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PERSPECTIVE

A deep dive into functional ribosome specialization

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Ribosome specialization, whereby ribosomes of distinct composition translate different sets of mRNAs, is a concept that has garnered both wide-spread excitement and skepticism from the translation field. The controversy is rooted in experimental challenges, which make rigorous controls difficult and not obvious to the nonexpert. In addition, considerations of translation mechanisms and ribosome homeostasis also suggest heterogeneity to be limited, fueling doubt. Lastly, the mechanisms by which heterogeneity can lead to specialization are often challenging to imagine and not spelled out. In this perspective, we define ribosome heterogeneity and specialization, use examples to examine both the technical challenges and potential solutions. We then consider the mechanism-based challenges with the goal of proposing biologically relevant circumstances where ribosome specialization might exist and how it might affect translation in an mRNA-specific manner. It is hoped that this article will help resolve the controversy around the subject, in addition to providing a guide for scientists entering the field, so they can concentrate their efforts fruitfully and rigorously.

Introduction

In all cells, ribosomes translate mRNAs to synthesize protein. Moreover, cells utilize the signal from stalled translation complexes to detect damaged mRNA, altered metabolic states, or other stresses (D'Orazio and Green, 2021; Filbeck et al., 2022; Kim and Zaher, 2022; Meydan and Guydosh, 2021). While it was originally envisioned that ribosomes, composed of both ribosomal RNA (rRNA) and ribosomal proteins (RPs), were translating each mRNA uniformly, it has become clear that different mRNAs are translated with different efficiencies (Ingolia et al., 2009; Kozak, 1984; Kozak, 1986). Ribosome heterogeneity, whereby different ribosomes have different compositions, has been described using mass spectrometry, RNA-sequencing approaches, and cryo-EM. This led to the idea that the observed heterogeneity might have functional consequences, whereby different ribosomes specialize in the translation of different mRNAs (reviewed by Barna et al. [2022]; Emmott et al. [2019]; Ferretti and Karbstein [2019]; Gay et al. [2022]; Genuth and Barna [2018]; Joo et al. [2022]; Li and Wang [2020]; Martinez-Seidel et al. [2020]; Mauro and Edelman [2002]; Murphy et al. [2023]; Norris et al. [2021]; Simsek and Barna [2017]; Sloan et al. [2017]; Xue and Barna [2012]).

Here we will explain the terms "ribosome heterogeneity" and "ribosome specialization" based on a brief review of sources for heterogeneity and using examples from the literature. Next, we examine the properties of ribosomes and translation mechanisms that are likely to limit the occurrence of ribosome specialization. Using these concepts, we will then infer biological circumstances where these obstacles to ribosome heterogeneity might not be present and where one might thereby uncover functional specialization. Finally, we review examples from the literature and first-principle considerations to suggest mechanisms that can explain how ribosome heterogeneity can lead to functional specialization that results in mRNA-specific differences in protein production. Of note, we do not aim for this perspective to be a comprehensive review of ribosome heterogeneity and specialization (which have been expertly and recently reviewed [Aspden et al., 2025; Barna et al., 2022; Emmott et al., 2019; Ferretti and Karbstein, 2019; Gay et al., 2022; Genuth and Barna, 2018; Joo et al., 2022; Kyei-Baffour et al., 2025a; Kyei-Baffour et al., 2025b; Li and Wang, 2020; Martinez-Seidel et al., 2020; Mauro and Edelman, 2002; Murphy et al., 2023; Norris et al., 2021; Simsek and Barna, 2017; Sloan et al., 2017; Xue and Barna, 2012]) but rather use published examples to illustrate experimental hurdles in the field and the controls that can address these, as well as some suggestions for deeper study. It is hoped that this perspective will provide a useful guide for new scientists entering the field with the goal of uncovering specialization.

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Sources and definitions of ribosome heterogeneity and specialization

Ribosome components and sources of heterogeneity

The ribosomal core is conserved from bacteria to humans (Melnikov et al., 2012). Ribosomes are composed of three (in bacteria) or four (in eukaryotes[1]) rRNAs and 54 (in bacteria) or 79 (in eukaryotes) RPs (Yusupova and Yusupov, 2014). rRNAs are co-transcribed in operons that include either all rRNAs (bacteria) or all rRNAs, except 5S rRNA (in eukaryotes), which may help in ensuring that rRNAs are produced in equal numbers. Interestingly, the number of these operons varies from a few (in bacteria) to hundreds (in eukaryotes), where they form tandem repeats either on the same or multiple chromosomes (Hori et al., 2023). While the repetitive nature of the rDNA repeat has until very recently precluded its sequencing in eukaryotes, small differences between the operons in bacteria have been described (Kurylo et al., 2018; Song et al., 2019). Similarly, recent rRNA and rDNA sequencing has uncovered sequence variation in eukaryotic rDNAs (Fan et al., 2022; Nurk et al., 2022; Parks et al., 2018; Rothschild et al., 2024). Additionally, rRNAs are elaborated with modifications both on the phosphate backbone and the bases (Georgeson and Schwartz, 2021; Sharma and Lafontaine, 2015; Sloan et al., 2017). In eukaryotes, most of these modifications are directed by two classes of small nucleolar RNAs (snoRNAs), which specify the site of modification and recruit proteins to carry out the chemical transformations (Kiss, 2002; Webster and Ghalei, 2023). In addition, a few base modifications are carried out by stand-alone enzymes (Sharma and Lafontaine, 2015). These modifications are thought to be irreversible, as no enzymes are known that can remove them. Thus, removing a modification requires degrading the old ribosome and making a new ribosome.

Similar to rRNAs, many eukaryotic organisms also encode multiple paralogous copies of RPs, which tend to be very similar or even identical. E.g., many RPs in *Saccharomyces cerevisiae* are encoded by two genes. In human cells, a few RPs have paralogs, which can have tissue-specific expression (Grimes et al., 2023; Hopes et al., 2022; Jiang et al., 2017; Li et al., 2022; Milenkovic et al., 2023; Shiraishi et al., 2023; Wong et al., 2014; Xu et al., 2023) (reviewed by Martinez-Seidel et al. [2020]; Norris et al. [2021]). This variation is more extreme in plants where many RPs have numerous paralogs (Martinez-Seidel et al., 2020). Finally, functional posttranslational modifications have also been described for several RPs (Simsek and Barna, 2017), including histidine methylation of uL3/Rpl3 (Malecki et al., 2021; Matsuura-Suzuki et al., 2022; Webb et al., 2010) and proline hydroxylation of uS12/Rps23 (Singleton et al., 2014).

Importantly, as has been extensively reviewed, there is evidence (from mass spectrometry, RNA sequencing, and cryo-EM) for heterogeneity arising from each of these sources.

While ribosome-associated proteins can further modulate the function of the ribosome in an mRNA-specific manner, for the purpose of this discussion, we are ignoring such additional functionalization, instead focusing on heterogeneity that is intrinsic to the ribosome itself. We think this is justified not just to limit the scope of the perspective but also because ribosome-associated factors display different biochemical behavior, as they dissociate from the ribosome at moderately high salt, while ribosome components are salt-stable (Acker et al., 2007). Nonetheless, many of the points raised here will apply similarly to ribosome-associated proteins.

What is a standard ribosome?

To discuss ribosome heterogeneity and specialization across many genetic backgrounds and physiological or pathological situations, we want to first consider what the "standard" ribosome looks like. Mass spectrometry and structural studies show that the vast majority of ribosomes contain all RPs. Consistently, most RPs are essential (Steffen et al., 2012). In contrast, Asc1, eS7, eS12, eS25, eS31, eL12, eL22, eL24, uL24(Rpl26), eL29, eL31, eL38, eL39, eL41, Rpp1, and Rpp2 are nonessential in yeast, but deletion of both copies of eS7, eS12, eS31, eL12, eL31, Rpp1, and Rpp2 causes severe growth defects that cells resolve by rapidly acquiring suppressor mutations (Steffen et al., 2012). Similarly, most rRNA modifications are present in over 90% of the ribosomes (Marchand et al., 2016; Marchand et al., 2020; Krogh et al., 2016). Thus, it would be most intuitive that a ribosome containing all RPs and all the modifications encoded in an organism be the standard ribosome. In this definition, heterogeneity arises if a component (or modification) is lacking or a different rRNA or RP paralog is incorporated.[2]

There are instances where in "normal" conditions, individual modifications or RPs seem to be missing, which are then "induced" under specific physiologic conditions, apparently providing evidence that the standard ribosome is not so standard (Dopler et al., 2024; Ishiguro et al., 2025). We will discuss these in more detail below but suggest that some of these discrepancies might arise from laboratory conditions (e.g., rich media, aeration etc.) that are standard for use, but not reflective of the evolutionary pressures that the organisms evolved under.

What are ribosome heterogeneity and ribosome specialization?

Given that the standard ribosome in eukaryotes is made up of ~80 components, and that many of those are encoded by multiple nonidentical genes, and given that a single cell has hundreds of thousands (yeast) to millions (humans) of ribosomes, it is conceivable that individual ribosomes within a cell differ in their composition—whether it be their constituent rRNA isoforms, RPs and their paralogs, or modifications. This is referred to as ribosome heterogeneity. Indeed, there is now extensive evidence for such heterogeneity.

In a related scenario, if a modification enzyme or snoRNA, or even an RP paralog, is encoded in an organism, but is not ubiquitously expressed across all tissues, leading to the loss of that modification (or RP isoform) in some tissues, it is considered a

[1]Note that 5.8S rRNA, the fourth rRNA in eukaryotes, is fused to 23S rRNA in bacteria. Thus, eukaryotes have split the 23S rRNA into two molecules, leading to the existence of 4 rather than 3 rRNAs in eukaryotes.

[2]While this definition is intuitive for RPs, as these are universally conserved (within eukaryotes or bacteria, respectively), things are more complicated for snoRNA-derived modifications, for which there are more in higher eukaryotes. In these cases, a reasonable alternative for a standard definition would be the conserved subset. For our discussion, this does not matter as the critical point is that changing the complement of modifications requires making a new ribosome and degrading the old one.

tissue-specific ribosome. Again, there is now extensive evidence for such tissue-specific differences (Grobicki et al., 2025; Häfner et al., 2023; Hopes et al., 2022; Jiang et al., 2017; Li et al., 2022; Martinez-Seidel et al., 2020; Milenkovic et al., 2023; Norris et al., 2021; Shiraishi et al., 2023; Wong et al., 2014; Xu et al., 2023). These tissue-specific differences in ribosome composition might not reflect heterogeneity within cells, but between cells.

Ribosome specialization describes the idea that ribosomes with different compositions have different biochemical properties and abilities, which prime them for translation of different mRNAs. Thus, depletion of the ribosome subpopulation with the specialization would only, or preferentially, affect the translation of a subset of mRNAs. At its extreme, each mRNA could require a slightly different ribosome for its translation, as originally proposed by Crick (Crick, 1958) and then refuted experimentally by Brenner (Brenner et al., 1961).

Notably, the literature describes many pathologic conditions, collectively referred to as ribosomopathies, either caused by genetic defects or by somatically acquired differences, including from aneuploidy, in cancer cells. These deplete ribosomes but may also result in heterogeneity via the formation of ribosomes lacking a component (e.g., a RP or a modification) (Ajore et al., 2017; Blomqvist et al., 2023a; Dreggors-Walker et al., 2022; Guimaraes and Zavolan, 2016; Jansson et al., 2021; Khoshnevis et al., 2019; Kondrashov et al., 2011; Kulkarni et al., 2017; Zhou et al., 2023). In some instances, there is evidence that these changes differentially affect mRNAs translation (Ferretti et al., 2017; Jansson et al., 2021) or lead to fidelity defects (Collins et al., 2018; Khoshnevis et al., 2022; Parker et al., 2019). Ribosomes with altered RP stoichiometry might lack all function and be degraded, reducing ribosome numbers. Alternatively, they might retain partial functionality on a subset of mRNAs. Importantly, both scenarios, reduced ribosome numbers and altered ribosome composition, can affect translation in an mRNA-specific manner (Cheng et al., 2019; Ferretti et al., 2018; Ferretti et al., 2017; Ferretti and Karbstein, 2019; Gaikwad et al., 2021; Ivanov et al., 2022; Jha et al., 2021; Khajuria et al., 2018; Lodish, 1974; Luan et al., 2022; McNutt et al., 2023; Mills and Green, 2017).

We consider this form of "specialization" as defective ribosomes because the ribosomes are (1) missing a (typically essential) component present in most ribosomes across cells, tissues, and organisms; (2) because the missing component leads to a disease condition, rather than being a beneficial physiological response for the cell; and (3) because these ribosomes arise from genetic defects that enable bypass of quality control or overwhelm it (reviewed by Parker and Karbstein [2023]), rather than in response to a physiological cue. Defining residual activity (if any) of these defective ribosomes is nonetheless important, as they might alter protein homeostasis promoting the cancer phenotype that generates them in a targetable manner. Additionally, such preferentially translated proteins might serve as useful diagnostic tools.

Examples of ribosome heterogeneity and specialization

We will use examples from the literature that illustrate the challenges and important controls for the study of ribosome heterogeneity.

Examples of nonfunctional ribosome heterogeneity

As described above, rRNAs are transcribed from multiple loci, which in eukaryotes encode hundreds of rDNA genes. These can expand and contract and are generally kept rather homogeneous within one locus (Hori et al., 2023). Nonetheless, differences between rDNA sequences have been described using innovative sequencing technologies (Kurylo et al., 2018; Parks et al., 2018; Rothschild et al., 2024). Most of these variants are expressed at very low levels (<1% of rRNA molecules), indicating that either they arise from a single mutated rDNA repeat (1/~400 genes in humans: ~0.25%), or that their expression is actively suppressed, or that they are degraded. Moreover, mutations are not generally conserved between individuals (Rothschild et al., 2024) (at least for the single nucleotide variations [SNV], which occur less frequently), consistent with random mutations, rather than evolution toward functionality. Furthermore, of the 28 SNVs that occur in >60% of all sequenced individuals (and thus could be considered conserved), 24 are in regions of the rRNA that are disordered in cryo-EM structures (Table 1), indicating their structural flexibility. We suggest that the flexibility observed in cryo-EM structures is likely to reflect a lack of sensitivity towards mutations in that region, making it less likely that they will lead to functional alterations. The remaining four SNVs that are conserved in >60% of sequenced individuals are the C1597G and U1615C mutations in 18S rRNA and G60A and C4913U in 28S rRNA (Rothschild et al., 2024). However, we note that the low SNV frequency of 0.4 and 0.5% for the two 18S mutations suggests that they arise from mutation of one or two rDNA genes (out of ~400). Thus, it appears likely that the majority of the SNVs observed in rDNAs represent random mutations without functional roles.

In contrast, the G60A and C4913U mutations are present in ~38 and 18% of all 28S rRNAs (Table 1). Whether these lead to any changes in ribosome function has not been tested but would be exciting to investigate and thus may or may not be examples of functional heterogeneity.

Examples of functional ribosome specialization

One example for functional specialization is the translation of virulence genes in the human pathogen *Vibrio vulnificus*, which requires the expression of the minor I-rRNA operon (Song et al., 2019) (Fig. 1 A). Ribo-seq and proteomic data have identified genes whose expression requires the I-rRNA operon and shown that translational effects are specific for the I-rRNA operon and not caused by loss of ribosomes. These genes include HspA, a heat shock factor, and triose-phosphate isomerase. Consistently, the I-rRNA deletion strains, but not control strains, are sensitive to heat shock, unable to grow on glycerol, and have impaired virulence. Moreover, functional data have identified the regions in the rRNA and in the mRNAs, respectively, that confer specificity for I-rRNA and these mRNAs, demonstrating functional and phenotypic rescue. Thus, the work combines multiple approaches (RNA-seq, biochemistry, reporter assays, and mutational and physiological analyses), controls for the effect from ribosome numbers and differences in expression levels and provides clear evidence for physiological roles. Surprisingly, there is no evidence for upregulation of I-rRNA upon infection,

Table 1. **Variant residues in human rDNA conserved between individuals**

rRNA	Residue	Ref	SNV	% of individuals with SNV	Average SNV frequency	Location	Sequence in chrUN_GL000220v1
18S	699	C	G	60	0.004	Disordered	
18S	1,597	C	G	67	0.004	**Head/h41**	
18S	1,615	T	C	73	0.005	**Head/h42**	
28S	60	G	A	100	0.380	**j11/12**	
28S	628	C	G	73	0.009	Disordered	
28S	761	G	C	60	0.006	Disordered	
28S	762	C	G	93	0.013	Disordered	
28S	812	G	C	83	0.011	Disordered	
28S	862	C	T	83	0.021	Disordered	
28S	865	C	T	77	0.060	Disordered	
28S	868	T	C	97	0.069	Disordered	
28S	871	C	T	77	0.025	Disordered	
28S	874	C	T	67	0.009	Disordered	
28S	879	G	T	73	0.107	Disordered	
28S	883	C	G	80	0.085	Disordered	
28S	2,176	G	T	100	0.097	Disordered	
28S	2,189	C	T	100	0.039	Disordered	T
28S	2,194	G	C	93	0.017	Disordered	C
28S	2,195	C	G	67	0.009	Disordered	G
28S	3,040	A	G	100	0.300	Disordered	C
28S	3,338	A	C	97	0.016	Disordered	C
28S	3,349	A	G	67	0.067	Disordered	C
28S	3,455	G	C	87	0.010	Disordered	C
28S	3,513	G	A	100	0.391	Disordered	
28S	4,804	G	C	80	0.008	Disordered	C
28S	4,805	C	G	60	0.007	Disordered	
28S	4,817	A	G	80	0.082	Disordered	C
28S	4,913	C	T	100	0.180	**ES39B**	G

Data are reanalyzed from Rothschild et al. (2024). A total of 185 SNV are observed and this Table contains the 28 SNV conserved in at least 60% of the 30 individuals from the 1,000 genome project (Byrska-Bishop et al., 2022) analyzed by Rothschild et al. (2024). Note that the rDNA numbering in Ref (Rothschild et al., 2024) starts with "0" and is thus shifted by one nucleotide, which is corrected here. If the residue in the chrUN_GL000220v1 reference sequence differs from the reported reference sequence, it is notated in the last column.
ES39B: extension segment 39B; h41: helix 41; j11/12: junction between helices 11 and 12; Ref: Reference; SSU: small (ribosomal) subunit. Residues in bold are not disordered and discussed in the text.

consistent with the metabolic roles the proteins translated by these ribosomes play even outside a host.

A second example is the chaperone-mediated release of eS26/Rps26 from ribosomes when *S. cerevisiae* are exposed to high osmolarity or high pH stress (Ferretti et al., 2017; Yang and Karbstein, 2022) (Fig. 1 B). Reporter assays have shown that the resulting eS26-deficient ribosomes have lost preference for adenosine residues at position −4 of the Kozak sequence, just upstream of the start codon, consistent with a contact of eS26 with the −4 residue (Ferretti et al., 2018; Ferretti et al., 2017; Yang and Karbstein, 2022). Importantly, these functional effects are observed both when eS26 (but not a control RP from the same subunit) is genetically depleted or when it is released via high salt stress. Moreover, purification of eS26-containing and -deficient ribosomes from the same cells, followed by RNA sequencing, shows that these enrich different subsets of mRNAs. For eS26-deficient ribosomes these include mRNA encoding components from the Hog1 (high osmolarity glycerol) and Rim101 (pH response) pathways. Consistently, eS26-deficient yeast (but not control strains where another RP is depleted) demonstrate upregulated activation of these pathways, and are resistant to high salt and high pH, but not control stresses (Ferretti et al., 2017). Finally, mutation of the −4 residue can also program the sensitivity to eS26-depletion, producing predicted

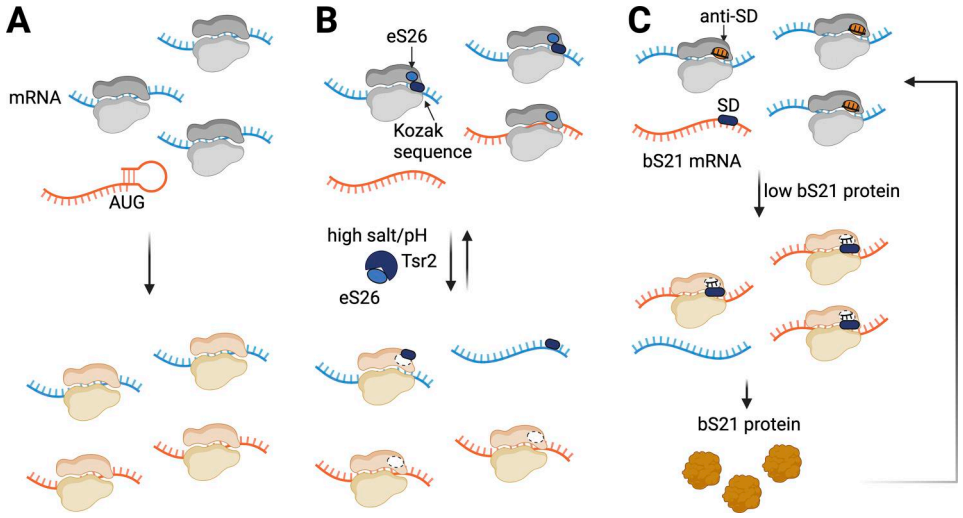

Figure 1. **Validated cases of ribosome specialization. (A)** In *V. vulnificus*, the I-rDNA operon produces ribosomes (I-ribosomes, in orange), which are required for the translation of a subset of mRNAs (in orange), whose start codon (AUG) is embedded in secondary structure (Song et al., 2019). Other mRNAs (in blue) do not require the I-ribosomes. Ribosomes produced from the other operons are in gray. **(B)** In *S. cerevisiae*, exposure to high salt or pH leads to the reversible, Tsr2-mediated dissociation of eS26 from ribosomes (Ferretti et al., 2018; Ferretti et al., 2017; Yang and Karbstein, 2022), to yield eS26-deficient ribosomes (in orange). These do not recognize part of the Kozak sequence. mRNAs without a strong Kozak sequence (orange) are not well translated by canonical ribosomes (in gray), which prefer Kozak-containing mRNAs (blue with the highlighted Kozak sequence). However, eS26-deficient ribosomes do not discriminate against weak Kozak mRNAs, enabling a relative increase in the production of their encoded proteins. **(C)** In *F. johnsoniae* and other *Bacteroides*, most mRNAs (in blue) do not have SD sequences and adding a SD sequence does not promote translation. This is because bS21 (shown in orange on the ribosome) sequesters the anti-SD. Ribosomes lacking bS21 do recognize the SD sequence. Because the b21 mRNA has a SD sequence, which—together with bS21-deficient ribosomes—is required for its efficient translation, this establishes a feedback loop to ensure all ribosomes contain bS21 (Jha et al., 2021; McNutt et al., 2023).

physiological outcomes (Ferretti et al., 2018). Thus, this is an example of how one can use multiple complementary methods (reporter assays, selective ribosome profiling, and physiological assays), different ways to induce the same stress (genetic eS26 depletion and high salt), and mutational analyses and rescue experiments to strengthen controls and avoid the pitfalls commonly observed when changing ribosomes and ribosome numbers.

A final example of functional ribosome specialization involves the autoregulation of translation of the RP bS21 in some bacteria. bS21 is a component of the so-called platform on the small ribosomal subunit (Fig. 1 C, [Jha et al., 2021; McNutt et al., 2023]). Most genes in *Bacteroides* do not contain a Shine–Dalgarno (SD) sequence, and consistently, ribosomes from *Flavobacterium johnsoniae* do not recognize SD sequences, even though the 16S rRNA contains an anti-SD sequence. Resolving this paradox, cryo-EM structures have shown that bS21, together with its binding partners bS18 and bS6, occlude access to the anti-SD (Jha et al., 2021). Weakening bS21 binding, or mutating its interaction with the anti-SD, allows for better recognition of the SD sequence in reporter assays and stronger binding of small subunits to mRNA in a reconstituted system *in vitro*, demonstrating these effects to be direct. Interestingly, bS21 is preceded by a strong SD (and the same is true less frequently for bS18, another platform component) and is poorly translated. However, bS21 production is increased in the bS21 mutant strains, suggesting a model whereby accumulation of ribosomes lacking bS21 (and/or bS18), upregulates the translation of bS21 mRNAs via recognition of the SD sequence, thereby ensuring sufficient production of bS21. Because in most organisms only bS21 has the type of strong SD sequence that is enabled by this switch (McNutt et al., 2023), it appears that this is an autoregulatory mechanism to ensure stoichiometric production of bS21 (and in some species bS18) to enable complete assembly of the platform. Thus, these specialized ribosomes essentially prevent ribosome heterogeneity or minimally keep it at a defined and regulated amount.

Surprisingly, two novel modifications in *Escherichia coli* 23S rRNA were recently described. These are only detected when the bacteria are grown with mild shaking in a low oxygen growth chamber (Ishiguro et al., 2025). Thorough examination revealed that the enzyme installing these modifications, named RlmX, contains an Fe-S cluster that is highly sensitive to oxidation and becomes inactivated under typical culturing conditions (vigorous shaking). The authors also provide strong evidence that the enzyme functions only during ribosome biogenesis and does not modify assembled preexisting ribosomes. Thus, this seems to be an example of a modification that is standard but was missed because standard laboratory growth conditions are not reflective of the growth environment of *E. coli* in the guts of mammals. Regardless, an important question remains as to how *E. coli* avoids ribosome subpopulations with distinct translation efficiencies from colliding, as cells transition from aerobic to anaerobic growth or vice versa (see section "Cells and cell states in which specialized ribosomes could play physiological roles").

In addition, recent work suggested that ribosomes in melanoma cells incorporate P-stalk proteins into translating ribosomes specifically after interferon treatment, as the ratio of bound/free Rpp1 increased substantially (Dopler et al., 2024). If true, this would be another example of a cellular (stress) state

where formation of a standard ribosome with the full complement of factors may be induced. However, a significant fraction of the P-stalk proteins are free in normal cells (Khalatyan et al., 2025), and a closer inspection of the data reveals that most of the increase observed by Dopler et al. is due to a loss of free Rpp1. Moreover, the quantification relies on a single peptide of Rpp1, which is not uncommon as RPs produce small fragments after trypsin digest, that are difficult to resolve in mass spectrometry experiments. Notably, data in yeast indicate that while P1 and P2 are not essential, growth of cells lacking them is significantly impaired and that suppressors readily arise (Steffen et al., 2012), indicating the importance of the P-stalk proteins for translation. Thus, taken together, the data for the existence of translating ribosomes without P-stalk proteins is not convincing—and more broadly, ribosomes lacking individual proteins are likely an exception, and not the normal state.

Examples of disease-associated ribosome heterogeneity—Defective ribosomes

There are several instances where alterations in ribosomes are associated with disease states, although it is difficult to ascertain whether the alterations in the ribosome are a cause or a consequence of the disease. Disease states can arise from defects in assembly that reduce ribosome numbers, which reduces overall translation and produces mRNA-specific effects, including on Hox genes (Cheng et al., 2019; Ferretti and Karbstein, 2019; Gaikwad et al., 2021; Ivanov et al., 2022; Khajuria et al., 2018; Lodish, 1974; Luan et al., 2022; Mills and Green, 2017).

Additionally, or alternatively, the ribosomes might also lack individual components (Ajore et al., 2017; Blomqvist et al., 2023a; Dreggors-Walker et al., 2022; Guimaraes and Zavolan, 2016; Jansson et al., 2021; Khoshnevis et al., 2019; Kondrashov et al., 2011; Kulkarni et al., 2017; Zhou et al., 2023), thus producing heterogeneity in the ribosome pool. Ribosomes that have lost the stoichiometric complement of RPs have been described in cancer cells (Guimaraes and Zavolan, 2016; Kulkarni et al., 2017), especially in cells lacking p53 (Ajore et al., 2017). These altered stoichiometries might arise from the widespread aneuploidy observed in cancer cells. Reduced expression of many RPs also results in cancer predisposition (Amsterdam et al., 2004), either due to the accumulation of ribosomes lacking the RP, the reduced abundance of ribosomes, or both.

Similarly, induction of the Myc oncogene increases the methylation of C174 in 18S rRNA, likely through overexpression of the snoRNA, which is encoded within an intron of a gene that responds to Myc levels (Jansson et al., 2021). Comparison of ribosome footprints in WT cells and cells where the snoRNA is deleted, identified about 2,000 mRNAs with altered ribosome load, although it is not known whether these effects arise from the deletion of the snoRNA and ensuing assembly defects, or the loss of the modification, or both.

Similarly, about half of all cancers display a loss of the 1-methyl-3-α-amino-α-carboxyl-propyl pseudouridine modification of the 18S rRNA residues U1248, which is located in the P-site (Babaian et al., 2020). Notably, loss of the modifying enzyme, Tsr3, in cancer cells does not seem to account for the loss in the modification, and consistently, upon knockout of Tsr3, no altered protein levels have been documented (although only ~14% of the proteome was detected).

Release of immature ribosome assembly intermediates into the translating pool, as occurs in the case of cancer-associated mutations in the late 40S assembly factor Pno1 (Parker et al., 2019), or upon overexpression of Rio1 (Parker et al., 2024), or loss of Tsr3 (Huang et al., 2022) might also give the appearance of ribosome heterogeneity if not all RPs are incorporated (the above examples lack eS26). Similarly, defects in rRNA processing, such as those caused by pathologic mutations in structural subunits of the RNA exosome, can also result in release of premature ribosomes into the translation pool (Fasken et al., 2023, *Preprint*; Sterrett et al., 2025). Finally, co-translational decay of 18S rRNA (Li et al., 2025; Parker et al., 2024) as well as ribosome repair (Yang et al., 2023) both transiently produce partially assembled ribosomes (Fusco et al., 2021; Sun et al., 2021), which are bound to mRNA. These repair or degradation intermediates look like heterogeneous translating ribosomes by mass spectrometry or structural tools, as well as ribosome profiling. And they might even be bound to specific subsets of mRNAs that are more likely to produce collisions, like well-translated mRNAs (where the spacing of ribosomes is reduced), or long mRNAs (where there is more time to get to a collision). Yet, the heterogeneity in these cases would be a product of the decay, which is induced on specific mRNAs, not the other way around. Thus, we would not consider decay intermediates specialized.

Examples for tissue-specific ribosome heterogeneity

Tissue-specific expression of ribosomes is well documented (Häfner et al., 2023; Hopes et al., 2022; Jiang et al., 2017; Li et al., 2022; Milenkovic et al., 2023; Shiraishi et al., 2023; Wong et al., 2014; Xu et al., 2023) (reviewed by Martinez-Seidel et al. [2020]; Norris et al. [2021]). It is tempting to assume that all these instances have functional consequences relevant for the specialized proteome of that cell. For example, a secretory cell in an endocrine organ has a proteome that is largely translated into the ER, while a red blood cell makes large amounts of hemoglobin. It is appealing to think that the ribosomes in these cells have adapted to these specific translation needs. However, data for such specialization are limited. One example is the tissue-specific expression of Rpl39-like (Rpl39L) in male germ cells, where it replaces the canonical Rpl39/eL39 (Li et al., 2022). Rpl39L, located in the peptide exit channel, changes the channel's dimensions and charge state relative to its paralog Rpl39, thereby influencing co-translational protein folding. As a consequence, the folding and stability (but not the translation) of about 220 sperm-specific proteins are dependent on the expression of Rpl39L and not rescued by expression of eL39 (Li et al., 2022). We consider this an excellent example of tissue-specific ribosome specialization that draws on a role of the ribosome (protein folding) beyond protein synthesis.

In contrast, the tissue-specific expression of Rps27/eS27 and Rps27L, Rpl3/uL3 and Rpl3L, or Rps5a and Rps5b (uS7) seems to be important to produce enough ribosomes in all tissues. In the case of the Rps27/Rps27L pair, the tissue-specific expression of the minor paralog (Rps27L) appears to compensate for the relatively reduced levels of the major

Table 2. **Changes in the abundance of tRNA^{Pro} isodecoders**

	Average tRNA reads		Rpl3 KO/WT*
tRNA	**WT**	**Rpl3 KO**	
Pro-AGG	4,469**	262	0.05***
Pro-TGG-1	212,266	181,531	0.94
Pro-TGG-2	169,863	1,072,546	6.5
Pro-TGG-3	27	37	1.5
Pro-CGG	**1,003,214**	**57,538**	**0.06**

Data are reanalyzed from Shiraishi et al. (2023). *Reads were normalized for total reads in each sample. **This number is driven by an outlier in one sample and likely much smaller. ***This number is driven by an outlier in one sample and likely close to 1. We have bolded the most abundant species in wt cells, which is reduced in the Rpl3 KO.

paralog in the tissues of question, which otherwise would reduce ribosome numbers (Xu et al., 2023). Thus, the observed effects on translation from the knockout of the minor paralog are rescued not only by re-introduction of the minor but also the major paralog.

In the case of Rpl3L, its knockout in mice leads to upregulation of Rpl3 in the heart and muscle cells where Rpl3L is typically expressed (although it remains unclear whether the total number of ribosomes is changed or not) (Milenkovic et al., 2023; Shiraishi et al., 2023). In addition, Rpl3L$^{-/-}$ mice have no (Milenkovic et al., 2023) or few (Grimes et al., 2023; Shiraishi et al., 2023) significant phenotypes, and translation of only two mRNAs is affected by the Rpl3L knockout, indicating that there is no mRNA specific translation by Rpl3L ribosomes (Milenkovic et al., 2023; Shiraishi et al., 2023). One study showed that in Rpl3L$^{-/-}$ mice ribosomes stall with empty A-sites encoding proline codons (Shiraishi et al., 2023). However, because the decoding site is on the small subunit, but Rpl3(L) on the large subunit, these effects are likely indirect: the most abundant proline tRNA is downregulated by ∼95% in Rpl3L$^{-/-}$ mice (Table 2 [Shiraishi et al., 2023]). Codon-specific pausing at proline codons that is dependent on the levels of proline isodecoders, and the preceding amino acid has been demonstrated in bacteria (Krafczyk et al., 2021), providing a precedent for mRNA-specific pausing caused not by ribosome alterations but changes in tRNA isodecoder levels. Altogether, this careful study provides a powerful illustration for the caveats when comparing two different cells lines.

Finally, a recent study in *Drosophila melanogaster* investigates tissue-specific expression of RP paralogs systematically (Grobicki et al., 2025). The authors find that most paralogs arose randomly via retro-transposition and do not retain coding capacity. Of those that do, all but one are nonessential. The one essential paralog, Rps5b (uS7), is expressed in the germline and required for oogenesis. Notably though, the careful work of the authors revealed that Rps5a and Rps5b are functionally interchangeable and show that instead Rps5b is essential because germlines do not make sufficient Rps5a. Thus, addition of Rps5a complements the deletion of Rps5b, demonstrating that there is no specialized role for this tissue-specific RP.

These four examples indicate that tissue-specific heterogeneity sometimes, but not always, leads to functional specialization. Then why does the tissue-specific expression of RPs exist? One possibility consistent with existing data (Jiang et al., 2017; Milenkovic et al., 2023; Shiraishi et al., 2023; Xu et al., 2023) is that tissue-specific changes in chromatin or transcription factors led to silencing of an essential RP in that tissue. Because of the large growth defects associated with such a change, evolutionary pressure would have selected for the transcriptional activation of one of the many pseudogenes, which evolved to the minor paralog, a mechanism also suggested by the authors of the Rps5 work (Grobicki et al., 2025). This model is consistent with data from yeast and mammals, which have shown that cells rapidly acquire genetic changes upon deletion of genes encoding RPs or other components of the translational machinery (Hughes et al., 2000; Johnson et al., 2020; Steffen et al., 2012).

Notably, a similar observation has been made for tRNA expression (Gao et al., 2024). While the expression of individual tRNAs can change substantially between different cell types, leading to significantly different tRNA pools, the anticodon pools, which matter for decoding, remain relatively constant. This is ensured via the constitutive expression of high-abundance housekeeping tRNAs that decode each codon. In contrast, the expression of individual tRNA isodecoders can be induced "coincidentally" in some cell types via the presence of nearby cell type–specific enhancer elements (Gao et al., 2024).

Akin to tissue-specific expression of RPs, tissue-specific differences in RNA 2′-*O* methylation have been observed in different brain regions in mice (Häfner et al., 2023), and expression of four ribosome-targeting snoRNAs (snoRA81, snoRA19, snoRD36A, and snoRD111B) has been described (Fafard-Couture et al., 2021), suggesting tissue-specific differences at their modification sites might be possible (although there is not necessarily a correlation between snoRNA levels and modifications [D'Souza et al., 2018]). Nonetheless, two of these snoRNAs share the same target site with additional snoRNAs, which could limit differences between tissues.

Technical and experimental hurdles in studying functional ribosome specialization

While the idea of ribosome specialization appears conceptually simple, there are significant technical hurdles that complicate its experimental study, as detailed below. Addressing them is difficult and might not always be possible, but important controls are outlined below.

Changes in ribosome number are common and modulate translation in an mRNA-specific manner

Because ribosomes are so central to life, any alterations in ribosomes have both direct and indirect effects on cellular homeostasis. These are important to control for when trying to support the conclusion that an observed ribosome heterogeneity has functional consequences.

One of these complications is that most changes in ribosome composition or assembly are accompanied by changes in ribosome numbers. This can even be true when there is no

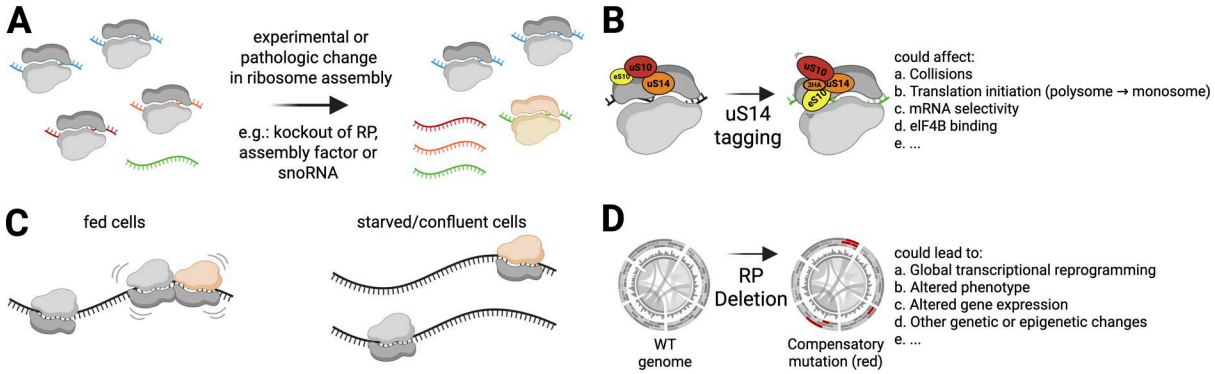

Figure 2. **Situations that must be addressed by control experiments. (A)** Most alterations in the ribosome assembly machinery (including the deletion of snoRNAs) produce both altered ribosomes (in orange), as well as reduced ribosome numbers, which both can have mRNA-specific effects on translation. **(B)** Tagging of RPs can perturb ribosome function, affecting mRNA recruitment, or ribosome numbers, thereby affecting translation in an mRNA-specific manner. **(C)** Reduced translation in starved or confluent cells will preclude collisions, preserving ribosome heterogeneity artificially. **(D)** Alterations in the translational machinery can lead to compensatory genetic or epigenetic changes.

discernible growth defect (Huang et al., 2022). This is significant because theoretical considerations (Ferretti and Karbstein, 2019; Lodish, 1974; Mills and Green, 2017) and experimental evidence (Cheng et al., 2019; Gaikwad et al., 2021; Ivanov et al., 2022; Khajuria et al., 2018; Luan et al., 2022) have shown that alterations in ribosome numbers can have mRNA-specific effects on translation (Fig. 2 A). Effects on translation that arise from the change or defect in ribosome assembly that is under investigation can be difficult to disentangle from effects arising from reduced ribosome content.

Thus, studies addressing the roles of ribosomes lacking an RP, or a modification/snoRNA, or an RP isoform should carefully examine if ribosome numbers are affected and carry out controls to ensure that the observed effects are specific to the deletion of the particular subunit and not observed with a different RP or snoRNA. These could include depleting a different protein from the same subunit. Moreover, rescue experiments are critical to demonstrate that any phenotypes under investigation arise from the RP alterations and not secondary effects (Johnson et al., 2020).

For studies addressing the role of one RP isoform, it is particularly important that all conditions have the same total amount of this RP, as one isoform is often a lot more abundant than the other (Ghulam et al., 2020). Moreover, different cellular states or stresses (or tissues) can change the relative abundance of these isoforms (Ferretti and Karbstein, 2019; Gasch et al., 2000; Parenteau et al., 2025). Thus, studies that address effects under stress must also ensure that under stress conditions the same number of ribosomes are produced. Again, rescue experiments with the two isoforms can be helpful controls. Moreover, RP mutations might lead to the loss of the RP from ribosomes (McNutt et al., 2023), which could produce exaggerated, or entirely different phenotypes.

Deletion of snoRNAs abrogates rRNA modification and can affect RNA folding and ribosome production

One type of change in ribosome composition that has aroused significant recent interest (Georgeson and Schwartz, 2021; Janin et al., 2020; Sloan et al., 2017) are changes in rRNA modifications installed via snoRNAs. These modifications are quite abundant (53 or 111 2′-O-methylations and 44 or 104 pseudouridylations in yeast and humans, respectively [Marchand et al., 2016; Marchand et al., 2020; Krogh et al., 2016]), and their concentration near the active sites has been taken as support for the functional importance of the modification. Moreover, even in WT cells there is now substantial evidence that a small subset of the modifications are not installed on every ribosome, providing evidence for modification heterogeneity (Häfner et al., 2023; Jansson et al., 2021; Krogh et al., 2016; Marchand et al., 2016; Marchand et al., 2020; Sharma et al., 2017; Zhou et al., 2023). Notably, modifications that are stoichiometric, i.e., found in every ribosome, are concentrated in the functional core. Moreover, depletion of fibrillarin, the rRNA 2′-O-methylase (Sharma et al., 2017), or of snoRNA assembly factors (Dreggors-Walker et al., 2022; Khoshnevis et al., 2022) depletes modifications outside of the functional core, while those in the core remain stable. While this has been interpreted as evidence for the importance of the modification, an alternative hypothesis is that snoRNA binding and/or the modifications in the core are so essential that unmodified rRNAs are rapidly degraded either during or after assembly, depleting the rRNAs that lack these modifications (Bailey et al., 2022). In this model, loss of specific modifications would deplete the rRNA and reduce the ribosome levels, which would both cause growth defects and mRNA-specific translational defects (Ivanov et al., 2022; Khajuria et al., 2018; Luan et al., 2022). Indeed, deletion of snR35 leads to rRNA misfolding, causing reduced 18S levels and reduced growth rates (Blomqvist et al., 2023a; Huang and Karbstein, 2021). Moreover, deletion of many snoRNAs leads to small growth defects in *S. cerevisiae* (Blomqvist et al., 2023a; Li et al., 2009; Thompson et al., 2016). Similarly, deletion of snR24, which methylates C1437, C1449, and C1450 in 25S rRNA reduces 25S rRNA levels and produces a temperature-sensitive phenotype (Li et al., 2009; Thompson et al., 2016). Deletion of snoRD52, which modifies U3904 in human 28S rRNA depletes ribosomes (Häfner et al., 2023). Thus, many snoRNAs may play structural

roles by preventing the premature formation of secondary structure during assembly (Karbstein, 2011). This would be akin to U3 and snR30 snoRNAs, which evolved from canonical modification snoRNAs, form analogous base pairs that chaperone RNA folding, but without specifying modifications (Fayet-Lebaron et al., 2009; Venema and Tollervey, 1999). Notably, many snoRNA binding sites are clustered around helical junctions, which are prone to misfolding (Holmes and Culver, 2004; Huang and Karbstein, 2021). Thus, when deleting a snoRNA to abrogate a modification, the modification is lost, but the potential for rRNA misfolding must also be taken into consideration, as this could either cause or contribute to any observed phenotype.

To disentangle these effects, we suggest reintroducing not just the WT snoRNA to complement the snoRNA deletion but also an engineered snoRNA that can modify a neighboring residue. Because modified residues should make specific interactions that mediate their function, while the base pairing between snoRNAs and rRNA are extensive (Kiss, 2002; Webster and Ghalei, 2023) and much less likely to be affected by a move to a neighboring residue, folding defects are likely to be rescued by an engineered snoRNA, while functional defects from the modification are not expected to be rescued in this manner. A potential drawback from this method might be that an artificial snoRNA might perturb assembly per se, as previously shown (Lebaron et al., 2012). We think this is less likely at a site adjacent to an existing modification site, as defects during assembly when the snoRNA is present would be unlikely. Indeed, proof of concept has shown that such introduction of artificial snoRNAs is possible (Huang et al., 2011) and can replace the modification from a standalone enzyme (Yelland et al., 2023).

Affinity tags on RPs perturb their structure and function

An excellent demonstration of specialized ribosomes involves the separation of ribosome populations and the subsequent sequencing of bound mRNAs. However, separation of ribosome populations relies on affinity tags, often on RPs. These affinity tags can perturb ribosome function (Fig. 2 B), even if they are small, as suggested by genetic interactions displayed by 3x-HA–tagged uS14/Rps29, but not 1x-HA–tagged uS14 (Collins et al., 2018), and differences in levels of tagged and untagged Rps27/eS27 and Rps27L (Xu et al., 2023). Moreover, the gradient sedimentation of fluorescently tagged uS5/Rps2 and eL29/Rpl29 differs from the endogenous untagged protein, with tagged-RPs accumulating in 80S ribosomes (Zhang et al., 2025). Similarly, purified Rpp1-HA–tagged ribosomes are monosomes (as they do not co-purify untagged Rpp1), while untagged Rpp1 sediments in polysomes, suggesting that the HA-tag on Rpp1 perturbs translation (Dopler et al., 2024). Finally, tagging several proteins from the large subunit perturbs the accessibility to nucleases as read out in rRNAseq data (Alkan et al., 2022). These functional alterations to RPs might cause or affect the observed behavior of those ribosome populations (Fig. 2 B).

To address these concerns, growth rates should be carefully measured, and ribosomes purified from strains or cell lines with or without the purification tag should be assessed for RP composition. In addition, one might consider measuring genetic interactions between the perturbation under study and the introduction of the affinity tag. Ideally, one would also add additional controls using tags on different subunits and/or combining the utilized tag with test and control alterations to observe commonalities (arising from the tag) and differences (arising from the perturbation under study).

In addition to structural perturbations from the tag, separation of ribosomes requires quantitative binding to the affinity matrix (Xu et al., 2023). Moreover, if affinity tags on one RP are combined with depletion of another component (RP or snoRNA), and the subunits lacking the depleted component are less stable (because they are either degraded, labile, or immature), then the "purification" will not enrich the desired product. Thus, this approach requires careful controls and alternative purification strategies. For example, when purifying late 40S assembly intermediates from cells lacking Tsr1 using an affinity tag on Rio2, only WT intermediates enrich. This is because loss of Tsr1 weakens Rio2 binding, leading to its loss from ribosomes. Thus, the "correct" intermediates (those lacking Tsr1) also lack Rio2-TAP and are therefore never captured, requiring the use of an affinity tag on another factor, which is unaffected by Tsr1 depletion (Strunk et al., 2011).

Manipulating rDNA sequence is difficult

Because rDNA loci are tandem repeats of hundreds of genes their perturbation is difficult. In yeast, a workaround has been a system, where rRNA transcription is shut off using a temperature-sensitive mutation in an RNA polymerase I mutant (Nogi et al., 1993). This is complemented by RNA polymerase II–driven expression of the rDNA operon from a plasmid (Nogi et al., 1991). Alternatively, there is a strain without the rDNA repeat (Wai et al., 2000). The problem with these tools is that the yeast grow extremely slowly (Nogi et al., 1991; Parker et al., 2024; Wai et al., 2000), due to insufficient rRNA transcription, which selects for recombinants between the plasmid and the genome (Lamanna and Karbstein, 2011; Parker et al., 2024).

In metazoans, such a system has not been developed, although plasmids exist that allow for the expression of rDNA variants in addition to endogenous RNAs (Coria et al., 2025; Quinodoz et al., 2025). However, one could imagine using RNA-directed CRISPR (van Beljouw et al., 2023) or RNaseH to specifically target rRNA isoforms under study. This should be facilitated because many rRNA variants are low abundance (see above).

The experimental system could produce artifacts from indirect effects

Given the importance of ribosome concentration-dependent effects, it is also important to ensure that an experimental system does not produce artifacts. For example, if mammalian cells in culture are grown to high density, their translational activity decreases, leading to polysome collapse and a large increase in the 80S monosomes peak. Under those conditions, collisions are expected to be impaired (as they require more than one ribosome to bind a single mRNA), which could enable the persistence of ribosome populations that might otherwise be removed in a collision-dependent manner (Li et al., 2025; Parker et al., 2024).

It could also hide functional differences between ribosome populations that arise from differential susceptibility to collisions (Fig. 2 C). Thus, it is critical to show polysome profiles of the cells under study for the audience to evaluate the translational status of the cell.

Another example of alterations that can occur due to differences in growth state are the recently discovered oxygen-sensitive modifications in *E. coli*, which were previously missed because standard laboratory growth conditions generally do not reflect the environment in the "wild" (Ishiguro et al., 2025).

To compensate for reduced ribosome numbers observed in many genetic backgrounds where assembly factors, snoRNAs or RPs are knocked out, transcriptional upregulation of ribosome biogenesis is often observed (Cheng et al., 2019; Milenkovic et al., 2023; Shiraishi et al., 2023). In addition, more peculiar and idiosyncratic responses have been observed, which may not be rescuable by re-expression of the depleted component. For example, yeast and mammalian cells respond to depletion of components of the translational machinery by genomic alterations to change copy number (Hughes et al., 2000; Steffen et al., 2012), or rewiring of the cell state, resulting in altered transcriptomes (Johnson et al., 2020). Similarly, when exposed to stress, *E. coli* upregulate one of their 7 rRNA operons to specifically produce the *rrsH* 16S rRNA (Kurylo et al., 2018). This produces a distinct transcriptional response. In addition, as described above, knockout of Rpl3L in mice alters the levels of proline decoders, which is likely to affect proline decoding (Shiraishi et al., 2023). Thus, when comparing translation in these strains, differences may be found. However, these changes do not necessarily arise from discrete differences in the ribosome, but the cellular response to these genetic alterations. To control for these types of artifacts, it is critical to carry out add-back experiments to ensure that any phenotypes observed can be rescued. In addition, whole-genome sequencing will reveal genomic alterations. Finally, another way to ensure that any observed effects arise from the altered ribosomes is to utilize these altered ribosomes in reconstituted *in vitro* translation systems (Filipek et al., 2024).

Reasons why ribosome specialization is likely limited

While it is tempting to speculate how much specialized ribosomes may contribute to the regulation of gene expression, our knowledge of ribosome homeostasis suggests that functional ribosome specialization is limited. Below, we examine these arguments and use them as a starting point to consider situations where exceptions might exist.

Ribosomes are stable and assembly is blocked in all studied cellular stresses

To change the composition of the ribosome pool, existing ribosomes must be degraded, and new, distinct ribosomes must be assembled. However, ribosomes are extremely stable, with half-life times that far exceed the doubling times of the cells that harbor them (Yang and Karbstein, 2024). Thus, preexisting ribosomes can only "disappear" from a population by cell division (Yang and Karbstein, 2024). This very slow response is biologically insignificant, especially in cells with long doubling times. Moreover, other changes in regulated gene expression typically occur in response to a change in the environment, which manifests as stress. However, reflecting the large energetic cost of ribosome production, ribosome assembly is downregulated under all studied stress conditions (Gasch et al., 2000), thus limiting the production of new ribosomes. Together, these considerations suggest that alterations in ribosome populations arising from modulation of assembly are slow and unlikely to occur during cellular stress. Notably this includes all rRNA modifications as these are not known to be reversible.

Quality control during ribosome assembly

Instead of producing a different set of ribosomes only under stress conditions, one might imagine that cells constantly produce different sets of ribosomes that could have specific functions, as exemplified by the I-ribosomes in *V. vulnificus*. However, there is significant evidence that assembly pathways are hierarchical and built to limit heterogeneity (Blomqvist et al., 2023a; Blomqvist et al., 2023b, *Preprint*; Huang et al., 2020; Huang et al., 2022; Lo et al., 2010; Parker et al., 2019; Zhou et al., 2019). Moreover, many early-binding RPs are essential for rRNA processing and subsequent maturation steps (Ferreira-Cerca et al., 2005; Ohmayer et al., 2013), making it hard to envision how assembly without these RPs could proceed. In addition, quality control pathways exist to sieve out ribosomes that are not fully functional (Blomqvist et al., 2023b, *Preprint*; Ghalei et al., 2017; Huang et al., 2020; Parker et al., 2019; Strunk et al., 2012; Yelland et al., 2023), and quality control would be very challenging if there are many different correct ribosomes. Thus, the cell's need to make fully functional ribosomes likely limits heterogeneity during assembly.

Dysfunctional rRNA decay purifies the ribosome pool

Both prokaryotic and eukaryotic cells sense ribosome collisions induced by aberrant translation (D'Orazio and Green, 2021; Filbeck et al., 2022; Kim and Zaher, 2022; Meydan and Guydosh, 2021). Similar mechanisms limit the heterogeneity of ribosomes (at least in yeast) if some translate more slowly than others (Li et al., 2025; Parker et al., 2024). When a faster (canonical) ribosome catches up with a slower (heterogeneous) ribosome, a collision occurs that leads to the decay of the slower ribosome (Fig. 2 A, [Li et al., 2025; Parker et al., 2024]). Similarly, both in mammalian and yeast cells, ribosomes defective in recruiting the first charged tRNA are stalled at the start codon and degraded there (Cole et al., 2009; Garshott et al., 2021; LaRiviere et al., 2006; Sugiyama et al., 2019). One might argue that heterogeneity does not necessarily need to indicate a "defect" such as slower translation. However, if "specialized ribosomes" were doing better over certain sequences than canonical ones, this would lead to the decay of the canonical ones in a collision-mediated manner, again homogenizing the ribosome pool (albeit to an altered ribosome).

Cells and cell states in which specialized ribosomes could play physiological roles

Next, we consider hypothetical situations where mechanisms that limit heterogeneity are disrupted. Importantly, the documented instances of ribosome specialization all fit these exceptions. This demonstrates the value of considering the limitations to heterogeneity and specialization to discover new instances where specialization plays important physiological roles.

Chaperone-mediated release of RPs from fully matured subunits can rapidly produce alternate ribosomes while maintaining quality control

While slow ribosome turnover likely limits ribosome specialization as a means of regulated gene expression, we have shown that in a few cases RPs can be released while retaining the ribosome: under high salt and pH stress, eS26/Rps26 can be rapidly and reversibly released from ribosomes by its chaperone Tsr2 ([Ferretti et al., 2017; Yang and Karbstein, 2022], Fig. 1 B). Moreover, oxidatively damaged uL16/Rpl10 can be similarly released in a chaperone-dependent manner (Yang et al., 2023). 13 additional RPs have chaperones, most of them located on the surface of the ribosome (Yang and Karbstein, 2024), suggesting the possibility that ribosomes lacking some of these RPs might also exist.

Rapidly dividing cells might be defective in dysfunctional rRNA decay and could change ribosome content quickly

Rapidly dividing cells have fewer ribosomes (Genuth et al., 2022; Leesch et al., 2023; Ni et al., 2025; Papagiannopoulos et al., 2022), as assembly might not be able to fulfill the demand from the rapid cell division. Because collisions are concentration-dependent (Simms et al., 2017), reduced ribosome numbers will limit the collision-mediated decay that occurs when two populations coexist (Li et al., 2025; Parker et al., 2024), indicating that such cell types and states might allow for functional heterogeneity.

Moreover, even though ribosome turnover is slow as it occurs by cell division as described above, rapidly dividing cells, such as blood or skin cells, or cells during development might be able to change their ribosome pools on a biologically relevant timescale. Indeed, there are many examples of developmentally associated heterogeneity occurring at the level of RP incorporation, rRNA isoform expression and modification (Joo et al., 2022; Li and Wang, 2020; Norris et al., 2021).

For example, in zebrafish, different rDNA repeats with slightly different sequences are transcribed in the oocyte and later developmental stages (Locati et al., 2017), although no functional differences for these have been described.

Similarly in mice, RP occupancy in polysomal ribosomes changes during embryogenesis with about 40% of RPs changing (Genuth et al., 2022). Among these are Rpl10A/uL1, Rpp1 and Rpp2, and eS25/Rps25, which all decrease in ribosome residence during mesoderm development, indicating that ribosomes lacking these components accumulate. Importantly, translation by ribosomes lacking uL1/Rpl1 has been previously observed (McIntosh et al., 2011; Musalgaonkar et al., 2019; Shi et al., 2017). Similarly, eS25 is nonessential in yeast and translation by ribosomes lacking this protein is possible (Landry et al., 2009). Curiously, deletion of Rpl10A/uL1 in mice (thus mimicking the biological situation) produces phenotypes, suggesting that the formation of these ribosomes is not physiological but deleterious, and thus confusing the interpretation of the data (Genuth et al., 2022).

Moreover, the analysis of changes in ribosomes during early embryogenesis is further complicated by a recent finding in worms that demonstrate that intact L1 larvae can develop without any production of new ribosomes (Cenik et al., 2019). This finding suggests that at least in worms, production of distinct ribosomes is not necessary for early embryogenesis (Cenik et al., 2019). Similar findings have been made in zebrafish, where RP occupancy does not change during the first 24 h after fertilization (Leesch et al., 2023). Moreover, ribosome levels remain constant despite the increasing number of cells in the developing embryo (Leesch et al., 2023), indicating that akin to worms, early developmental stages use preexisting ribosomes. While it is not clear when *de novo* ribosome assembly is required for embryogenesis in mammals, it is noteworthy that at least in worms transcription of embryonic rRNA is uncoupled from their active use in translation (Cenik et al., 2019). This observation indicates the need to determine when *de novo* assembled ribosomes are utilized in important model organisms, as well as the importance of specifying the exact parental lineage of heterozygous animals, as embryogenesis might be driven largely by maternally deposited ribosomes.

Specialized ribosomes unmix by recruiting distinct mRNA sets or in distinct cellular locales

Ribosome collisions purify the ribosome pool of defective, and likely also of heterogeneous, ribosomes ([Li et al., 2025; Parker et al., 2024], Fig. 3 A). However, if the two subsets of ribosomes do not bind the same mRNAs, then collisions cannot identify the specialized subset (Fig. 3 B). Indeed, this scenario is likely what allows the eS26-deficient ribosomes in yeast, the I-ribosomes in *V. vulnificus* and the bS21-deficient ribosomes in *Bacteroides* to persist (Fig. 1).

Relatedly, if different subsets of ribosomes are localized to different cellular spots, they might also not bind the same mRNAs, even if they lack mRNA selectivity, thus again stabilizing these ribosome populations. Such scenarios could include specialized ribosomes at the ER or mitochondrial membrane or in dendrites or other locales of highly polarized mammalian cells. Indeed, recent work that takes advantage of expansion microscopy, where fixed cells are "stretched" within a gel, suggests that ribosomes (lacking either eL29 or eS25) exist on mitochondria (Zhang et al., 2025). The interpretation of the images is complicated by the fact that the technique was calibrated on scales ~100-fold larger than ribosomes. This might obscure artifactual dislocation of ribosomes on a much smaller scale, which could lead to the appearance of enrichment at mitochondria, plausible as the cytosol is packed with ribosomes. In addition, the visualization uses tags on uS5 and eL29 that are much larger than the RPs, which perturb ribosome function as described in detail above (Affinity tags on RPs perturb their structure and function). Nonetheless, it would be exciting (though challenging) to replicate similar studies by cryo-ET, which utilizes live cells and at least in principle could be done without tagging of RPs.

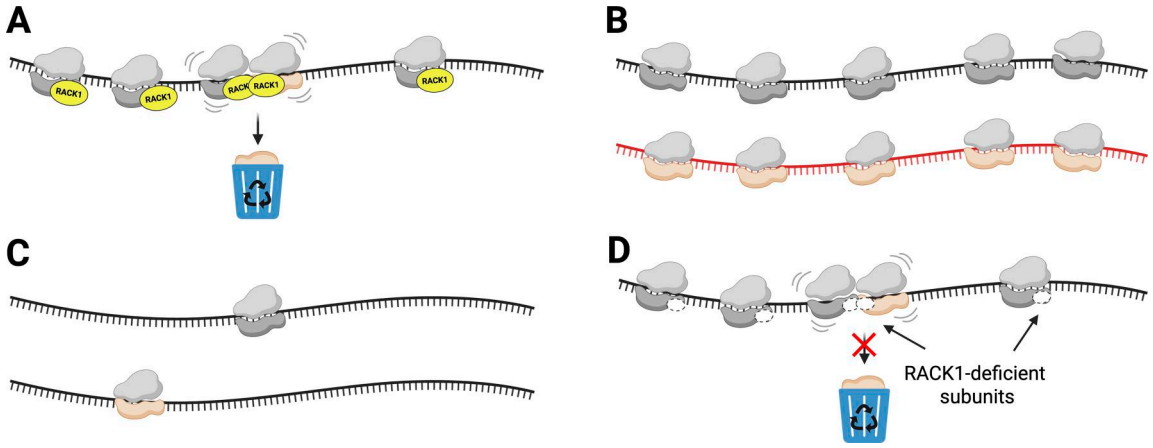

Figure 3. **Ribosome collisions purify the ribosome pool under some conditions. (A)** Ribosome collisions identify defective ribosomes (in orange) via the collisions that they incur with canonical ribosomes, which then lead to the degradation of the defective ribosome (Li et al., 2025; Parker et al., 2024). The collided interface that is recognized by the degradation machinery includes the RP Asc1/RACK1 (in yellow). **(B)** Collisions between defective or heterogeneous ribosomes (in orange) and canonical ribosomes (in gray) cannot occur if the different ribosomes sort on different mRNAs (black or red), either because of strong mRNA specificity or due to subcellular localization. **(C)** Collisions between defective or heterogeneous ribosomes (in orange) and canonical ribosomes (in gray) cannot occur in cells with low ribosome levels or low translation (initiation), which both limit ribosomes to one or a few on each mRNA, thus precluding collisions. **(D)** Collisions between defective or heterogeneous ribosome species (in orange) and canonical ribosomes (in gray) can occur but are not recognized by the degradation machinery if the collision interface is destabilized by the absence of nonessential RPs like Asc1 (Ikeuchi and Inada, 2016; Limoncelli et al., 2017; Sitron et al., 2017; Wang et al., 2018) (shown in yellow in A) or mutation of other interface components (Matsuo et al., 2017; Narita et al., 2022).

Low ribosome content or reduced translational activity limit collisions

Because collisions that purify the ribosome pool require at least two ribosomes on an mRNA and increase in likelihood with the translational load (Simms et al., 2017), cells with low translational activity are likely limited in their ability to use collisions to purify the ribosome pool (Fig. 3 C). Examples include neurons, where much of translation occurs in 80S monosomes (Biever et al., 2020), as well as during development (Hopes et al., 2022), when cells are rapidly dividing and may be unable to produce enough ribosomes (Ni et al., 2025; Papagiannopoulos et al., 2022), thus limiting ribosome collisions (Simms et al., 2017). They could also include cells in culture grown to high confluency.

Asc1/Rack1-deficient ribosomes preclude collisions

The RP Asc1 (or Rack1 in human cells) is located at the interface formed by collided ribosomes (Ikeuchi et al., 2019; Juszkiewicz et al., 2018). Deletion of this nonessential RP stabilizes defective ribosomes (Collins et al., 2018; Ikeuchi and Inada, 2016; Sitron et al., 2017; Wang et al., 2018; Wolf and Grayhack, 2015). Thus, cells, tissues, or cell states that reduce Asc1/Rack1 expression might also stabilize specialized (and defective) ribosomes, as collided disomes are not degraded in these cells (Fig. 3 D).

How could specialized ribosomes affect translation of mRNA subsets?

Different ribosomes have different mRNA selectivity during translation initiation

One possibility is exemplified by the cases above, where the distinct ribosomes have different mRNA selectivity, thereby enabling the translation of different subsets of mRNAs (Fig. 1). Notably, differential translation of different mRNAs as revealed to be widespread by ribosome profiling (Ingolia et al., 2009) and implied by Kozak's original observations (Kozak, 1984; Kozak, 1986) likely reflects not the initial binding step, which occurs at the cap, and is likely not selective. Instead, the observed differences in translation efficiency must report on a step after initial mRNA selection that occurs at or near the start side, thereby producing sequence selectivities around that site (Dvir et al., 2013; Kozak, 1984; Kozak, 1986) as ribosomes either start translation or resume scanning. This selection step could be altered for small ribosomal subunits that differ in the composition of their mRNA-binding channel (like Rps26-deficient ribosomes) or the binding sites for translation initiation factors that contribute to recognition of the start codon. For example, mutations in eIF2α preferentially destabilize the so-called "closed" translation initiation complex, thereby affecting translation of mRNAs with AUG codons in poor Kozak context, as these require the additional stabilization by the eIF2α residues (Thakur et al., 2020). Thus, mutations that weaken or misposition this factor are expected to affect mRNAs with weak Kozak sequences more than those with strong Kozak sequences. Similarly, mutations of the −3 residue of the Kozak sequence affect the release of eIF5B and the transition from initiation to elongation (Wang et al., 2019), marking a potential checkpoint for these sequences.

Ribosomes defective in stabilizing collided disomes

Ribosomes that are defective in the formation of the collided ribosome interface, e.g., by the absence of Asc1/Rack1, or in binding of the disome sensor Hel2/ZNF598, can read through stall sequences, thereby producing more of the resulting protein product (Sundaramoorthy et al., 2017) (Fig. 4 A). In addition to Asc1/Rack1 deletion, such variants could include mutants in eS10/Rps10, uS10/Rps20, and uS3/Rps3. These alterations all stabilize defective mRNAs or rRNAs toward degradation (Collins

et al., 2018; Ikeuchi and Inada, 2016; Ikeuchi et al., 2019; Juszkiewicz et al., 2018; Limoncelli et al., 2017; Parker et al., 2024; Sugiyama et al., 2019; Wang et al., 2018). Notably, yeast have ~100 mRNAs with stretches of 11 adenosines. In addition, ribosome mutants defective in binding Gcn1 or Gcn2 (via alterations at the interface or the binding site) would be unable to launch the integrated stress response upon encountering collisions, thereby again affecting a subset of mRNAs.

Similar considerations arise for ribosomes defective in binding eIF5A, which will affect mRNAs differentially (Gutierrez et al., 2013; Schuller et al., 2017).

Ribosomes with differences in co-translational protein folding

Another mechanism supported by experimental data is the role of RPs in the exit channel in helping with co-translational folding of nascent proteins (Li et al., 2022). This affects neither mRNA selectivity or translation rates but protein stability and is therefore also not sensitive to collisions (Fig. 4 B). Again, similar considerations exist for RPs at the exit tunnel that bind ribosome-bound factors that handle the nascent proteins in ways that affect their stability, including folding chaperones, methionine aminopeptidases, N-acetyltransferases, etc. Of note, this mechanism enables specialization by 60S RPs.

Ribosomes with defects in subunit joining

Another possible mechanism could include mutations that destabilize formation of 80S ribosomes, which would lead to leaky scanning, as stalled pre-initiation complexes that cannot form 80S complexes might continue scanning with some frequency (Fig. 4 C). This would affect the regulation via upstream ORF. As above, this mechanism also enables specialization from 60S RPs (in addition to 40S RPs).

Conclusions and perspectives

There are variety of ways that ribosomes may be heterogeneous (Barna et al., 2022; Emmott et al., 2019; Ferretti and Karbstein, 2019; Gay et al., 2022; Genuth and Barna, 2018; Joo et al., 2022; Li and Wang, 2020; Martinez-Seidel et al., 2020; Mauro and Edelman, 2002; Murphy et al., 2023; Norris et al., 2021; Simsek and Barna, 2017; Sloan et al., 2017; Xue and Barna, 2012). Nonetheless, existing data also indicate that not every instance of heterogeneity leads to specialization, and data in the literature point to important caveats that can arise from the necessary experimental manipulations.

We anticipate that our examination of when ribosome specialization is unlikely to happen will allow for a more targeted search to uncover instances of ribosome specialization, which should then be supported by careful experimental controls, that are outlined here and elsewhere (Ferretti and Karbstein, 2019). We hope that this guide will help interested researchers develop the best possible experiments to address this exciting area of research.

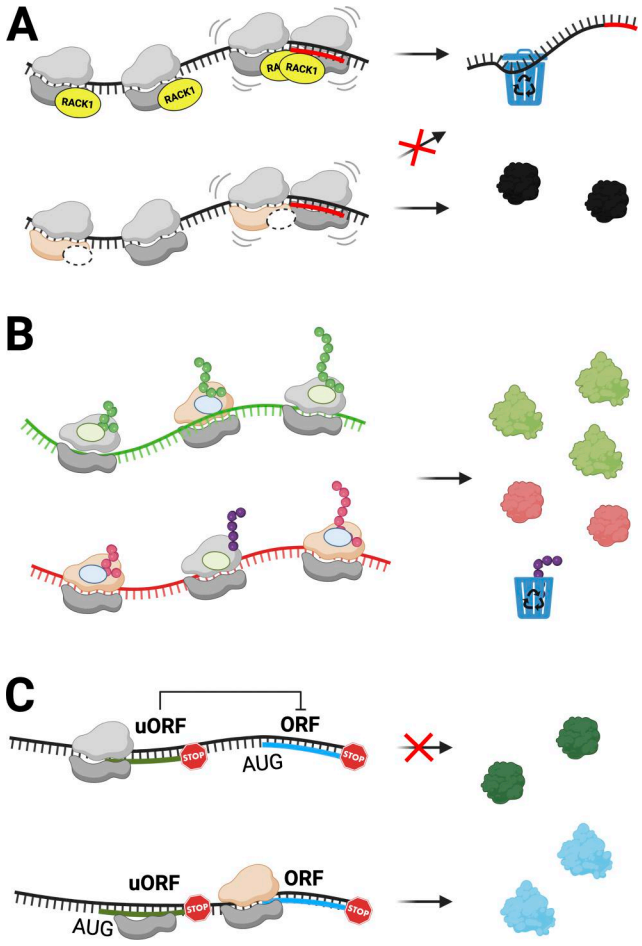

Figure 4. **Mechanisms by which ribosome heterogeneity might affect translation in an mRNA-specific manner. (A)** Top: Defects in mRNAs or specific stall sequences (shown in orange on the mRNA), lead to stalling of the ribosome and collision of the trailing ribosome. The collided interface is recognized to assemble a complex, which ultimately degrades the bound mRNA (D'Orazio and Green, 2021; Filbeck et al., 2022; Kim and Zaher, 2022; Meydan and Guydosh, 2021). Bottom: specialized (or defective) ribosomes with mutations at the collided interface fail to degrade the mRNAs with stalling sequences, leading to the increased production of proteins from such mRNAs. **(B)** Top: Some nascent proteins fold efficiently in exit channels of standard ribosomes. Bottom: Other nascent proteins require specialized ribosome channels for correct co-translational folding. Misfolded proteins are degraded. **(C)** Top: translation of upstream ORFs (uORFs, dark blue) often inhibits the translation of the downstream ORF (cyan). Bottom: Ribosomes with defects at the subunit interface (in orange) are expected to be prone to leaky scanning, if the time it takes for subunit joining is too long.

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