp., particularly prominently in *Blastocrithidia triatomae* isolated from the bug *Triatoma infestans* in Argentina in 1971 (Cerisola et al. 1971; Tieszen et al. 1986; Jensen and Schaub 1991). This discovery was accidental as these bugs were routinely used in xenodiagnostic transmission experiments of the etiological agent of Chagas disease, *Trypanosoma cruzi*, and were laboratory-screened to make sure they were trypanosomatid-free. Instead, a new trypanosomatid species, *B. triatomae* was discovered, becoming the model for numerous in vivo experiments (Cerisola et al. 1971). Because *B. triatomae* and *T. cruzi* share the same insect host, this *Blastocrithidia* species was extensively investigated in the past with an idea to use it for biological control against *T. infestans*. It has been shown that both flagellates colonize the whole intestinal tract and the Malpighian tubules but prefer the rectum (Böker and Schaub 1984; Schaub et al. 1992). Importantly, the prevalence of *T. cruzi* infection was reduced (but not eliminated completely) when *B. triatomae* was also present (Schaub and Lösch 1989).

The next *Blastocrithidia* species with an analyzed life cycle was *Blastocrithidia miridarum*. Experimental infections of *Adelphocoris quadripunctatus* confirmed that it colonizes the whole intestine and forms straphangers (mostly) in the rectum (Frolov and Skarlato 1988). But the most comprehensive analysis has recently been reported for *B. papi* (Frolov et al. 2018). Its life cycle is synchronized with its virtually omnipresent firebug host (*Pyrrhocoris apterus*)

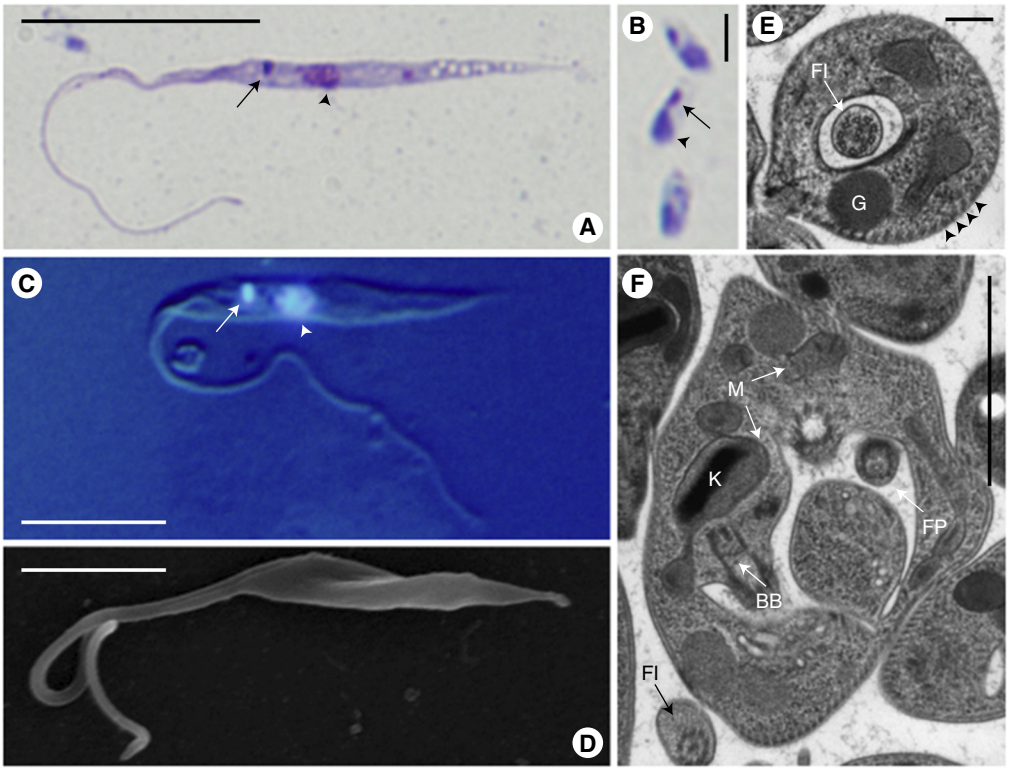

Figure 2. Morphology of *Blastocrithidia nonstop*. (*A*) Light microscopy of a Giemsa-stained promastigote stage. (*B*) Light microscopy of Giemsa-stained cystic stages (straphangers). (*C*) Fluorescent microscopy of a 4′,6-diamidino-2-phenylindole (DAPI)-stained promastigote stage. In *A* through *C*, the nucleus and the mitochondrial (=kinetoplast) DNA are labeled with arrowhead and arrow, respectively. (*D*) Scanning electron microscopy of the promastigote stage highlighting the thick flagellum equipped with a paraflagellar rod. (*E*) Transmission electron microscopy of the cross-sectioned promastigote stage. Note the flagellum (Fl) in the flagellar pocket (FP), glycosome (G), and the corset of subpellicular microtubules (arrowheads). (*F*) Transmission electron microscopy through the FP of the promastigote stage. Note the electron-dense, disk-shaped kinetoplast DNA network (K) within a reticulated mitochondrion (M), and the adjacent basal body (BB) of the flagellum. The cross-sectioned external flagellum (Fl) is supported by the paraflagellar rod. Scale bars, 10 µm (*A*,*C*,*D*); 2 µm (*B*); 1 µm (*F*); 200 nm (*E*).

and includes an obligate developmental stage in the Malpighian tubules of the insect that exited the winter diapause (Lukeš et al. 2018). In the short period before the mating of overwintered males and females, *Blastocrithidia* cells penetrate the Malpighian tubules, attach to the epithelial surface, and form straphangers in large quantities. The absence of peristaltic movement in the Malpighian tubules allows the parasites to accumulate, while the absence of peritrophic structures makes the extensive discharge of straphangers directly into the hindgut lumen possible. During oviposition, straphangers attach to the surface of eggs with feces and become engulfed by the newly hatched nymphs (Frolov et al. 2018). Under the laboratory conditions, *B. papi* may be transmitted vertically via contaminated surface of eggs and horizontally via contaminated substrate and/or necrophagy (Frolov et al. 2017).

The last unique example is the development of *Blastocrithidia raabei* in the dock bug, *Coreus marginatus*. It was shown that the refractory nature of the host provokes very aggressive behavior of the parasite and makes its life cycle more complex, reminiscent of that in some dixenous

kin. In the anterior midgut, the epimastigotes of *B. raabei* attach to the epithelium and divide similarly to other insect trypanosomatids. The flagellates progress from the anterior to the posterior region of the midgut, where they produce straphangers (Frolov et al. 2020).

CULTIVATION, TRANSFORMATION, TAGGING, KNOCKOUTS, AND INFECTION OF INSECT HOSTS

Members of the genus *Blastocrithidia* are notoriously difficult to cultivate (Peng and Wallace 1981). Early experiments with species available at the time relied on cells isolated directly from the infected hosts or cultivated in vitro in *Helicoverpa zea* or *T. infestans* cell lines (Podlipaev 1988; Reduth et al. 1989). Later on, methods of axenic cultivation were developed and are in use (with modifications) until now (Afonin et al. 2024; Záhonová et al. 2025), but only recently we have demonstrated that *B. nonstop* is amenable to genetic modifications. Using a *Blastocrithidia*-tailored CRISPR-Cas9-based system for gene editing, we successfully ablated and tagged the target gene (Galan et al. 2025). We have chosen this gene to ensure that the resultant cell line is viable. Our approach relied on the intergenic regions of the moderately expressed genes encoding a putative member of the glycoside hydrolase family 3 and a putative L15 ribosomal protein, along with the Cas9 ribonucleoprotein complex delivered exogenously. It proved to be significantly more effective than any traditional technique, probably because the amount of Cas9 delivered to the cell was substantially higher than what could be achieved by endogenous expression of this protein. Deletion of both alleles was complete, as verified by genome sequencing and other validation techniques (Galan et al. 2025).

The wild-type or genetically modified *Blastocrithidia* cells can be used for further molecular and biochemical studies or can serve as an inoculum for infection. Recently, adult hemipterid bugs of the genus *Dysdercus* have been successfully infected with culture-derived promastigotes of *B. nonstop* in vitro. Their capacity to infect the intestine of these insects was monitored with specific FISH probes, revealing the establishment of a stable, long-term infection, which must have allowed reproduction of the whole life cycle (Š Zeman, M Kaltenpoth, J Lukeš et al., unpubl.).

GENOMIC FEATURES ASSOCIATED WITH THE NONCANONICAL CODE

The *Blastocrithidia* model is unique because of its genetic tractability that allows ablations, knock-ins, and tagging of proteins associated with the code reassignment. However, to design informative experiments, it is critical to identify all key genetic elements associated with the altered genetic code. At its center, there are three standard stop codons (UAA, UAG, and UGA) reassigned to specific amino acids, with only UAA serving as the universal termination signal, which has, thus, acquired a dual meaning (Kachale et al. 2023). This pangenomic feature, for which the term "codon homonymy" has recently been introduced, describes cases where one codon acquires multiple meanings, which the given translation system distinguishes based on its context (J Lukeš, M Eliáš, A Kachale et al., unpubl.).

Interestingly, there is another case of codon homonymy in *B. nonstop*, namely, that of UGA. All trypanosomatids, including *Trypanosoma brucei*, encode at least three selenocysteine-containing proteins (Lobanov et al. 2006; Bonilla et al. 2016) and *B. nonstop* is no exception (Záhonová et al. 2025). In a wide majority of selenoprotein-carrying organisms, the UGA codon is homonymous in the sense that it plays a dual role, and, depending on the sequence context, encodes either a stop codon or, less frequently, selenocysteine, the 21st amino acid (Stadtman 1996). However, while UGA underwent a wholesale reassignment from serving as a stop codon to encoding exclusively tryptophan in *B. nonstop* (Kachale et al. 2023), its role as a triplet specifying the incorporation of selenocysteine (Turanov et al. 2009) has been retained. Thus, the flagellate dissected herein is uniquely homonymous in UAA (glutamate and universal stop) and UGA (tryptophan and selenocysteine).

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Several genomic features distinguish *B. nonstop* from other trypanosomatids with the standard genetic code. Firstly, it is the nonrandom distribution of in-frame reassigned stop codons, the frequency of which inversely correlates with the abundance of their protein products. It reveals certain translational burdens associated with these codons. Secondly, the frequency of UAA codons significantly increases in the 3′ untranslated region (UTR) following the genuine UAA stop. Thirdly, the eukaryotic release factor 1 (eRF1) has unique mutations and the eRF3 carries an extended amino-terminal prion-like region (Kachale et al. 2023; Záhonová et al. 2025; see below). Finally, *B. nonstop* has evolved specific tRNAs to decode all three stop codons, all of which are described in more detail below.

Importantly, the relevance of all these features for code reassignment has been markedly strengthened by the recent assembly and annotation of high-quality genomes of *Blastocrithidia frustrata*, *B. raabei*, and *B. triatomae*, which are closely related to *B. nonstop* (Záhonová et al. 2025). Although all four flagellates have been isolated from distinct host species and from different continents, their unique genomic features, most likely associated with the reassignment, are virtually identical. Underscoring the uniqueness of this evolutionary phenomenon, all these unifying features are absent in the morphologically indistinguishable and evolutionarily closely related *Obscuromonas* spp., which have a standard genetic code (Záhonová et al. 2025). The high level of similarity of the *Blastocrithidia* genomes is compatible with an evolutionary scenario positing a cascade of steps either leading to or immediately preceding the genetic code switch that must have been wholesale and mutually interdependent in their common ancestor. Although very plausible, given the absence of species with any intermediate stage, such a scenario remains speculative.

CRACKING THE CODE—THE POWER OF tRNAs

In studies of the unique features of noncanonical genetic code expression, the tRNA repertoire and the mechanisms involved in decoding stop codons are typically the primary focus. Effective recognition of stop codons by suppressor tRNAs is essential for their reassignment as sense codons. In the course of evolution, suppressor tRNAs may emerge from mutations in the anticodon, allowing them to decode premature stop codons (PSCs) and mitigate the effects of nonsense mutations. Additionally, near-cognate tRNAs can also recognize the stop codons, explaining almost all known cases of the nuclear stop codon reassignment (Salman et al. 2024).

Ciliates, a diverse group of single-celled eukaryotes, exhibit remarkable flexibility in their genetic codes, often reassigning standard stop codons to encode amino acids. This adaptability is facilitated by the presence of suppressor tRNAs, which recognize these redefined codons and incorporate specific amino acids during protein synthesis. In many ciliate species, the canonical stop codons UAA and UAG have been reassigned to code for glutamine (Swart et al. 2016; McGowan et al. 2024). For instance, expressing a ciliate gene in *E. coli* required the use of a suppressor tRNA capable of reading UAA and UAG as glutamine codons, enabling proper translation in this bacterial system (Cohen et al. 1990). A recent study has uncovered multiple independent genetic code changes within the Phyllopharyngea class of ciliates. Notably, certain uncultivated ciliate species from marine environments have reassigned the UAG codon to encode leucine, with novel suppressor $tRNA^{Leu}_{CUA}$. On the other hand, other Phyllopharyngea ciliates, such as *Hartmannula sinica* and *Trochilia petrani*, have independently reassigned UAG to glutamine, highlighting the dynamic evolution of genetic codes in these organisms (McGowan et al. 2024).

Significant progress has recently been made in understanding the codon reassignment mechanisms in *B. nonstop*, and this species has emerged as an important model in this context (Fig. 3). Sequencing of its nuclear genome revealed 70 tRNA genes, including newly evolved $tRNA^{Glu}_{CUA}$ and $tRNA^{Glu}_{UUA}$, which can decode both UAA and UAG stop codons (Kachale et al. 2023). These tRNAs are not pseudogenes, as they are expressed and charged, and the phy-

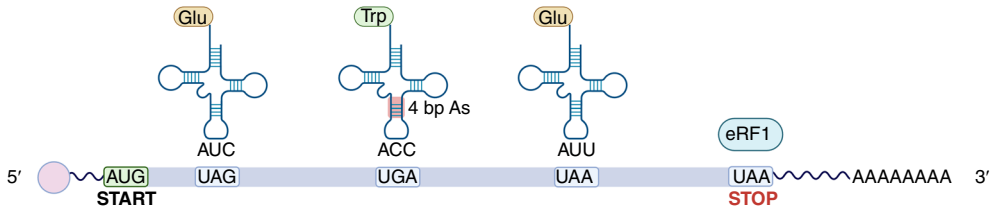

Figure 3. *Blastocrithidia nonstop* uses an unconventional messenger RNA (mRNA) decoding strategy that relies on specialized transfer RNAs (tRNAs) and a distinctive termination system. This includes tRNAs with anticodons that match UAG and UAA, which carry the amino acid glutamate (Glu). Additionally, the stem of the tryptophanyl-tRNA is a single nucleotide pair shorter than usual, resulting in this tRNA being able to decode UGA as tryptophan (Trp). The termination machinery triggered by release factor (eukaryotic release factor 1 [eRF1]) stops protein synthesis only when UAA is near the end of the open reading frame (ORF).

logenetic analyses revealed that they have evolved from the canonical $tRNA^{Glu}$ isoacceptors. Interestingly, *B. nonstop* encodes only a single glutaminyl-tRNA synthetase (RS), which must charge both canonical and suppressor $tRNA^{Glu}$ species. This is similar to what is observed with other aminoacyl-RSs such as LeuRS, ArgRS, and GlyRS, which efficiently charge multiple isoacceptors regardless of anticodon variation (Giegé and Eriani 2023).

A puzzling question arose regarding the identity of a tRNA that would decode the UGA codon as tryptophan, as in the ciliate *Condylostoma magnum*, which has the same UGA codon reassignment, a cognate $tRNA^{Trp}$ was not found (Swart et al. 2016). A possible explanation draws a parallel to the mitochondria of related trypanosomatids, such as *Leishmania tarentolae* (Alfonzo et al. 1999) and *T. brucei* (Wohlgamuth-Benedum et al. 2009), where in the mitochondrially encoded transcripts the UGA codons are decoded through a C-to-U editing event of the wobble position of $tRNA^{Trp}$. This generates the edited $tRNA^{Trp}_{UCA}$ that is cognate to the UGA tryptophan codon. Although *B. nonstop* possesses the same cytidine deaminase responsible for mitochondrial editing in *T. brucei* (Paris et al. 2021), in both organisms editing occurs exclusively in the mitochondrion with no evidence for this activity in the cytoplasm of *B. nonstop*, which would affect the cytosolic translation of UGA (Kachale et al. 2023).

Finally, the UGA codon is decoded by the near-cognate $tRNA^{Trp}_{CCA}$ due to its unique structure (Fig. 3). The secondary structure predictions revealed that the anticodon stem (AS) of this *B. nonstop* (as well as *C. magnum*) tRNA is only 4 bp long, whereas their closely related trypanosomatids and ciliates universally possess a canonical 5 bp AS, similarly to the majority of the eukaryotic world. Experimental testing of both $tRNA^{Trp}$ variants in heterologous systems of *T. brucei* and *Saccharomyces cerevisiae* documented a significant increase in UGA readthrough induced by the 4 bp version, unraveling a novel mechanism for UGA reassignment (Baranov and Atkins 2023; Kachale et al. 2023).

Modifications of tRNAs should also be considered as an important factor, since they can be essential for various types of previously described codon reassignments, enabling modified tRNAs to recognize the noncanonical codons, such as the stop codons (Smith et al. 2024). For example, pseudouridine (Ψ) at position 35 in $tRNA^{Tyr}$ in eukaryotes allows for suppression of the UAA and UAG stop codons (Blanchet et al. 2018). Additionally, tRNAs can "superwobble," a phenomenon in which modified nucleotides, such as inosine, 5-formylcytidine, and carboxymethoxyuridine at the wobble position allow a single tRNA to recognize all four codons that differ only in their third nucleotide (Kothe and Rodnina 2007; Weixlbaumer et al. 2007). All these mechanisms expand the decoding capacity of the tRNAs, enabling them to decode multiple codons with different specificities that, in turn, may also facilitate codon reassignment (Lei and Burton 2022).

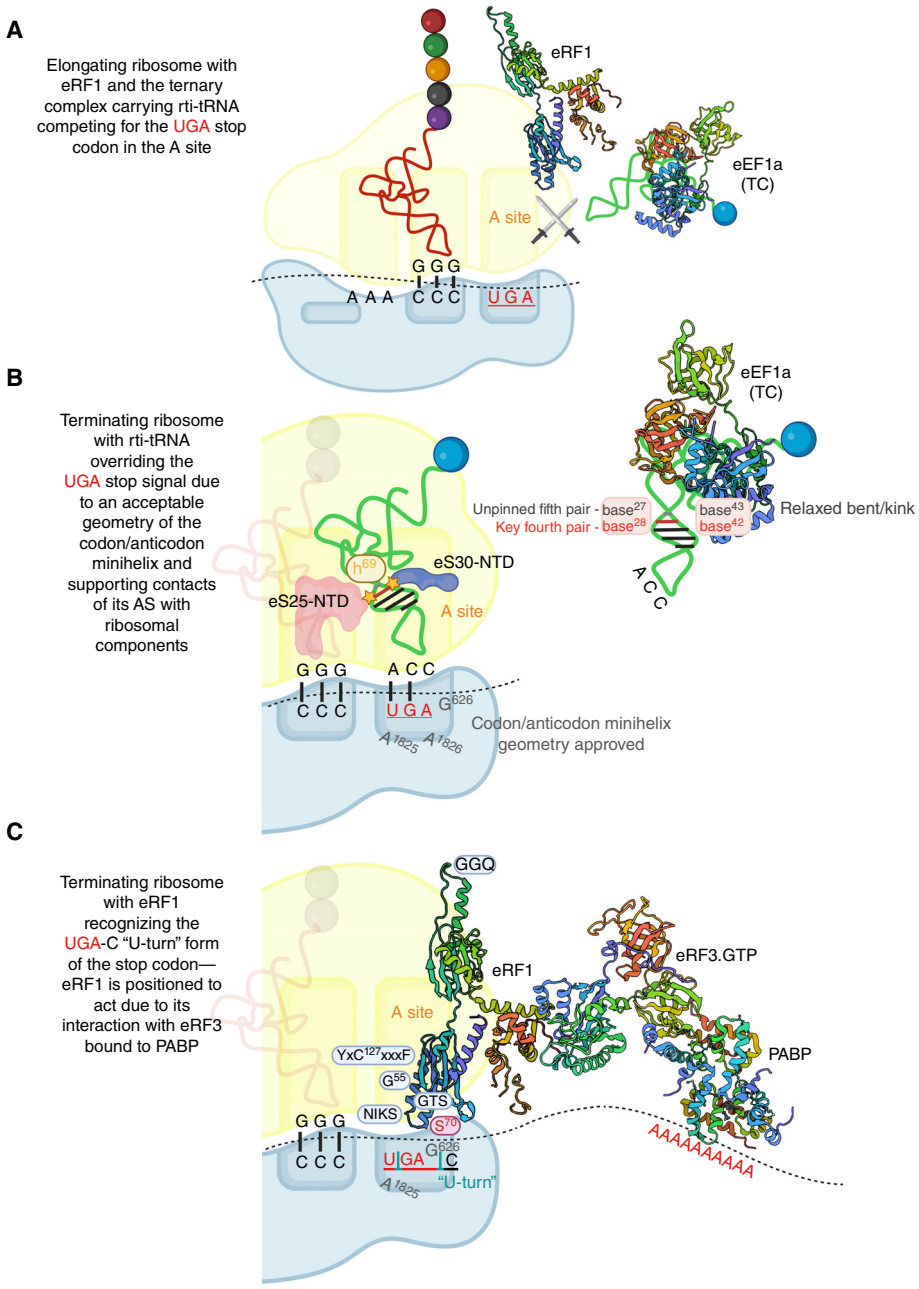

Figure 4. Translation termination and stop codon readthrough in eukaryotes. (*A*) The ternary complex carrying readthrough-inducing (rti)-tRNA competes with eukaryotic release factor 1 (eRF1) for the UGA stop codon situated in the A site of the pretermination ribosome. (A site) Aminoacyl-tRNA-binding site. (*B*) Terminating ribosome with rti-tRNA overriding the UGA stop signal due to (1) an acceptable geometry of the codon/anticodon minihelix, which is monitored by the G626, A1825, and A1826 bases of 18S ribosomal RNA (rRNA), and (2) supporting contacts of its AS with ribosomal components. The key AS base pairs greatly increasing the efficiency of stop codon readthrough of at least some rti-tRNAs are depicted on a model tRNA associated with the eukaryotic elongation factor 1α (eEF1A) (TC). (*C*) Terminating ribosome with eRF1 recognizing the UGA-C "U-turn" form of the UGA stop codon via the G626 and A1825 bases of 18S rRNA. eRF1 is positioned next to the genuine stop codon to act promptly upon the arrival of the elongating ribosome due to its interaction with eRF3 bound to PABP. The eRF1 motives and individual residues critical for stop codon recognition and polypeptide release (GGQ) are highlighted. (PABP) Poly(A)-binding protein.

The follow-up research on *B. nonstop* has uncovered remarkable insights into how the mitochondrial genome and expression remain insulated from the extensive codon reassignment occurring in its nuclear genome (Afonin et al. 2024). Despite the nuclear genome's unique recoding of all three canonical stop codons to sense codons, the mitochondrial genome maintains a typical trypanosomatid genetic code. The situation becomes more complex when tRNAs are taken into account. In trypanosomatids, there are no mitochondrial encoded tRNAs and, consequently, the complete set of all tRNAs required for mitochondrial mRNA translation has to be imported from the cytosol (Hancock and Hajduk 1992; Schneider et al. 1994). In *B. nonstop*, however, the tRNA import mechanism appears to be nonselective, with potentially harmful readthrough tRNAs entering the mitochondrion at equal or even higher rates than the canonical $tRNAs^{Glu}$ (Afonin et al. 2024). Therefore, it is surprising that there is no noticeable "footprint" of these imported suppressor tRNAs (e.g., the use of stop codons in the protein-coding sequences) within the mitochondrial genome. A possible explanation for this phenomenon is that these tRNAs are modified or otherwise "deactivated" upon entering the mitochondrion, preventing them from participating in the organellar translation. This could also involve changes to their aminoacylation status. However, the exact process of putative deactivation of these tRNAs remains to be investigated further.

Recently, a focus has been directed toward tracing the evolutionary history of the stop codon reassignment within the *Blastocrithidia* lineage. As described above, this was achieved by comparing the genomes of four *Blastocrithidia* and four *Obscuromonas* species, which use the noncanonical and canonical genetic codes, respectively (Záhonová et al. 2025). Notably, no intermediate stage was identified within the *Blastocrithidia* lineage, as all analyzed genomes exhibit the same degree of stop codon reassignment. The analysis revealed the presence of cognate suppressor $tRNAs^{Glu}$ for the reassigned UAA and UAG codons, with UAA serving also as the sole stop codon. These were exclusively found in *Blastocrithidia* spp. and were completely absent in their *Obscuromonas* relatives. Similarly, the $tRNA^{Trp}_{CCA}$ variant featuring a shortened 4-bp-long AS, previously shown to be critical for efficient UGA decoding as tryptophan in *B. nonstop* (Kachale et al. 2023), was restricted to *Blastocrithidia* spp. In contrast, the *Obscuromonas* spp. maintain the canonical 5-bp-long AS $tRNA^{Trp}$ variant. Furthermore, we documented variability in the number of predicted tRNA genes across the *Blastocrithidia* genomes, ranging from 70 to 95 (Záhonová et al. 2025). However, this variability is consistent with the range of tRNA genes in other trypanosomatid genomes and, probably, does not yield any functional consequences in terms of translation of the reassigned genomes. A potential adaptation in tryptophanyl-RS (TrpRS), the enzyme responsible for charging $tRNA^{Trp}$ with tryptophan, also has to be considered. All *Blastocrithidia* spp. possess both the cytosolic (TrpRS1) and mitochondrial (TrpRS2) isoforms, with TrpRS1 exhibiting four unique amino acid substitutions in the anticodon-binding domain. These variations may enhance the enzyme's capacity to recognize and load the structurally altered 4-bp-long AS $tRNA^{Trp}_{CCA}$, thereby ensuring efficient decoding of the reassigned UGA codon (Záhonová et al. 2025).

tRNAs WITH THE 4-bp-LONG ANTICODON STEM—OCCURRENCE VERSUS EXPERIMENTAL DESIGN

The exact mechanism by which unpinning the top bp in the AS of the *B. nonstop* $tRNA^{Trp}$ with a natural CCA anticodon outcompetes eRF1 and thus enhances the UGA stop codon readthrough (Fig. 4A) has yet to be elucidated. Nonetheless, the importance of no-base-pairing at this position in facilitating this peculiar protist to read through in-frame UGA stop codons is underscored by the fact that a similar readthrough phenomenon has also been observed for several other tRNAs in bacteria and eukaryotes (Komine and Inokuchi 1990; Schultz and Yarus 1994a,b; Ortiz-Meoz and Green 2010; Kemp et al. 2013; Kachale et al. 2023).

Historically, the very first example of a mismatched top bp (G27:A43) in the AS that is functionally important was observed in the naturally occurring *E. coli* $tRNA^{Thr}$. When the CGU anticodon was replaced with the amber CUA, the native tRNA with a 4-bp-long AS suppressed the UAG stop codon more efficiently than its 5-bp-long AS variants (G27:C43 or U27:A43) (Komine and Inokuchi 1990). Two other examples relate directly to $tRNA^{Trp}$. When Schultz and Yarus (1994a) screened for more efficient *E. coli* $tRNA^{Trp}$ suppressors bearing mutated anticodon from natural CCA to CUG, they detected elevated UAG readthrough (with the first U-G wobble position) for $tRNAs^{Trp}$ with mutations at the top AS bp. Interestingly, only one of these highly active mutants, C27G:G43C, preserved the Watson–Crick base pair, while the other seven had the 27-43 mispair. Noteworthy, one of them was identical to the natural C26-U42 mispair of *B. nonstop* (Kachale et al. 2023). In addition, they demonstrated that tRNAs with the 27-43 mispair, when introduced into $tRNA^{Trp}$ bearing the CCA anticodon mutated to CUA, displayed similar suppression efficiency also at the UAA stop codon (with the third position A-C wobble) (Schultz and Yarus 1994b). Note that in contrast to the above-described *E. coli* mutant $tRNA^{Trp}$, the *B. nonstop* $tRNA^{Trp}$ retained the native CCA anticodon to read through the UGA stop codon (with the third position A-C wobble) (Kachale et al. 2023).

Among the most frequently observed mutations in the in vitro screening for novel miscoding variants of *E. coli* $tRNA^{Trp}$ with preserved CCA anticodon were those at the junction of the anticodon and D stems. These include C27A of the top AS pair, the so-called Hirsh mutation (G24A) (Hirsh 1971), and a mutation in the T loop (G59A) (Ortiz-Meoz and Green 2010). In fact, the Hirsh mutation in the D stem of $tRNA^{Trp}_{CCA}$, which occurs naturally in one *E. coli* strain where the U11:24G pair is replaced with U11:24A, was the first one to spark a debate as to why a single base change outside the anticodon loop could affect the stop codon decoding (Hirsh 1971). Although the single C27A mutation disrupting the canonical C27:G43 Watson–Crick bp at the top of the AS displayed no miscoding phenotype on its own, it was essential for efficient readthrough of the triple mutant G24A C27A G59A, which worked as the best UGA miscoder (Ortiz-Meoz and Green 2010). In support of the important role of the AS length in decoding in unrelated organisms, a U27G mutation unpinning the top AS bp of $tRNA^{Gln}_{CUG}$ was identified as an UAG nonsense suppressor in *S. cerevisiae* (Kemp et al. 2013). However, substituting A43 of the fifth AS bp of $tRNA^{Gln}_{CUG}$ did not have this effect (Z Paris, N Al-Chamy, F Brazdovic et al., unpubl.), suggesting that the contribution of the top AS bp to UAG decoding is a more complex issue. Indeed, we systematically unpinned the top AS bp of all near-cognate tRNAs and found only a very few tRNAs, where specific mutations resulted in an increased SC-RT, but only at some stop codons and in a completely unpredictable manner (N Al-Chamy, F Brazdovic, and LS Valášek, unpubl.). Therefore, further mechanistic and structural insights are needed to understand the underlying principle of these observations.

Interestingly, in addition to *B. nonstop*, there are a few other organisms with all three stop codons reassigned in their nuclear genomes (Heaphy et al. 2016; Swart et al. 2016; Bachvaroff 2019; DeMontigny and Bachvaroff 2025). One of them is a ciliate *C. magnum* that independently adopted a similar mechanism for reassignment of the in-frame UGA stop codons to tryptophan. Its genome contains three copies of $tRNA^{Trp}_{CCA}$ with the unpinned top AS bp (i.e., 4-bp-long) as the sole source of $tRNA^{Trp}$ (Heaphy et al. 2016). Importantly, we demonstrated that restoring the fifth AS bp by mutation dramatically compromised UGA readthrough in model organisms *T. brucei* and *S. cerevisiae* (Kachale et al. 2023). Therefore, at least for $tRNA^{Trp}$, this peculiar AS adaptation appears to be well conserved.

tRNAs WITH THE 4-bp-LONG ANTICODON STEM—WHAT ABOUT THE MECHANISM?

Even though the underlying molecular mechanisms remain elusive, we are not completely clueless and several possible scenarios have

been proposed. When the aminoacyl-tRNA (aa-tRNA) in the ternary complex with eukaryotic elongation factor 1α (eEF1A) and guanosine triphosphate (GTP) enters the ribosomal A site, it adopts a distorted conformation to sample the mRNA codon. During this process, aa-tRNA remains attached to eEF1A, which interacts mainly with the large ribosomal subunit. This A/T conformation requires bending of the anticodon arm at the junction of the anticodon and D stems defined as the "kink" (Valle et al. 2002). This kink is made possible through distortion of the D stem and the upper part of the AS (Valle et al. 2003). Considering that the discussed bp at the AS top resides in the distortion hot spot, one can assume that its unpinning may help 4-bp-long AS tRNAs to adopt the distorted A/T-conformation more easily, so that codon sampling may become productive even without reaching a perfect codon–anticodon complementarity match (Fig. 4B). Along these lines, Schultz and Yarus (1994b) suggested that alterations of the 27:43 AS bp may induce an altered L conformation (i.e., tRNA may have a more flexible angle between the anticodon arm and the center of the tRNA molecule as the 27–43 bp is just at their junction). An altered or more flexible conformation of the L-shaped tRNA was proposed to decrease the rate of tRNA rejection from the ribosome, and a similar mechanism was also proposed for the Hirsh G24A mutation of $tRNA^{Trp}$. In fact, the contacts between the large ribosomal subunit (helix 69) and mutations at the $tRNA^{Trp}$ top AS bp alone or in combination with the Hirsh mutation promote accommodation of these mutant tRNAs in the A site during decoding (Ortiz-Meoz and Green 2010).

To provide experimental support for these suggestions, the crystal structure of the 70S ribosome in complex with EF-Tu and $tRNA^{Trp}$ mutant variants revealed that the Hirsh G24A mutation indeed helps $tRNA^{Trp}$ to adopt the distorted bent A/T conformation through formation of the additional internal interactions, but with no effect on the interactions between $tRNA^{Trp}$ and the ribosome (Schmeing et al. 2011). However, comparing the decoding structures of $tRNA^{Trp}$ and $tRNA^{Thr}$, it became obvious that none of the observations can be generalized because each tRNA adopts its own unique conformation upon delivery to the ribosome to ensure accurate decoding. Thus, the structural insight into the impact of the unpinned fifth AS bp on tRNA accommodation in the A site remains to be shown.

In addition to the top AS bp, we recently demonstrated that the elevated readthrough-inducing potential of the wild-type *S. cerevisiae* M isoacceptor of $tRNA^{Gln}_{CUG}$ ($tRNA^{Gln}_{CUG}$[M]) depends on the specific pyrimidine 28:purine 42 bp constituting the fourth AS bp (Fig. 4B; Čapková Pavlíková et al. 2025). All isodecoders of the UUG isoacceptor family of $tRNA^{Gln}_{UUG}$ have a purine 28:pyrimidine 42 bp instead, which prevents an efficient stop codon readthrough (SC-RT). The same principle applies to $tRNA^{Gln}$ in *T. brucei*. Importantly, the mutational analysis revealed that this specific pyrimidine 28:purine 42 bp of $tRNA^{Gln}_{CUG}$ interacts with Arg10 of the small ribosomal protein eS30 (and the same pyrimidine:purine fourth bp of $tRNA^{Cys}$ with histidine 5 of eS30) and that these contacts are crucial for elevated readthrough activity of these tRNAs (Fig. 4B). A similar functionally important interaction for efficient SC-RT was also observed between $tRNA^{Tyr}$ and the extreme amino terminus of eS25. Collectively, we proposed that, in addition to the degree of complementarity of the codon–anticodon minihelix and its interactions with the decoding residues A1824, A1825, and G626 of 18S rRNA (A1492, A1493, and G530 of bacterial 16S rRNA; Ogle et al. 2001, 2002), which are primarily monitoring the geometry of the minor groove of the minihelix (Fig. 4B), tRNAs establish additional contacts with the decoding center components through their backbone, in particular through their AS (Čapková Pavlíková et al. 2025). These so far unrecognized interactions could further propagate a tighter fit around the codon–anticodon minihelix, thereby increasing the likelihood that both cognate but mainly specific near-cognate aa-tRNAs reach an activated state to become selected (Fig. 4B; Čapková Pavlíková et al. 2025). Interestingly, when we mutated Arg10 of eS30, the SC-RT potential of the 5-bp-long AS $tRNA^{Trp}_{CCA}$ but not of its un-

pinned 4-bp-long variant was significantly reduced (Čapková Pavlíková et al. 2025). These results suggest that the distortion of the upper part of the AS stem induced by unpinning of the top AS bp compensates for the otherwise weak interactions that some tRNAs establish with the rRNA and protein components of the A site. As a result, the unpinning increases their odds of being selected during codon sampling.

Evidence for a contribution of the tRNA backbone dynamics to efficient decoding or SC-RT has also been demonstrated in mammalian cells. Based on the in vivo analysis of various human $tRNA^{Cys}$ isodecoders, it has been proposed that position 16 in the D loop of $tRNA^{Cys}$ influences SC-RT (Valášek et al. 2023). In addition to that, a robust suppression analysis of various tRNA isodecoders has revealed that the long variable arm plays a critical role in the stop codon suppression by $tRNA^{Leu}$ and $tRNA^{Ser}$ isodecoders (Geslain and Pan 2010). Given what we have learned about readthrough-inducing tRNAs (rti-tRNAs) (Beznosková et al. 2019, 2021; Čapková Pavlíková et al. 2025), it is plausible that the disparities in the SC-RT efficiency among the isodecoder tRNAs arise not only from variable tRNA folding due to their differing sequences but also from the interactions that some of them establish with the decoding site components. For the sake of completeness, it is important to note that the sequences in the AS loop outside the anticodon are known to greatly influence SC-RT efficiency too. Their contributions were covered by others (Yarus 1982; Raftery and Yarus 1987; Kleina et al. 1990; Olejniczak and Uhlenbeck 2006; Grosjean and Westhof 2016).

Taken together, it seems that it is "a little bit of everything" that determines the efficiency of proofreading during decoding and SC-RT including, for example, interactions of tRNAs with rRNA and ribosomal proteins in the A site, altered tRNA flexibility mainly at the "kink hot spot," rearranged tertiary interactions within the tRNA molecule itself, and tRNA modifications (Fig. 4B). To make the researcher's life uneasy, the importance of these individual requirements seems to dramatically differ for different tRNAs.

WHAT DOES IT TAKE TO RESTRICT UGA DECODING BY eRF1?

To capture protein synthesis, occurrence of one of the three stop codons (UAA, UAG, and UGA) in the ribosomal A site is recognized by the complex of release factors eRF1 and eRF3 (Fig. 4C; Alkalaeva et al. 2006; Wong et al. 2012; Kryuchkova et al. 2013). This triggers GTP hydrolysis by eRF3, inducing a conformational change that leads to eRF3 dissociation, permitting the "omnipotent" eRF1 to accommodate fully in the A site (Valášek et al. 2017; Hellen 2018). This conformational change is also thought to bring a universally conserved GGQ motif close to the ester bond between the nascent polypeptide and peptidyl-tRNA sitting at the P site, thereby stimulating its hydrolysis and subsequent release of the newly born polypeptide from the ribosome. The GGQ repositioning is promoted by the eukaryotic ribosome recycling factor ABCE1 (Pisarev et al. 2010; Shoemaker and Green 2011), which subsequently, together with eRF1 and in an ATP-dependent manner, splits the 80S post-termination complexes into individual ribosomal subunits (Preis et al. 2014; Heuer et al. 2017; Mancera-Martínez et al. 2017).

Structural studies of the eukaryotic termination complex revealed that eRF1 recognizes a specific U-turn structure established by the stop codon and the immediately following fourth base (+4 relative to +1 base of a stop codon) in the decoding center (Fig. 4C; Preis et al. 2014; Brown et al. 2015; Matheisl et al. 2015). In particular, entry of eRF1 into the decoding site flips nucleotide A1825 in helix 44 (h44) of human 18S rRNA so that it stacks on the second and also the third stop codon bases. Importantly, this configuration pulls the fourth base into the A site, where it is stabilized by stacking against G626 of 18S rRNA. These two interactions thus drive the critical mRNA compaction, so that second and third stop codon bases are decoded as a single unit. This solves the long-standing mystery of how guanosine can occur at either the second or third position, but never at both; two successive guanosines would lead to repulsion between their O6 atoms and critical Glu55 (human numbering) (Brown et al. 2015; Matheisl et al. 2015).

Therefore, UGG encoding tryptophan cannot be recognized by eRF1.

The universality of uridine in the first position of stop codons is defined by its extensive hydrogen bonding with essential eRF1 residues of the TASNIKS motif. In the case of the second and third stacking bases, the conserved GTS motif plays the discriminatory role by adopting two different conformations that are interdependent with the position of the YxCxxxF motif and the aforementioned Glu55, depending on whether adenosine or guanine are in the second position (Fig. 4C; Brown et al. 2015; Matheisl et al. 2015).

Molecular dynamics simulation confirmed that in the case of UAG, the Glu55 attraction with the second position A is more or less balanced by its repulsion with G in the third position (Lind et al. 2017). However, the Glu55 repulsion is about twice as strong with G in the second position compared to G in the third one, making its overall interaction with UGA unfavorable. This is, however, greatly compensated by Cys127 from the YxCxxxF motif that has a distinctly favorable backbone interaction with G in the second position. In essence, the ability to read three out of four purine–purine combinations at the second and third stop codon position (UGG is forbidden) is largely ensured by two key universally conserved residues, namely, Glu55 providing repulsion and Cys127 providing attraction to the critical stop codon guanines (Fig. 4C). Of note, based on earlier biochemical analyses, recognition of the UGA codon was proposed to be promoted by the highly conserved S33 and S70 residues (human numbering) (Kryuchkova et al. 2013), which are juxtaposed from the parallel β sheet (S33) and α helix (S70) (Song et al. 2000).

In the context of a genome that uses a restricted set of stop codons, it is important to stress that the structural work combined with these simulations postulated that eRF1 binding to the three stop codons is characterized by uniform binding affinity with little energetic preference for any particular codon (Brown et al. 2015; Matheisl et al. 2015; Lind et al. 2017). Thus, it is clear that the entire termination process is very well balanced for all three stop codons, whose presence in the A site is closely monitored by a few evolutionarily conserved amino acid residues. Thus, it seems highly likely that any substitution of these residues must always affect recognition of all three stops. In stark contrast to this assumption are cases of the cytosolic translation systems with a limited number of true stop codons, using UAA in combination with either UAG or UGA, or even just UAA or UGA alone (Swart et al. 2016; Pánek et al. 2017; Kachale et al. 2023), all with peculiar substitutions of the given key residues of eRF1. One example is the trypanosomatid *B. nonstop* that managed to completely restrict UGA recognition by a single substitution of the otherwise highly conserved Ser74 (S67 and S70 in *S. cerevisiae* and *Homo sapiens*, respectively) with the Gly residue (S74G) without any impairment of UAR recognition (Záhonová et al. 2016; Kachale et al. 2023). An inverse situation is represented by the ciliates *Paramecium* and *Stylonychia*, whose eRF1 only allows UGA decoding (Lekomtsev et al. 2007), while UAA and UAG are repurposed as sense codons (Eliseev et al. 2011). In the case of *Paramecium tetraurelia*, the highly conserved residues Glu55 and Thr58 are mutated to Asn and Glu, although Cys127 remains unchanged. Thus, all these findings greatly support the unexpected flexibility of eRF1 with respect to specific decoding of individual stop codons. Therefore, the pressing question is as follows: What does it really take to restrict eRF1 from decoding individual stop codons in organisms with stop-to-sense reassignments? In particular, from UGA decoding in case of the UGA = W reassignment?

In addition to the work with *Blastocrithidia* sp. ex *Ligus* (UGA = W) revealing that its eRF1 has S74G (Záhonová et al. 2016), earlier comparative analyses of eRF1 had already revealed that Ser74 (*B. nonstop* numbering) in the ciliates *Blepharisma* (UGA = W) and *Euplotes* (UGA = C) is substituted with Ala (S74A) (Inagaki et al. 2002; Eliseev et al. 2011). Experiments carried out in *S. cerevisiae* with mutant yeast eRF1-S67G (Kachale et al. 2023) and eRF1-S67A and with mutant *H. sapiens* eRF1-S70A (Kryuchkova et al. 2013; Blanchet et al. 2015) clearly confirmed the importance of these substitutions in achieving ~100-fold increase of UGA SC-RT in

vivo. Furthermore, the S67G mutation potentiated UGA readthrough by the 4-bp-long AS $tRNA^{Trp}_{CCA}$ from sevenfold to >200-fold compared to the wild-type situation (Kachale et al. 2023). As mentioned above, all these findings entitled us to propose that G74 in *B. nonstop* (and A74 likely in *Blepharisma* and *Euplotes*) is primarily responsible for the inability of eRF1-G74 to recognize UGA, and thus represents one of the key factors of the efficient UGA = W reassignment. This conclusion was further supported by the comparative analysis of four *Blastocrithidia* species, all of which have eRF1-S74G and share the UGA = Trp reassignment, and four representatives of the closest known sister genus *Obscuromonas* species with the standard nuclear genetic code and the wild-type eRF1-Ser74 (Záhonová et al. 2025). Interestingly, sequence alignment also revealed six additional positions that are fully conserved in all trypanosomatids except members of the genus *Blastocrithidia*. Although they occur outside of all known functional motifs, five of them map to two antiparallel helices harboring the stop codon recognition motifs. Therefore, their potential roles must be explored before the incompetence of *Blastocrithidia* eRF1 to recognize UGA can be fully attributed to the G67 residue.

In addition to *Blepharisma* and *Euplotes*, the S74A substitution (*B. nonstop* numbering) was found also in eRF1 of the ciliate *C. magnum*, which uses UGA as a homonymous codon decoded as tryptophan or stop in a position-dependent manner, and its genome contains three copies of $tRNA^{Trp}_{CCA}$ with unpinned top AS bp as the sole source of $tRNA^{Trp}$ (Heaphy et al. 2016; Kachale et al. 2023). Therefore, it is tempting to speculate that the S74A substitution is also important for this particular organism to read the in-frame UGA codons only as tryptophan by this specific $tRNA^{Trp}$. However, since unlike *B. nonstop*, *C. magnum* uses UGA also as a stop codon, so the S74A substitution could at the same time interfere with proper UGA recognition at the end of coding sequences.

The situation is even more complicated, as the possibility of the general importance of a natural Ser74 substitution was recently challenged because of the following two examples (Swart et al. 2024). One consists of the ciliate class Karyorelictea, the distantly related ciliate *Plagiopyla frontata* (class Plagiopylea), and the dinoflagellate *Amoebophrya* sp. ex *Karlodinium veneficum*. They also display UGA = W/stop homonymy, but their eRF1 carries either the wild-type Ser74 or a potentially functionally equivalent Cys74 (Bachvaroff 2019; Seah et al. 2022; McGowan et al. 2023). It is important to note, however, that it is not known whether or not $tRNA^{Trp}$ can decode UGA in most, if not all, of these protists. It is therefore possible that they use $tRNA^{Trp}$ with the UCA anticodon fully cognate to UGA, which would serve as a strong competitor for eRF1, thereby obviating the need for S74 substitution with Ala or Gly.

The second example concerns the eukaryotes whose eRF1 carries Ala74 but whose genetic code is perfectly standard or at least with UGA preserved as the stop codon, namely, the ciliates *Fabrea salina* and *Climacostomum virens*, and the diplomonad *Giardia intestinalis* (Slabodnick et al. 2017; Seah et al. 2022). Thus, it is clear that, at least in these organisms, S74 either allows the UGA = W/stop homonymy, or substitution of Ser74 for Ala does not restrict UGA decoding by their eRF1. An open question remains whether or not they bear additional mutations that either functionally mimic the S67A/G replacement and render eRF1 incapable of efficient UGA recognition or compensate for the S67A replacement, thereby maintaining efficient UGA recognition. Collectively, these observations might point to an emerging complex identity pattern within the amino-terminal residues of eRF1, whose specific combinations might be required for efficient versus inefficient UGA decoding.

POSITION-SPECIFIC TERMINATION

Based on our thorough bioinformatic analysis, UAA has been conclusively determined to be the only true stop codon in *B. nonstop* (Kachale et al. 2023). The fact that it serves the dual meaning, as it is decoded as Glu in the coding sequences by $tRNA^{Glu}_{UUA}$ with fully cognate anticodon, its "meaning" is clearly position-dependent. The unanswered questions are as fol-

lows: What drives eRF1 to genuine UAA stop codons and how does eRF1 avoid decoding the in-frame UAA codons specifying Glu? In case of the latter, the obvious factor is the presence of a newly evolved gene expressing sufficiently high levels of $tRNA^{Glu}_{UUA}$ to serve as a strong competitor for eRF1 (Kachale et al. 2023). In case of the former, one possibility could be that some specific sequence signatures occur near genuine stops that specifically increase the termination efficiency at the stop codons in organisms with these codons reassigned to sense codons. However, no such case has been found so far (Heaphy et al. 2016; Swart et al. 2016). Instead, we and others have proposed that the underlying mechanism could rely on the known eRF1–eRF3 interaction with a poly(A)-binding protein (PABP) (Fig. 4C; Heaphy et al. 2016; Záhonová et al. 2016; Alkalaeva and Mikhailova 2017; Kachale et al. 2023). Some of the species where all 64 codons specify amino acids, namely, the ciliates, were shown to possess extremely short 3′ UTRs (20–30 nt long) (Heaphy et al. 2016; Swart et al. 2016), which in principle ensures that the poly(A) tails immediately following these 3′ UTRs are juxtaposed to the terminating ribosomes. Given that poly(A) tails are covered by PABP (Deo et al. 1999) and PABP is an interaction partner of eRF3 (Alkalaeva et al. 2006; Cheng et al. 2009), the PABP–eRF3 partnership could play an instrumental role in promoting the position-specific termination. In essense, eRF1 bound to the eRF3–PABP complex would essentially wait at the right place for the ribosome to reach the genuine stop codon (Fig. 4C). Indeed, it has been shown that PABP enhances the productive binding of the eRF1–eRF3 complex to the ribosome, via it interaction with the amino-terminal domain of eRF3, which in turn increases the termination efficiency (Ivanov et al. 2016). Analogously, the proximity of PABP and poly(A) tails to stop codons and the proximity of the PTCs to poly(A) tails were shown to increase termination efficiency, strongly supporting the earlier postulated "faux 3′ UTR termination" theory (Mangus et al. 2003; Amrani et al. 2004; Wu et al. 2020). With all these measures in place, it is conceivable that the termination activity of even a noncanonical form of eRF1, which might be partially impaired in function, as occurs in some reassigned organisms, would be highly potentiated.

Somewhat inconsistent with this model, we found that the average length of 3′ UTRs (~400 nt) is in the fully reassigned *B. nonstop* similar to that of other trypanosomatids (Kachale et al. 2023). Thus, the poly(A) tails would not be anywhere near the genuine stop codons. However, we also found that the intergenic regions of *B. nonstop* are dramatically AU-rich compared to other trypanosomatids, with A being the most abundant nucleotide in the first ~39 nt after the genuine stop codon, followed by a predominance of U. Since PABP was shown to promiscuously interact with the AU-rich sequences in other eukaryotes (Sladic et al. 2004; Baejen et al. 2014; Kini et al. 2016), robust AU-richness of the sequences following the true stops in *B. nonstop* could serve the same purpose as the short 3′ UTRs in ciliates (i.e., to ensure that PABP presents the eRF1/eRF3 complex directly to the ribosome terminating at the true stop codons).

Position-specific termination could explain the situation in the aforementioned organisms with UGA = W/stop homonymy and wild-type eRF1-Ser74; such a mechanism could prevent eRF1 sampling at the in-frame UGA codons and restrict its action only to genuine UGA stops. Thus, the presence of unmutated Ser74 would be irrelevant for in-frame stops but very critical for UGA termination. On the other hand, in the case of *B. nonstop*, which does not use UGA as a true stop codon and does not have a fully cognate $tRNA^{Trp}$ to UGA, the S74G substitution, along with this specific targeting mechanism, may serve as a fail-safe modus operandi to ensure that the 4-bp-long AS $tRNA^{Trp}$ with its genuine anticodon, and thus much weaker affinity for UGA, eliminates any competition for the in-frame UGA stops.

In any case, the likelihood of the physiological relevance of the proposed PABP–eRF3-driven, position-specific termination mechanism increases with the existence of a similarly operating mechanism. The presence of a specific, so-called SECIS hairpin encompassing the UGA ensures its translations as selenocysteine (Kryukov et al. 2003). Like the binding of PABP

to poly(A) tails, the SECIS element is bound by the SPB2 protein, which brings a selenocysteine-specific translation elongation factor in the complex with GTP and $tRNA^{Sec}$ to UGA in a precisely position-specific manner (Forchhammer et al. 1989; Allmang et al. 2009). While the analogy is obvious, future experiments will show whether this model really manifests itself in these alien organisms.

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