The ribosome epitranscriptome: inert—or a platform for functional plasticity?

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ABSTRACT

A universal property of all rRNAs explored to date is the prevalence of post-transcriptional ("epitranscriptional") modifications, which expand the chemical and topological properties of the four standard nucleosides. Are these modifications an inert, constitutive part of the ribosome? Or could they, in part, also regulate the structure or function of the ribosome? In this review, we summarize emerging evidence that rRNA modifications are more heterogeneous than previously thought, and that they can also vary from one condition to another, such as in the context of a cellular response or a developmental trajectory. We discuss the implications of these results and key open questions on the path toward connecting such heterogeneity with function.

Keywords: epitranscriptome; modifications; rRNA; review; ribosome

Ribosomes are massive macromolecular complexes, often conceptualized as uniform entities and "factories" of protein translation. The assumption that ribosomes are an invariable homogenous entity suggests that they lack an inherent ability of *regulating* the translational output of a cell or organism. In recent years, however, it has become clear that ribosomes are considerably more heterogeneous than had previously been thought. Specifically, it was shown that ribosomes can differ in their primary ribosomal RNA (rRNA) sequence, in the composition of ribosomal proteins, as well as in the post-translational modification profiles of the ribosomal proteins (López-López et al. 2007; Slavov et al. 2015; Brown et al. 2017; Shi et al. 2017; Simsek et al. 2017; Fujii et al. 2018; Małecki et al. 2021); this has been extensively reviewed (Sergiev et al. 2011; Xue and Barna 2012; Kobayashi 2014; Sloan et al. 2016a; Bates et al. 2018; Genuth and Barna 2018a,b; Gerst 2018; Ferretti and Karbstein 2019). An emerging layer of heterogeneity between ribosomes lies in diverse post-transcriptional modifications to which the rRNA is subjected. This layer, and its potential for regulating the functional properties of the ribosome, form the topic of this mini-Review.

rRNA MODIFICATIONS: DISTRIBUTION AND BIOGENESIS

Ribosomes are composed of a highly conserved catalytic rRNA core and dozens of auxiliary proteins. A universal property of all rRNAs explored to date is the prevalence of post-transcriptional ("epitranscriptional") modifications, which expand the chemical and topological properties of the four standard nucleosides (Sharma and Lafontaine 2015; Sloan et al. 2016a). Dozens to hundreds of residues are modified across ribosomes from different domains of life, with *E. coli* rRNA harboring 36 modifications (Golovina et al. 2012), yeast 112 (Taoka et al. 2016), human 228 (Taoka et al. 2018), and the protist *Euglena gracilis* rRNA containing 350 modified sites (Schnare and Gray 2011). A core set of rRNA modifications is conserved across the three domains of life (Sergiev et al. 2018), and these modifications typically cluster around the functional centers of the ribosome (Ben-Shem et al. 2011; Sloan et al. 2016b). There is a remarkable diversity of modifications that adorn the ribosome, including diverse forms of methylation, acetylation, and pseudouridylation. In terms of density of modification patterns, rRNA is second only to tRNA.

rRNA modifications are catalyzed through a diverse set of RNA-modifying enzymes. The vast majority of enzymes directly recognize and modify their targets. In eukaryotes and archaea, a subset of RNA modifying enzymes are recruited to their targets via small nucleolar RNAs (snoRNAs), which harbor stretches of complementarity toward specific rRNA targets and thereby guide the modifying enzyme into place (Kiss-László et al. 1996; Ganot et al. 1997; Ni et al. 1997; Sharma et al. 2017b). The appearance of snoRNAs in evolution correlates with a dramatic

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expansion of rRNA modifications guided by them, primarily pseudouridine and ribose methylations (Lafontaine and Tollervey 1998). This may be due to the relative ease with which snoRNAs can evolve or duplicate and acquire new targets, in comparison to the greater difficulty of an entire enzyme evolving new specificity without compromising its catalytic activity.

rRNA MODIFICATIONS: FUNCTIONS

Although the first rRNA modifications were already discovered six decades ago (Lane and Allen 1961; Lane 2014), and have been subjected to considerable investigation ever since, their functions are understood only to a limited extent. We currently lack a systematic understanding of which properties are conferred via which modifications, and how. Nonetheless, it is becoming increasingly clear that there is no single function of rRNA modifications. This need not come as a surprise, given the highly heterogeneous chemical nature of rRNA modifications, the widely distinct machineries giving rise to their formation, the different regions of the ribosome at which they are catalyzed, the variability in the relative timing of their deposition with respect to ribosome biogenesis and the different cellular compartments at which they are deposited.

Given the paramount importance of the ribosome, the universality of rRNA modifications across all domains of life, their enrichment at functional regions of the ribosome, and their high conservation between very distantly related species, it is to be expected that rRNA modifications would be critical components of cells, and that their absence results in dramatic phenotypes. Indeed, such is the case for a number of modifications, where for example in yeast, absence of a single modifying enzyme installing modifications at one of several sites required for 18S biogenesis is lethal (Lafontaine et al. 1994, 1995; Liang et al. 2009; Schilling et al. 2012; Peifer et al. 2013; Ito et al. 2014; Zorbas et al. 2015). Yet, genetic dissection of roles played by rRNA modifications have consistently given rise to two surprises. First, despite the high conservation of many modifications and associated modifying enzymes, their disruption often results in subtle or even indiscernible phenotypes. For example, elimination of one or even two modifications in helix 69 of the ribosome, which interacts with both A and P site tRNAs, resulted in no discernible phenotype (Liang et al. 2007). Only when three or more modifications were eliminated from this helix, did phenotypes become apparent. In one case, a lethal phenotype caused by deletion of an rRNA modifying enzyme involved in ribosome biogenesis could even be *rescued* by deletion of additional genes involved in rRNA modifications (Buchhaupt et al. 2006, 2007; García-Gómez et al. 2011). A second—related—surprise, consistently manifesting itself in yeast studies, is that catalytically defective rRNA modifying enzymes often give rise to much milder phenotypes than deletions of the entire genes (Lafontaine et al. 1995, 1998; Zebarjadian et al. 1999; Sardana and Johnson 2012; Peifer et al. 2013; Sharma et al. 2013a; Gigova et al. 2014; Zorbas et al. 2015; Liger et al. 2016; Shen et al. 2020). These studies suggest that in many cases rRNA modifications are dispensable (or partially so), whereas the rRNA modifying enzyme is not.

These insights from genetic studies coupled with extensive follow-ups have given rise to two broad roles of ribosome modifying enzymes—modification dependent functions and modification independent ones. A first set of roles, which is often at least partially modification independent, is in facilitating the multistep rRNA maturation. rRNA is typically transcribed as a single precursor, which is subsequently subject to complex exo- and endonucleolytic cleavage events, giving rise to the large and small rRNA subunits that concomitantly need to fold and assemble into their proper structure (Demirci et al. 2010; Polikanov et al. 2015; Jiang et al. 2016; Sloan et al. 2016a; Aubert et al. 2018; Birkedal et al. 2020). Loss-of-function assays have revealed that some rRNA modifying enzymes are required for this processing (Lafontaine et al. 1995; Liang et al. 2009; Sharma et al. 2013b) and their loss results in accumulation of ribosome precursors. The fact that rRNA modifying enzymes, but often not the modifications themselves, are required for mediating this role suggests that in these contexts the enzymes serve a scaffolding or chaperoning function, assisting in the proper folding of the ribosome (Lafontaine et al. 1995; Shen et al. 2020). Indeed, a general chaperoning function was recently also proposed for snoRNAs (Huang and Karbstein 2021). Such a modification-independent function of RNA modifying enzymes is reminiscent of modification-independent chaperone-like roles for tRNA modifying enzymes. Among the best characterized examples for this is TruB, a highly conserved tRNA pseudouridine synthase, installing pseudouridine at position 55 of tRNA. Remarkably, studies in bacteria found that the catalytic activity of this enzyme is dispensable for bacterial fitness, whereas the RNA-binding domain of this enzyme is essential for proper folding and aminoacylation of the tRNA (Gutgsell et al. 2000; Keffer-Wilkes et al. 2016), strongly suggesting that the primary function of this enzyme is to chaperone the folding of the tRNA. A second set of roles, dependent on the modifications, is in facilitating RNA:RNA or RNA:protein contacts between the key components of the ribosome—rRNA, tRNA, mRNA, and proteins (Sergiev et al. 2011; Polikanov et al. 2015; Sharma and Lafontaine 2015; Sas-Chen et al. 2020). In principle, both modification-dependent and independent roles can manifest in the broad range of functional outcomes associated with disruption of diverse rRNA modifying enzymes, including aberrant assembly, aberrant structures, aberrant translational activity, reduced translational output, reduced amino acid incorporations, increased stop codon readthrough, and modulation of frameshift rate (King et al. 2003; Liang et al. 2007, 2009;

Baudin-Baillieu et al. 2009; Sloan et al. 2016a; Sergiev et al. 2018). Yet, due to the subtlety of the phenotypes and the difficulties in dissecting them, the functions of most modifications remain to a large extent elusive (Lafontaine et al. 1995; Phillips and de Crécy-Lagard 2011; Spenkuch et al. 2014; Sharma and Lafontaine 2015; Popis et al. 2016; Ayadi et al. 2019).

HETEROGENEOUS AND DYNAMIC rRNA MODIFICATIONS

In recent years, compelling evidence has accumulated for heterogeneity in ribosome composition at various levels. In zebrafish, ribosomes are expressed from two separate genomic loci, differing significantly in sequence, whereby one locus serves for transcription of maternal rRNA and the other for zygotic rRNA (Locati et al. 2017). Similarly, in *Plasmodium falciparum*, different diverging copies of rRNA are encoded, one of which is utilized during the mosquito-stage and another during the human-stage of the infection (Rogers et al. 1996; Vembar et al. 2016). At the level of protein composition, compelling evidence has emerged that ribosomes lacking specific ribosomal proteins exist within mouse embryonic stem cells (Slavov et al. 2015; Shi et al. 2017), yeast (Ferretti et al. 2017; Collins et al. 2018; Samir et al. 2018) and bacteria (Loveland et al. 2016), though questions remain as to the functionality of such ribosomes. Moreover, there are numerous amino acids within ribosomal proteins that accomodate post-translational modifications, affecting stability, structure, localization, and function (Hornbeck et al. 2015; Simsek and Barna 2017; Emmott et al. 2019; Li and Wang 2020). Many post-translational modifications are heterogeneous, and are responsive to stimuli like growth signals (Imami et al. 2018), and immune response (Mukhopadhyay et al. 2008), often leading to preferential translation of mRNAs. Protein and rRNA paralogs provide yet another source of heterogeneity, where gene duplication has resulted in very similar or identical isoforms but expressed from different genomic loci (Gerst 2018; Segev and Gerst 2018; Nurk et al. 2021).

These discoveries, combined with advances in genomic and mass-spectrometry based approaches for systematically measuring RNA modifications, have spurred explorations into the extent of heterogeneity of rRNA modifications. Such heterogeneity is of interest at two levels: First, how heterogeneous is an rRNA modification *within* a specific condition, that is, what is the stoichiometry of that modification in the ribosomes. Second, how heterogeneous is an rRNA modification *between* samples, for example, across different conditions, stimuli or pathological states. These two levels are not completely unrelated: sites that are substoichiometric within a sample also tend to change across samples (Sharma and Lafontaine 2015; Ayadi et al. 2019). To date, both dimensions have been sampled relatively sparsely, and our knowledge is hence partial at best. Below we review some of the key themes that have been uncovered to date.

1. **Less than half of modified sites in yeast and human ribosomes are substoichiometric:** To date, most studies have focused primarily on yeast and human ribosomes. Quantitative mass-spectrometry based measurements revealed that in yeast, 12 (of 112) sites have stoichiometries ranging from 50%–80% and 28 sites from 80%–95%. In addition, one site (25S:U2345) can be either modified with a ribose methylation or with a pseudouridine or both (Taoka et al. 2016), highlighting that heterogeneity can stem from a single position harboring multiple modification types. In human the heterogeneity is even more dramatic, whereby almost half the sites are modified at substoichiometric levels: 22 (of 228) sites are modified at levels ranging from 5%–49%, 23 sites from 50%–79%, and 64 sites falling in the range of 80%–95% (Taoka et al. 2018). Similar findings, pertaining to a subset of sites exhibiting substoichiometric pseudouridines and 2′-O methylation sites were found by later studies (Sharma et al. 2017a; Marchand et al. 2020).
2. **A minority of modified sites exhibit changes in modification levels across different conditions in human and yeast:** Variability in rRNA modification levels across conditions have been observed, to date, in a minority of instances in human and yeast. In a study in *S. pombe*, modification stoichiometries were sampled via mass-spectrometry across a range of different temperatures, and of the 40 sites that could be quantified across all temperatures, six sites displayed a >20% difference in stoichiometries across conditions, all of which harbor pseudouridines (Taoka et al. 2015). An analysis of pseudouridine on rRNA during human chondrogenic differentiation revealed that a small subset of sites displayed relatively moderate changes in stoichiometry over the course of this process. The same study reported that typically subtle changes in pseudouridylation levels were present across many sites, when comparing fibroblasts, HEK293, and HeLa cells (Marchand et al. 2020). Another recent study observed a change in pseudouridylation levels of one site during stem cell differentiation, corresponding to induced levels of the snoRNA targeting it (McCann et al. 2020). Differences in ribose methylation levels were also observed in p53 knockout models, whereupon 13 (of 106) sites in human were hypomodified in comparison to WT (Sharma et al. 2017a). Furthermore, it was recently observed that many types of cancer show substoichiometric levels of the highly conserved hypermodified base m1acp3Y (Babaian et al. 2020). Finally, it was found that the universally conserved tandem adenosines at the 3′ end

of 18S rRNA, thought to be constitutively di-methylated (m6,6A), can also be mono-methylated (m6A), and that the extent of monomethylation versus dimethylation is regulated by sulfur levels (Liu et al. 2021).

3. **The majority of substoichiometric and "dynamic" sites are ribose methylations and pseudouridines:** A shared finding in many of the above studies is that the majority of sites reported to be either "substoichiometric" or "dynamically regulated" in human and yeast are modified either with pseudouridine or with 2′-*O*-methylation. To some extent, this mirrors the relative abundance of these modifications, and that the techniques used by some studies were directed exclusively against these modifications and hence blind to all others. Nonetheless, this conclusion is also based on mass-spectrometry based approaches, which do not suffer from these limitations, and hence may suggest that these two modifications may be inherently less "constitutive" in human and yeast. Given that these two modifications are both guided by snoRNAs, it is tempting to speculate that the substoichiometric modifications associated with a subset of them may reflect this snoRNA-mediated biogenesis. By separating the catalytic from the targeting machinery, individual snoRNAs may have acquired more flexibility in evolving optimal affinities toward their targets (whereby "optimal" can, at times, also be substoichiometric) and may have attained more freedom in evolving optimal expression levels for their individual targets. With respect to the latter, snoRNAs certainly provide a *potential* platform via which rRNA modifications could be controlled, given that dramatic variations in snoRNA levels have been observed between different tissues, stimuli and disease states (Jorjani et al. 2016; Gong et al. 2017; Warner et al. 2018; McCann et al. 2020). However, only few studies have linked such heterogeneous expression with differences in modification levels (Khoshnevis et al. 2019; McCann et al. 2020). This notwithstanding, additional mechanisms for achieving substoichiometric levels of these modifications have been documented. Depletion of fibrillarin—the rRNA ribose methyltransferase—impacts methylation of different sites in varying ways, establishing that alterations of fibrillarin levels could serve as a potential mechanism for achieving heterogeneous levels of methylation across sites (Erales et al. 2017; Sharma et al. 2017a). It was also shown that methylation at a subset of sites requires the nucleolar protein Nucleophosmin (NPM1), and hence disruption of NPM1, as occurs in dyskeratosis congenita, a rare bone marrow disease, can lead to substoichiometric modification of its targets (Nachmani et al. 2019). Heterogeneity in rRNA modifications was also shown to be associated with the relative timing at which a modification is deposited, with modifications arriving late in ribosome biogenesis being more prone to substoichiometric levels (Birkedal et al. 2015). In this context, it is important to rule out that the observed heterogeneity is present in mature ribosomes and does not merely reflect the relative composition of mature and immature ribosomes in a sample.
4. **Two examples for dramatic and systematic changes in rRNA modification levels:** As indicated above, in human and yeast, differences in modification levels of rRNA across conditions are relatively rare and often subtle. Two cases have been reported, to date, in which rRNA modifications are dramatically and systematically altered in response to an environmental cue. *Trypanosoma brucei*, the parasitic kinetoplastid responsible for widespread sleeping sickness in sub-Saharan Africa, was found to have life-cycle dependent rRNA pseudouridylation patterns. In total, 68 pseudouridine modifications were identified on rRNA in both the procyclic and bloodstream forms, and 21 of these sites were hypermodified (>1.3-fold) during the bloodstream form, during which the corresponding H/ACA snoRNAs were also induced (Chikne et al. 2016). It is thought that this is a developmentally regulated adaptation resulting from the 10°C difference between the tsetse fly vector and human host, and interestingly enough the sites cluster around functional domains of the ribosome. The second case, discovered in our laboratory, is a dramatic induction of cytidine acetylation (ac4C) in ribosomes of an archaeal hyperthermophile in response to increased growth temperatures. *T. kodakarensis* ribosomes grown under 55°C are modified at only seven sites whereas under optimal growth conditions of 85°C the ribosomes undergo acetylation at >170 sites. Consistently, loss of the single acetyltransferase enzyme, required for acetylation of all sites, led to growth defects at higher—but not at lower—temperatures. Acetylation at a minority of the target sites contributed to RNA:RNA and RNA:protein interactions, or to interactions with solvents, whereas the vast majority of sites were proposed to contribute to thermostabilization of the RNA structure at higher temperature (Sas-Chen et al. 2020). Common to both cases is that a single modification was found to be dramatically and systematically induced across the majority of harboring sites.

HORIZONS

Our understanding pertaining to rRNA modifications is by and large limited to few modifications, few species, and few conditions. Many fundamental questions thus remain wide open. Key questions include the following:

1. **Extent of heterogeneity:** To what extent are substoichiometric modifications an exception or a rule? And

to what extent are dynamically modulated rRNA modifications an exception or a rule? How abundant are these phenomena across evolution? And how abundant are they across different tissues or across physiological and pathological responses? We anticipate that exploration of these dimensions will be hugely facilitated by the ever-expanding arsenal of genomic methodologies for assaying distinct modifications (Schaefer et al. 2009; Meyer et al. 2012; Ryvkin et al. 2013; Carlile et al. 2014; Schwartz et al. 2014a; Birkedal et al. 2015; Hauenschild et al. 2015; Linder et al. 2015; Zheng et al. 2015; Marchand et al. 2016; Dai et al. 2017; Li et al. 2017; Safra et al. 2017; Marchand et al. 2018; Enroth et al. 2019; Lin et al. 2019; Pandolfini et al. 2019; Zhang et al. 2019; Sas-Chen et al. 2020).

2. **Rules of heterogeneity:** Can rules be defined pertaining to which modifications are prone to be substoichiometric or regulated, versus constitutive? Are certain modifications more prone to be so than others? Might modifications catalyzed by snoRNAs be more heterogeneous than ones catalyzed by site specific enzymes? Are modifications within certain domains of the ribosome more prone to be regulated or substoichiometric? In our studies into dynamic ac4C in archaea, we found, for example, that a small subset of sites clustered around functional regions of the ribosome were invariably acetylated across all temperatures, typically at relatively high stoichiometries, whereas the vast majority of the remaining sites were distributed randomly throughout the ribosome and only catalyzed, typically at lower stoichiometries, under higher temperatures (Sas-Chen et al. 2020). Similarly, in an analysis of substoichiometric 2′-O methylated sites it was found that nucleotides participating directly in translation at the A- and P-sites, intersubunit bridges, and peptide exit tunnel were susceptible to variation in methylation, while the peptidyl transferase center and decoding center were not affected (Erales et al. 2017; Sharma et al. 2017a). These findings suggest that there may be a structurally coherent set of constitutive modifications.
3. **Sources of heterogeneity:** What regulates heterogeneity in rRNA modifications? Why are some sites substoichiometric? What gives rise to changes in rRNA modifications across different conditions? Is this due to changes in the levels of modifying enzymes? Or in their activity? Or also due to variability in the accessibility of the rRNA between conditions? In assessing these questions it will be critical to consider the populations of ribosomes in which such heterogeneity is observed. Is heterogeneity observed in total RNA? Or in rRNA purified from polysomes? In the case of the former, such heterogeneity can potentially also reflect a mixture of mature and immature ribosomes, modified at varying levels.
4. **Consequences of heterogeneity:** Arguably the most important question is whether differentially modified ribosomes give rise to differential functions. Such functionality should ideally be established at multiple levels, among which are the structural level (how does the modification impact rRNA structure), the molecular level (how does the modification impact the catalytic properties of the ribosome) and the phenotypic level (what fitness benefit is provided by the modification), with the ultimate, highly challenging goal of drawing a causal, connecting line between these three layers. The challenges of drawing such a causal line from heterogeneity to function is by no means unique to rRNA modifications. Indeed, in the vast majority of cases in which ribosome heterogeneity has been unequivocally observed, directly linking such heterogeneity to a function has proven to be challenging (for review, see Ferretti and Karbstein 2019). Such difficulties are a consequence of the complex nature of the ribosome and of its processing and assembly pathways, rendering it highly challenging to address functions in vitro, but also limiting the conclusions that can be drawn from in vivo studies. One powerful game-changer in recent years are the major leaps in cryoEM (Kirmizialtin et al. 2015; Shalev-Benami et al. 2016; Natchiar et al. 2017; Nikolay et al. 2021), permitting the relatively rapid acquisition of ribosome structures at low Å resolutions.
5. **Evolution of heterogeneity:** Finally, a fascinating dimension to explore is how rRNA modifications in general, and heterogenous modifications in particular, evolved over the course of evolution. How did they emerge? How did the machineries regulating them emerge? From an evolutionary perspective, are substoichiometric or dynamic modifications relatively recently acquired sets of modifications that have not yet undergone fixation? Or, in contrast, did they originate from evolutionary-fixed stoichiometric modifications, and evolve to become heterogeneous over time? A comprehensive dissection of these questions will require measurements of rRNA modifications across a wide set of species. Such endeavors, conducted via cryoEM and mass-spectrometry based approaches (Taoka et al. 2016, 2018), are likely to also give rise to discoveries of new rRNA modifications, of which our knowledge is likely still incomplete, as suggested by the continuous discovery of new forms of modifications (Boccaletto et al. 2018; Flynn et al. 2021).

From a historical perspective, it is surprising that investigations into substoichiometric and dynamically modified modifications on ribosomes are somewhat lagging behind with respect to their counterparts on mRNA (Schwartz et al. 2014b; Darnell et al. 2018), given that modifications on rRNA were discovered and explored decades before their

mRNA counterparts. To some extent, this may reflect the different disciplines, and associated philosophies, through which these modifications were studied: rRNA modifications were classically studied through the lens of structure and of post-transcriptional processing, whereas mRNA modifications—coined "RNA epigenetics" (He 2010) and the "epitranscriptome" (Meyer et al. 2012)—were intuitively connected with "epigenetics," wherein key components are dynamics and reversibility. We anticipate that new insights into ribosome heterogeneity in recent and forthcoming years, combined with the advent of new tools for systematically interrogating rRNA modifications, will allow revisiting this exciting field, pertaining to the regulatory potential of the core translational apparatus.

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