Trends in Biochemical Sciences

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REVIEWS

Opinion

Ribosome Stoichiometry: From Form to Function

Edward Emmott,[1] Marko Jovanovic,[2] and Nikolai Slavov[1,3,*]

The existence of eukaryotic ribosomes with distinct ribosomal protein (RP) stoichiometry and regulatory roles in protein synthesis has been speculated for over 60 years. Recent advances in mass spectrometry (MS) and high-throughput analysis have begun to identify and characterize distinct ribosome stoichiometry in yeast and mammalian systems. In addition to RP stoichiometry, ribosomes host a vast array of protein modifications, effectively expanding the number of human RPs from 80 to many thousands of distinct proteoforms. Is it possible that these proteoforms combine to function as a 'ribosome code' to tune protein synthesis? We outline the specific benefits that translational regulation by specialized ribosomes can offer and discuss the means and methodologies available to correlate and characterize RP stoichiometry with function. We highlight previous research with a focus on formulating hypotheses that can guide future experiments and crack the ribosome code.

Introduction

Ribosomes, the cellular machinery of protein synthesis, are present at up to 10 million copies per cell in mammals. Despite their abundance and the wide array of known modifications to both **RPs** (see Glossary) and rRNA, study of the direct role of the ribosome in tuning cellular translation has until recently taken a back seat to post-transcriptional regulation at the level of translation initiation. The hypothesis that ribosomes actively regulate protein synthesis as part of normal development and physiology dates back to the 1950s [1]. In the ensuing decades, numerous, albeit inconclusive, observations have supported this hypothesis, and a subset of those are shown in Figure 1.

For many years, the dominant 'abundance' model suggested a limited role for ribosomes in translational regulation [2]. In this model, if ribosomes have different initiation affinities for different transcripts, a global decrease in the availability of free ribosomes selectively decreases the initiation rates of different transcripts to varying degrees [2]. This mechanism, recently reviewed by Mills and Green [3], relies on the nonlinear dependence of translation initiation on free ribosomes. Recent research suggested this mechanism could explain the failure of erythroid lineage commitment seen with Diamond–Blackfan anemia (DBA) [4]. Under this model, cell or tissue specificity of **ribosomopathies** such as DBA (Box 1) is explained as a cell type-specific response to ribosome-induced cell stress [3]. However, other experiments performed in yeast suggested that this model alone is insufficient to fully capture the details of ribosome-mediated translational control in wild-type cells [5]. Translational regulation by the abundance model is limited in magnitude by changes in total ribosomal content and in flexibility since it provides unidirectional regulation for all proteins.

The Concept of Ribosome Specialization

In the 'specialized' ribosome model (Box 2), ribosomes do not possess constant structure or composition (Figure 2A,B) and instead exhibit altered stoichiometry of what were previously

Highlights

New and emerging methods permit the identification of ribosomes with distinct ribosomal protein stoichiometry from eukaryotic cells and the exploration of ways in which ribosome-mediated regulation could contribute to the control of protein synthesis.

Two models for ribosome-mediated translational regulation exist: (i) the abundance model whereby the abundance of ribosomes in the cell can exert bidirectional global regulation of mRNAs; and (ii) the specialized ribosome model in which changes to ribosome stoichiometry or post-translational modifications permit finer control of ribosome function.

Regulation by specialized ribosomes could include mRNA-specific or functional class-specific ribosomes and modification of ribosome elongation and error rates in response to stress or stimuli.

This Opinion article proposes a path to unambiguously identify functional ribosome specialization within and between mammalian cells.

[1]Department of Bioengineering, Northeastern University, Boston, MA, USA
[2]Department of Biological Sciences, Columbia University, New York, NY, USA
[3]Department of Biology, Northeastern University, Boston, MA, USA

*Correspondence: nslavov@alum.mit.edu (N. Slavov).

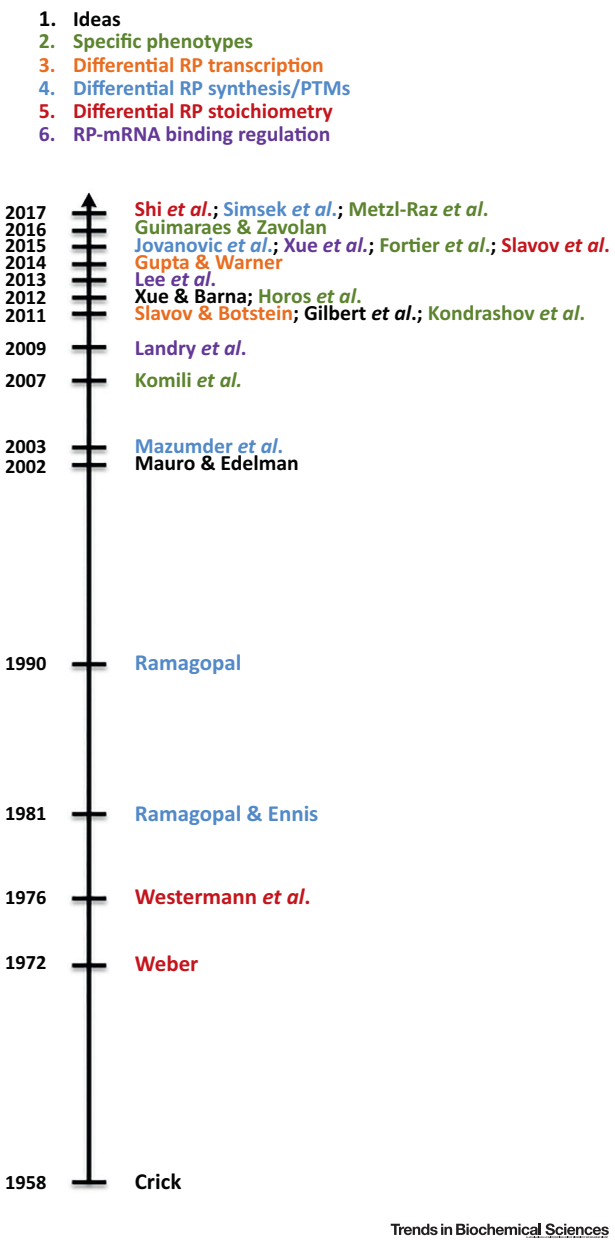

Figure 1. Timeline. The concept of eukaryotic ribosome specialization has existed for decades, and recent methodological advances have resulted in renewed interest and the ability to explore and characterize these phenomena. In this timeline, a few key manuscripts are colored by the areas of ribosome heterogeneity they have described.

thought to represent 'core' RPs (Figure 1) [6–11,98]. In this model, different ribosomal compositions are functional and have specific roles in translation. Specialized ribosomes could coexist within cells or between different cells or tissues [12,13]. Ribosome heterogeneity is a related concept where ribosomes within or between cells can have altered stoichiometry or composition but do not necessarily play a functional role.

Glossary

40S: the small ribosomal subunit in eukaryotic ribosomes. The prokaryotic equivalent is the 30S subunit.
60S: the large ribosomal subunit in eukaryotic ribosomes. The prokaryotic equivalent is the 50S subunit.
80S: see monosome.
Bottom up (mass spectrometry): bottom-up proteomics is the analysis of protease-digested protein samples. These methods measure peptides, not full-length proteins, and the peptide-level data is used to infer protein levels.
Elongation rate: the rate at which the ribosome is able to extend the growing polypeptide chain as it proceeds along an mRNA.
Error rate: the rate at which incorrect amino acids are misincorporated into elongating polypeptide chain by the ribosome.
Extraribosomal: some RPs are proposed to have roles in the cell separate from their role in forming part of the ribosome. When these proteins are found outside the ribosome they are termed extraribosomal.
Heterogeneous ribosomes: ribosomes possessing variation in their RP, rRNA, or PTM stoichiometry. Specialized ribosomes are a subset of heterogeneous ribosomes where the heterogeneity has been linked to specific functions.
Histone code: the hypothesis that PTMs of histone proteins function combinatorially to provide highly customizable control of transcription.
Housekeeping proteins: proteins required for the basic functioning of the cell; constitutively expressed, often at high levels.
Internal ribosome entry site (IRES): an RNA sequence allowing cap-independent translation of a target mRNA containing the IRES.
Kinetic proofreading: a method for correcting errors in biochemical reactions. By separating a reaction into multiple irreversible intermediate steps, error rates far lower than would otherwise be possible with a single-step reaction can be achieved.
Label free: methods of analyzing one sample at a time that does not contain isotopic labels. These methods can be DDA or DIA.

Box 1. Ribosomopathies

Ribosomopathies are conditions resulting from abnormalities in RPs, rRNAs, or a subset of related genes. These abnormalities may impact ribosome availability, function, or both [3]. Typically, these result from haploinsufficiency of a RP where one copy of a RP gene is knocked out or nonfunctional and the single remaining copy is insufficient for normal ribosome function [3]. Ribosomopathies target different tissues, each ribosomopathy targeting only one or a few specific tissues, and show a large degree of variation even between patients with the same condition [3,86]. Some of the conditions associated with aberrant ribosomes and RP mutations include the following.

- DBA: A group of conditions defined by specific reduction of erythrocytes. Approximately half of patients exhibit other symptoms, such as cleft palate and cardiac defects. Typically diagnosed early in life, DBA was recently suggested to be a result of low ribosome abundance, with a number of RP mutations linked to the condition, including RPS19, RPS17, and RPS24 [4,24].
- Isolated congenital asplenia: Characterized as the absence of the spleen at birth in the absence of other developmental issues. Linked to haploinsufficiency of RPSA [87,88].
- Neurodevelopmental disorders: RPL10 mutations have been linked to neurodevelopmental conditions including autism spectrum disorders and microcephaly [89].
- Cancer: Patients with ribosomopathies appear predisposed to certain cancers [3]. In addition, ribosomal genes are frequently dysregulated during cancer and mutations in RPs may promote cellular transformation; for example RPL5, RPL10, and RPL21 [35,90,91].

Box 2. Specialized Ribosomes and the Ribosome Code

Recent data support the idea that ribosomes are not simply passive recipients in the translation control process, but in fact play a more central role in translational regulation.

- Ribosome abundance: A major model, also termed the ribosome concentration hypothesis [3], that explains how ribosomes could exert control over host translation by the modulation of ribosome abundance in a cell. As long as ribosome association with mRNAs is a nonlinear function of ribosome concentration, this function will have different slopes for different ribosome concentrations. Thus, the translation rates of mRNAs whose response functions are steeper over the range of concentration changes will be affected disproportionately [2]. This model could explain some of the translation control often attributed to specialized ribosomes. Still, it is limited in the extent to which it could provide regulatory functionality as it can only provide unidirectional translational regulation; that is, as ribosome levels decrease, the translation of all mRNAs decreases, albeit to a different degree [2]. This model may explain some tissue-specific phenotypes if mRNAs change their ribosome association rates in a tissue-specific manner; for example, a particular transcript has high translation-initiation rate in one tissue (and is thus not affected by decreased ribosome availability in that tissue) but a low translation-initiation rate in another tissue (and is thus affected by decreased ribosome availability in that tissue) [3].
- Ribosome heterogeneity: A broad concept reflecting that ribosome composition may vary across different ribosomes. This variation can include the absence of some RPs, modifications of RPs, or modifications of rRNAs [22,92]. This variation does not necessarily imply functional differences. Recent MS data provide strong evidence for ribosome heterogeneity by demonstrating that RPs in purified ribosome fractions are not all present at stoichiometric levels [22]. Since the isolated ribosomes originate from many cells, it remains unclear whether this variation in RP ribosome association extends to within-cell heterogeneity. Within-cell heterogeneity is suggested by single-cell protein measurements but remains inconclusive because the ribosomes were not isolated [64]. Additionally, the observation that some populations of ribosomes exhibit altered stoichiometry does not necessarily mean that such populations are functional or have distinct functions [3]. Where **heterogeneous ribosomes** are confirmed to have distinct functions, these are referred to as specialized ribosomes.
- Specialized ribosomes: The term specialized ribosomes refers to a subset of heterogeneous ribosomes where that heterogeneity has been demonstrated to result in functionally distinct ribosomes with specific roles [3]. Ribosome specialization may take the form of the 'ribosome code'.
- The ribosome code: Refers to the concept that different modifications to the ribosome, such as altered RP stoichiometry, different PTMs to the RPs, or the use of alternative rRNA transcripts or rRNA modifications all combine to combinatorially regulate ribosome function. This is analogous to the 'histone code' hypothesis introduced by David Allis [15]; however, at present the existence of a ribosome code remains unproven.
- We emphasize that the ribosome abundance and specialized ribosome models are mutually compatible. The ribosome abundance model rests on well-validated principles but does not necessarily exclude the existence of specialized ribosomes. Still, it must always be considered first in explaining ribosome-related phenotypes, before invoking the more complex ribosome specialization model.

Monosome: a single ribosome (80S) comprising both small (40S) and large (60S) ribosomal subunits. Isolated monosomes may not necessarily be associated with a translating mRNA and thus monosome populations cannot be assumed to be fully translationally active.
MS1: following liquid chromatography and introduction into a mass spectrometer, MS1 analysis of a peptide reveals its charge state and mass. While this might sometimes reveal the amino acid content of a peptide, sequencing at the MS2 level provides the amino acid order and more definitive identification.
MS/MS (MS2): a peptide of interest, identified at MS1 level, can be isolated in the mass spectrometer and fragmented and the fragment *m/z* ratios analyzed. These fragments form a ladder of ions that can be used to determine the sequence order of amino acids in the peptide.
Polysome: multiple ribosomes present on a single mRNA. Polysome fractions from cells are studied as, unlike monosome fractions, the presence of multiple ribosomes on an mRNA indicates active translation.
Post-translational modification (PTM): common PTMs of proteins include phosphorylation, acetylation, and methylation. The addition or removal of a PTM can cause changes to a protein's structure, binding partners, or function.
Proteoform: a term that describes different modification states of a single protein. For example, unmodified RPS6, and RPS6 phosphorylated at Ser-148 represent different proteoforms of the same protein and potentially possess distinct functions or behavior.
Ribosomal protein (RP): in humans there are approximately 80 RPs.
Ribosome code: the hypothesis that modifications to ribosome stoichiometry or the PTM state of individual RPs can function in a combinatorial manner to generate specialized ribosomes with a high degree of customizability. A similar concept is the histone code.
Ribosomopathy: a pathological condition resulting from a mutation or absence of a particular RP, rRNA, or ribosome biogenesis factor.

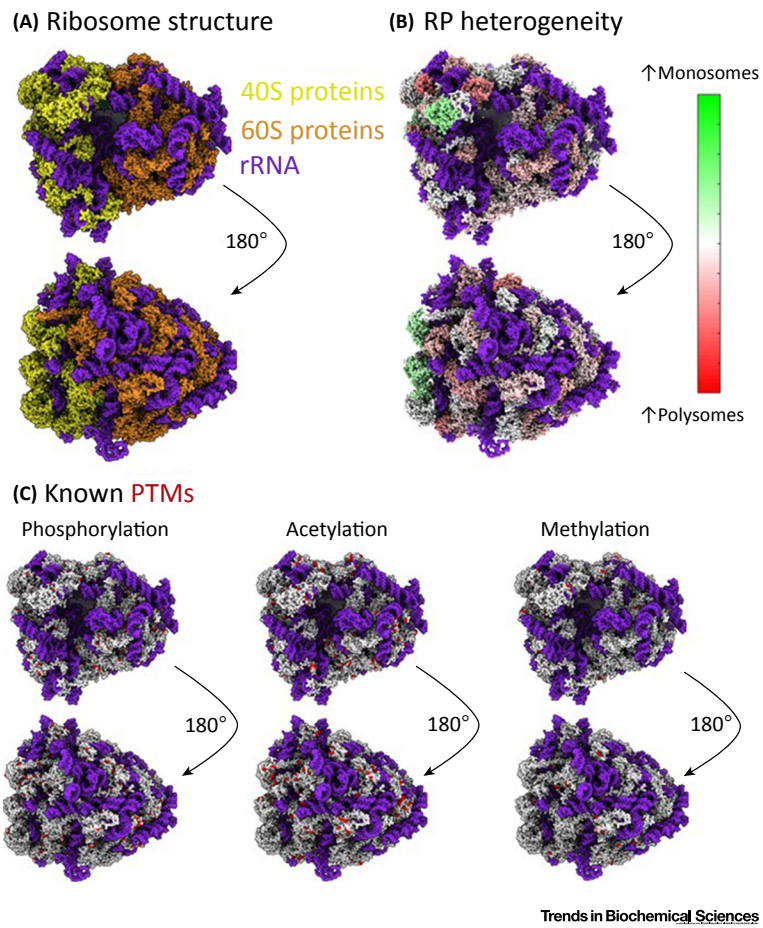

Figure 2. Heterogeneous Ribosomes and Their Post-translational Modifications (PTMs). (A) Ribosomes can be divided into the small (**40S**, shown in gold) and large (60S, shown in bronze) subunits, which in humans comprise four rRNAs (shown in blue) and 80 ribosomal proteins (RPs). (B) Mass spectrometric analysis of human ribosomes reveals that RPs are not all present at stoichiometric levels. (Levels in monosomes compared with polysomes, unpublished data, U-937 human monocyte cells.) (C) RPs are highly modified with over 2500 modifications listed in PhosphoSitePlus [66] as of January 2018. The most abundant RP modifications currently known are phosphorylation, acetylation, and methylation. Modification sites are shown in red. The human ribosome structures presented here were generated using PDB structure 5T2C [85] in the UCSF ChimeraX software.

Stable isotope labeling of amino acids in cell culture (SILAC): a metabolic labeling technique permitting relative quantification of the proteins in a sample by MS at the MS1 level.

Tandem mass tags (TMT): an isobaric labeling method allowing multiplexing and quantification of multiple samples by MS. Unlike SILAC quantification, quantification occurs at the MS2 level.

Top down (mass spectrometry): the analysis of intact proteins or complexes by MS, in contrast to the more common 'bottom-up' approach where proteins are first digested to peptides and proteins are identified and quantified based on these peptides.

A partial parallel for the specialization model is found with epigenetics and the '**histone code**', where different **post-translational modification (PTM)** states of the histone proteins can drive activation or repression of transcription [14–17]. Similar to histones, RPs are known to harbor a wide range of PTMs (Figure 2C). The key concept of the histone code hypothesis is that these modifications serve not only to modulate the specific interactions between histones and the DNA but also to recruit accessory factors that can recognize the modified histones, providing further functionality and regulation. These modifications are proposed to function combinatorially with these modified proteins or **proteoforms**, massively expanding the level of control that histones can exert over transcription.

A '**ribosome code**' could function similarly, with modifications to the ribosome-residence or PTM status of RPs, or rRNA modifications, either driving the recruitment of accessory factors

[18] or modifying the mRNA-binding biases of particular RPs and therefore the host ribosome. However, there are features that would distinguish these two regulatory codes. The ribosome combines the roles of both histones and RNA polymerase, and rather than acting in *cis* on the specific gene bound by the histones, the ribosome code would function in *trans* on its target mRNAs. By taking on the additional roles of the polymerase, the ribosome code would offer the potential to control ribosome **elongation** and **error rates** as well as subcellular localization of translation. While the authors of this Opinion article have focused on the roles of RPs and RP modifications, distinct rRNA transcripts and modifications may also contribute to ribosome specialization [7,12,19,20]. Regulation by specialized ribosomes can provide unique advantages for the cell, such as direct integration between cytoplasmic metabolites, and translational regulation [21], lower gene expression noise, spatial localization, and very short timescales (Figure 3).

We review the evidence for ribosome specialization and focus on experiments that can rigorously explore and discriminate between these two models. Future studies of ribosome specialization can benefit from well-formulated hypotheses about the degree of mRNA specificity, the timescale of regulation, and the potential regulatory benefits to the host cell of ribosome specialization.

Evidence for Ribosome Specialization

Wild-type cells make ribosomes with altered stoichiometry [22,23] (Figure 2). Genetic perturbations of RPs have highly specific phenotypes [24–26] (Figure 1 and Box 1), yet it remains possible that such specific phenotypes may be mediated by **extraribosomal** functions of RPs or a general depletion of functional ribosomes that decreases the translation of some transcripts more than others [2,3]. The biochemical evidence for specialized ribosomes fulfilling physiological roles in wild-type cells had until recently remained indirect, mostly limited to differential RP transcript levels. The lack of technologies to accurately identify and quantify proteins limited most early studies of RP stoichiometries in 30S and 50S fractions of bacterial ribosomes purified from sucrose gradients [27,28]. These fractions also contained immature ribosome biogenesis particles [29–31] that complicated the interpretation of measured stoichiometries. More recently, quantitative MS has begun to provide direct evidence for differential RP synthesis [32] and stoichiometry in isolated ribosomes [22]. In addition, advances in cryo-EM make it feasible to identify missing RPs [33].

Regulating Gene Expression

There is substantial evidence that modified ribosomes can specifically alter the translation of particular classes of mRNA or even individual transcripts (Figure 3A) [4,34,35]. Conceivably, each ribosomal structure – characterized by its rRNA and protein composition and their modifications – might be specific to a single mRNA transcript or even transcript isoform. In favor of broader specificity, wild-type cells with ribosomes enriched in RPL10A preferentially translate a subset of mRNAs containing **internal ribosome entry site (IRES)** elements [23]. Recent work in yeast identified RPS26-deficient ribosomes that preferentially bind mRNAs involved in select stress response pathways [36] and Horos *et al.* [37] reported that RPS19 affects the ribosomal density along hundreds of mRNAs essential for the differentiation of murine and human erythroblasts. Other studies also report that RP perturbations can affect the translation of hundreds of genes organized in coherent functional groups [23,38]. RPs are routinely dysregulated in the context of cancer [35,39] and adjusted throughout cell growth and metabolic cycles [40]; for example, by mTOR regulation via 5′ terminal oligopyrimidine (TOP) motifs in RP mRNAs [41].

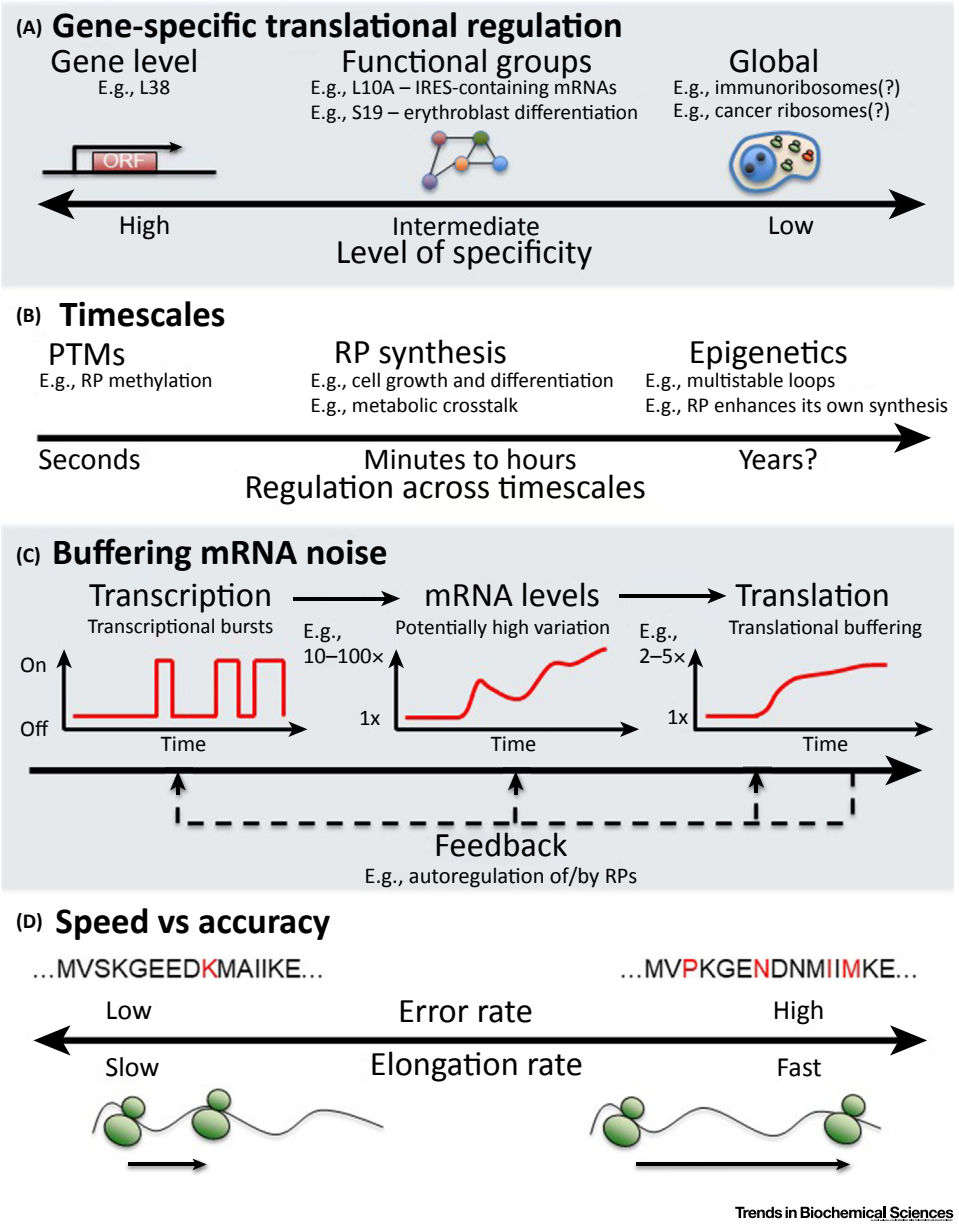

Figure 3. Ribosome Specialization. If populations of ribosomes exhibit distinct phenotypes, there are multiple ways in which these functional differences could exist. (A) Distinct ribosome subpopulations could have a range of specificities for their mRNAs. These could be from the individual mRNA level to global translational regulation. (B) The timescale at which changes to ribosomal protein (RP) stoichiometry or post-translational modifications (PTMs) could exert effects on translation can potentially range from extremely rapid/seconds (especially in the case of PTMs) to the very long term (e.g., years). (C) mRNA expression is noisy and buffered at the level of translation. (D) The elongation rate of a ribosome represents a tradeoff between speed and accuracy. Further, the elongation rate is not constant on a given mRNA, with some sections of an mRNA being translated more rapidly than others.

More limited examples exist for mRNA-specific ribosomal regulation of translation. Barna and colleagues have suggested that RPL38 affects specifically the synthesis of only three proteins. However, the authors did not measure genome-wide translation so the possibility that the synthesis of other proteins is altered as well cannot be excluded [38,42]. Loss of RPS25 also resulted in inhibition of viral IRES-based translation, although not cap-dependent cellular translation [43]. The interferon-gamma-regulated release of RPL13a from the ribosome is also postulated to impact the translation of around 50 genes [44,45]. These findings argue for a very high degree of specificity. This ability to preferentially translate individual or functional clusters of mRNAs could also allow the cell to help control localized translation by targeting the ribosomes responsible to specific subcellular destinations [46,47]. Targeting of mRNAs, either individually or in groups, can be achieved through recognition of mRNA motifs, such as the aforementioned TOP motifs [41], or structures in the untranslated regions of mRNAs [48].

Many of these data on the mRNA specificity of specialized ribosomes were obtained using sucrose gradient fractionation or immune enrichment and thus reflect population averages over all ribosomal structures in each sucrose fraction and are likely to capture only general trends that affect a large fraction of ribosomes and mRNAs, not ribosomal structures with single-gene specificity. Ribosomes vastly outnumber mRNA molecules in mammalian cells. If mRNA-specialized ribosomes play an important role, even modest changes to the ribosome population identified from sucrose gradient fractions could exert a significant effect.

A further benefit of mRNA-specific ribosomes could be in buffering mRNA noise (Figure 3C). Gene expression noise tends to be dominated by transcriptional noise due to transcriptional bursts and low-copy-number mRNAs. This can clearly be seen when examining transcriptomic and proteomic data from the same experimental system, with 10–100-fold changes in mRNA levels resulting in comparatively modest protein level changes. If post-transcriptional mechanisms did not actively buffer mRNA variability, these large-fold changes would propagate to the protein levels. One buffering mechanism may involve miRNAs or other translational regulators such as RNA-binding proteins (RBPs) [49,50]. Others may involve proteins interacting with specialized ribosomes and exerting direct feedback on the translation of their mRNAs. Good candidates for this mechanism of noise reduction are the RPs themselves.

RP levels correlate very poorly with their corresponding mRNA levels [51]. This poor correlation may reflect many post-transcriptional mechanisms, such as protein degradation. When RPL3 mRNAs is transcribed 7.5 times as much as in wild-type cells, RPL3 levels increase by less than 20% [52–55]. A particularly intriguing mechanism could be that some RPs, when incorporated into ribosomes, inhibit the translation of their own mRNAs, thus providing an efficient feedback loop.

Speed versus Accuracy: The Elongation and Error Rates of the Ribosome

While altered RP stoichiometry may influence which mRNAs a specific ribosome may bind, it could also allow the modulation of the ribosome's behavior once it has bound a target mRNA; for example, the elongation rate of the ribosome. The elongation rate is usually understood as a tradeoff between the speed of translation and accuracy, with improved accuracy benefiting from a lower elongation rate via **kinetic proofreading** [56,57] (Figure 3D). Elongation rates have been understood to be variable for decades [58], with cellular tRNA pools impacting the relative and local elongation rates [59,60].

Lipopolysaccharide treatment of monocytes altered the translation rates of hundreds of proteins [32]. This was especially true for **housekeeping proteins**, which are generally highly

expressed and understood to be more translationally robust [61,62]. While fold changes were dominated by altered mRNA levels, absolute protein abundance was dominated by altered translation and degradation rates. The ability to tune the elongation rate in response to changing conditions would give cells the ability to produce certain proteins more rapidly, albeit with higher error rates.

Identifying Ribosome Heterogeneity

Recent advances have begun to demonstrate functional specialization of ribosomes within species. We have demonstrated differences in RP stoichiometry in ribosomes purified from wild-type cells [22], although the functional specificity is implied by a correlation, not shown by direct measurement. Even the prominent example suggesting ribosome specialization, RPL38 regulation of HOX genes [38,42], falls short of direct proof since: (i) its exclusive specificity to 3 HOX is implied and not directly measured; and (ii) the existence of ribosomes lacking RPL38 in wild-type cells is assumed, not measured. However, more recent data identified distinct mRNA subsets exhibiting enriched or diminished ribosome association with ribosomes enriched for RPL10a [23], and Ferretti and colleagues demonstrated a specific role for RPS26-containing ribosomes [36]. A rigorous experimental proof of specialization should demonstrate functional specialization of distinct ribosomal structures/compositions found in wild-type cells. Dynamic settings, such as a time-course or differentiation protocol, offer the most straightforward means of inducing heterogeneity in a well-controlled framework, thus minimizing the potential for introduction of artifacts. A first requirement is to identify what variation in RP stoichiometry exists in the system under study.

The separation of translating ribosomes on a sucrose gradient is a long-established method in the translation field. It allows the isolation of intact ribosomes and by isolating individual peaks along the gradient, comparisons can be made between the composition of the various **monosome** and **polysome** fractions. MS approaches (Box 3) using isobaric or metabolic labeling can be applied to these fractions to yield data on the relative abundances of core and ribosome-associated proteins. We successfully applied this approach in [22], identifying differences in monosome and polysome RP stoichiometry as well as between fractions isolated from cells following stress, such as yeast grown in ethanol or glucose (Figure 4A). However, the method has limited resolution for separating ribosome populations and the underlying populations do not represent pure ribosomal populations, instead representing different levels of individual ribosome subpopulations.

Subcellular compartmentalization of specific ribosome populations is an emerging area for research. Advances in methods for detecting ongoing translation in cells have helped underline subcellular variation in translation [47]. While it is generally taught that ribosomes are free cytoplasmic or ER associated, translation can be found localized near synapses in neuronal cells, sequestered in virus factories following infection, or even in the nucleus. Subcellular fractionation or purification methods (e.g., LOPIT [63]) could therefore be applied to distinguish ribosome populations of interest. A further area where heterogeneity could exist is between individual single cells. Our group recently made advances in this area with the advent of a first method for performing single-cell MS on average-sized mammalian cells [64]. The data suggested altered RP stoichiometry between the two cell lines under study. However, in the case of both single-cell and subcellular localization-based approaches, it is uncertain whether the RPs demonstrating altered abundance are incorporated into fully assembled ribosomes, and therefore follow-up experiments would be required to determine whether changes determined in whole-cell lysates are representative of assembled ribosomes.

Box 3. MS Approaches to Studying Specialized Ribosomes

MS approaches offer a powerful tool with which to characterize functional specialization of ribosomes.

Identifying Heterogeneity

- Bottom up and top down: Methods based on liquid chromatography coupled to tandem MS (LC-MS/MS) allow the quantification of RPs with high throughput, specificity, and accuracy [22,23,92,93]. These methods can be broadly divided into two categories. The first is **bottom-up** proteomics, which involves quantifying peptides resulting from the digestion of proteins with one or more proteases such as trypsin or Lys-C [94]. The second is top-down proteomics, which involves quantifying whole proteins or complexes such as ribosomes. Most current research on ribosomes is conducted with bottom-up approaches since these methods are currently more robust and accessible than top-down methods [75].
- Quantification of relative RP abundance: Capturing the true heterogeneity of ribosomes poses a challenge for their LC-MS/MS analysis. Even when purified (e.g., by sucrose gradients), the ribosomes represent mixed populations. Measurements in such populations average across the heterogeneity. This averaging can substantially diminish the observed alterations to RP stoichiometry to the limits of detection and quantification of existing methods [22]. The three major methods for MS-based quantification are **label free**, metabolic labeling approaches such as **stable isotope labeling of amino acids in cell culture (SILAC)**, and isobaric labeling approaches such as **tandem mass tags (TMT)** or iTRAQ [94,95]. Metabolic and isobaric labeling approaches permit relative comparison of multiple samples in the same run. This enables both increased multiplexing and canceling out of sources of noise (e.g., variations in LC and ionization efficiency) when estimating the relative changes in a protein across samples [96]. Metabolic labeling approaches produce a shifted pattern of isotopic peaks for the peptide at the **MS1** level. Relative quantification is achieved by comparing the peak areas for the unlabeled and labeled peptides. Isobaric labeling approaches including TMT result in labeled peptides that migrate identically at the MS1 level but release unique reporter ions that can be quantified during **MS/MS** following peptide fragmentation [94].

Determining Functional Specialization

- Protein synthesis: Stable isotope-based pulse-labeling approaches provide the most direct means of measuring the synthesis and degradation rates of thousands of proteins when analyzed by MS [97]. The most recent iterations of this approach have combined SILAC pulse labeling with enhanced multiplexing through the use of TMT reagents [68].
- Mistranslation rates: Current estimates for mistranslation rates are on the order of 10^{-3} to 10^{-4}; however, a recent preprint has suggested that the sensitivity of modern mass spectrometers and data-processing algorithms can be used to investigate the error rate of protein synthesis [73].

Once RPs exhibiting altered stoichiometry are known, specific isolation of more homogenous ribosome populations can be attempted. Methods for this include affinity purification (Figure 4B). A caveat with this approach is that simple affinity purification will isolate both ribosome-associated and free RPs. Particularly in the case of epitope-tagged RPs, the incorporation of the tagged RP into the ribosome may be poor relative to the endogenous RP. Several means exist to ameliorate these issues. Prior removal of nuclei followed by affinity purification will reduce the background from incomplete/assembling ribosomes but still yield a mix of ribosome-associated and free RPs. An improved approach would combine sucrose gradient centrifugation and affinity purification, with the affinity purification being conducted on pooled or individual gradient fractions thus ensuring the isolated protein was derived from intact ribosomes (Figure 4C).

RPs are host to a huge array of PTMs [65], with over 2500 modifications of core human RPs known [66] (Figure 2C). The identification of RP PTMs represents an extension of the methods required to investigate RP stoichiometry. Large-scale PTM screens can be conducted by MS by enriching for individual PTMs such as phosphorylation, methylation, and acetylation, which represent a majority of currently known RP PTMs. One additional consideration when investigating RP PTMs is the case where the addition of a PTM induces the loss of ribosome association for the modified RP. A known example of this is L13a, where phosphorylation at Ser-77 is associated with dissociation from the **60S** ribosomal subunit [44]. Thus, the inclusion of either whole-cell lysate or soluble cytoplasmic extracts prepared from the same

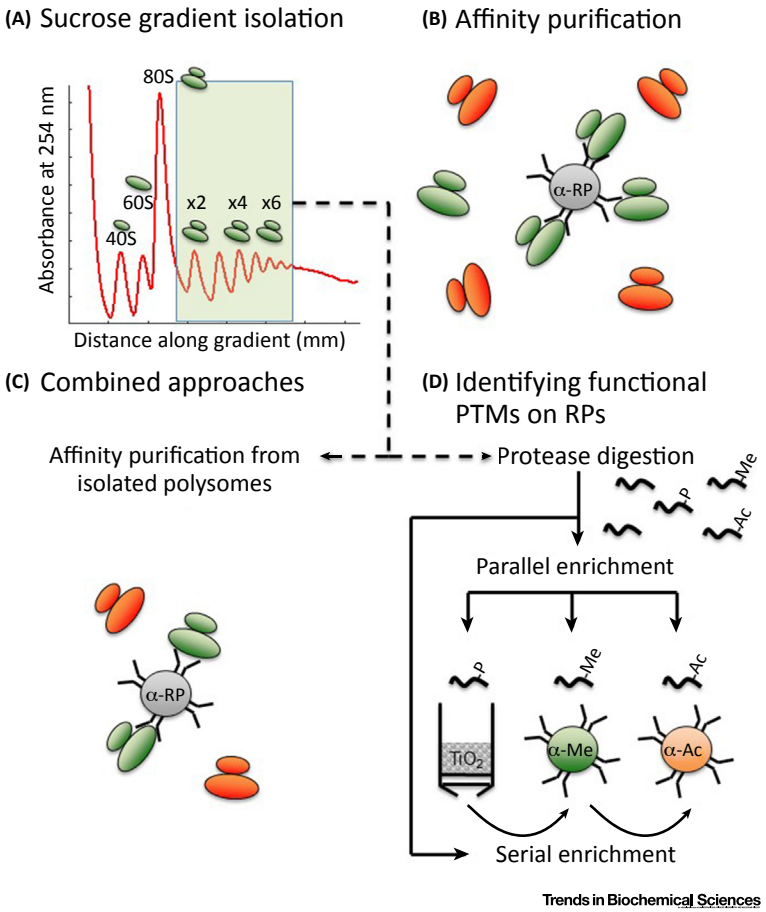

Figure 4. Identifying Altered Ribosomal Protein (RP) Stoichiometry and Post-translational Modifications (PTMs). (A) For decades, the gold-standard approach for isolating RPs in the context of intact, functional ribosomes has been sucrose gradient centrifugation. (B) Affinity purification is a powerful means of identifying differential RP association with complexes; however, it cannot definitively say whether an RP is resident in a ribosome or represents an extraribosomal population of the RP. (C) A combined approach whereby affinity purification is performed on sucrose gradient fractions allows the advantages of affinity purification to be applied to samples where the RP is known to be ribosome resident. (D) Heterogeneity among RP modifications is a promising new area of research, and the methods required to explore this are an extension of those for identifying changes in RP association. Protease-digested peptides from sucrose gradient fractions or affinity purification can be enriched for a particular PTM of choice either individually or serially, whereby the flow-through of one enrichment is applied to the next enrichment process.

cells used for sucrose gradient fractionation would allow determination of whether PTM status is affecting the ribosome association of the RP. Ideally, PTM enrichment should be performed on the same samples used for investigating RP stoichiometry, allowing the inference of PTM stoichiometry [67]. Functional validation of the impact of PTMs could be determined using inhibitors, knockout, or mutagenesis approaches and examining their impacts on the outputs described above.

Demonstrating Functional Specialized Ribosomes

A conclusive demonstration of altered RP stoichiometry does not prove functional specialized ribosomes. A key task is the identification of outputs that can be directly attributed to the ribosome itself rather than noise from transcriptional or translation initiation events, which may

also be influenced by a perturbation of choice. Ideally, several outputs would be examined, as illustrated in Figure 5A. Pulsed time-course experiments have been employed for decades in the study of protein synthesis and turnover and nonradioactive versions are amenable to MS-based analysis (Box 3). These approaches allow the investigation of the turnover and degradation rates of thousands of proteins, with a recent study characterizing the dynamics of over 6000 proteins [68,69]. However, protein synthesis rate per mRNA can change not only because of ribosome remodeling but also because of translation factors affecting translation

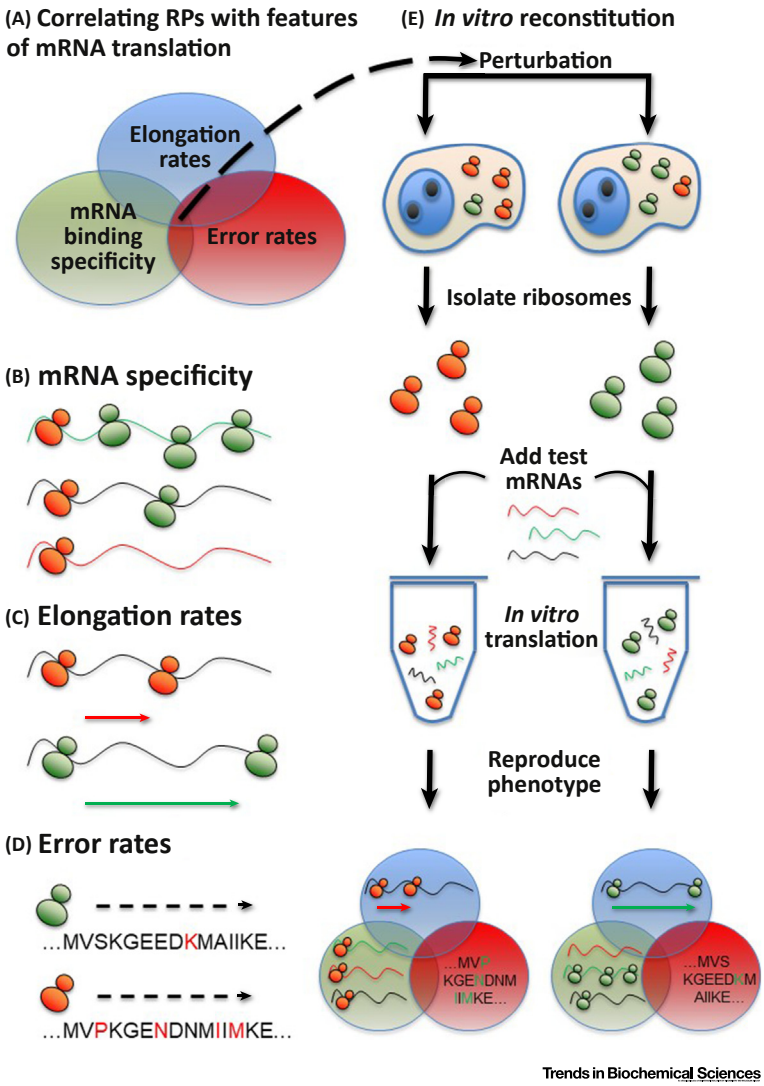

Figure 5. Testing for Functional Specialization. (A) To experimentally prove ribosome specialization, several outputs for measurement stand out. These are the mRNA binding specificity, elongation rates, and error rates. A conclusive demonstration of functional ribosome specialization will be likely to employ several or all of these. (B) If specific mRNAs are favored by individual ribosome conformations, this can be assessed by immunoprecipitation with tagged RPs. (C) Elongation rates for the ribosome on particular mRNA substrates can be estimated from pulse–chase data. (D) The error rate for individual ribosomes can be monitored using luminescent or fluorescent reporters for specific substrates or in a higher-throughput manner by mass spectrometry. (E) Functional validation of specialized ribosomes can be investigated through *in vitro* reconstitution of the phenotype.

initiation and elongation. It therefore may provide a potential functional readout rather than definitive confirmation of functional specialization. A similar claim can be made for investigating the association of specific mRNAs with ribosome subsets following a perturbation. mRNA specificity represents a key area where ribosome specialization could play a role (Figure 5B). However, the degree of association of an individual mRNA with specific ribosomes can be determined not only by increased affinity of specialized ribosomes for the mRNA but also by altered mRNA abundance and translation initiation factors.

For a definitive result, the elongation and error rates stand out for investigation because they relate directly to ribosome activity, although they can still be influenced by *trans* factors [70] (Figure 5C,D). One possibility makes use of the inhibitor harringtonine, which stalls translation at the initiation codon. Using a modification of the widely adopted ribosome profiling method [71], reduced ribosome density on a given mRNA at extending intervals after the addition of harringtonine are used to calculate the average time it takes a ribosome to completely traverse an mRNA. When the length of the mRNA is known this can be used to calculate the elongation rate [72], although this method has yet to be widely adopted by the translation community.

The error rate of the translating ribosome also offers a promising target for the investigation of ribosome specialization (Box 3). Typically, such assays are low throughput and rely on stop codon readthrough or frameshift/coding errors to generate a detectable signal, usually by a luciferase or fluorescent reporter. These methods are very context dependent and may therefore miss trends in error rates outside their specific context. However, a recent preprint has suggested a possible MS-based approach [73] to identify mistranslation products. While the sensitivity of the approach may limit it to studies of the more abundant mistranslation products, the authors' data included altered error rates following perturbations such as amino acid starvation and the addition of an antibiotic known to affect the ribosomal proofreading function, suggesting that the method holds promise as a high-throughput means of investigating ribosomal error rates.

Complementary Approaches

MS represents a powerful tool for the investigation of RP stoichiometry, although ultimately its conclusions are drawn from mixed, albeit enriched, populations of ribosomes. Single-molecule methods and imaging offer a powerful means of identifying the precise composition of individual ribosomes. Recent work highlighted how cryo-EM could be used to map the proportions of yeast ribosomes containing or lacking RPL10 and RPS1A/B [33]. Alternative approaches include super-resolution microscopy, which would allow imaging of ribosomes directly in cells. It does require fluorophore labeling, which can be limited in throughput by epitope occlusion or lead to artifacts if fluorescent proteins are used. Alternatively, **top-down** MS approaches, where intact proteins or complexes can be analyzed to determine structural and conformational information, have also begun to identify altered ribosome compositions [74,75]. This ability to precisely define specific, individual ribosome conformations will be invaluable in proving true RP heterogeneity in single cells.

Finally, while the above methods can validate the existence of altered RP stoichiometry and of functional ribosome specialization in cells, there remains a large degree of overlap where the impact of the ribosome and of other, linked translational events can contribute to this heterogeneity. The ability to extract specific ribosome conformations from cells and reproduce translational phenotypes *in vitro* is key (Figure 5E). Various methods for preparing translational components from cells are known, ranging from crude preparations [76] to methods requiring

extensive fractionation [77–81]. The reproduction of specific translational phenotypes present in cells, including mRNA specificity, elongation, and error rates, with specific ribosomes *in vitro* offers the most stringent demonstration of functional ribosome specialization.

Concluding Remarks: Ribosome Specialization – More Than Just RPs

We have focused on the impact of RP stoichiometry on ribosome function. However, equally important and interesting are modifications of the rRNAs that may also confer ribosome specificity, as discussed by Mauro and Matsuda [7]. rRNA isoforms are expressed in tissue-specific patterns [12], complementing observations of cell-specific RP transcripts [82,83]. rRNAs exhibit extensive and pervasive variation at the level of rDNA between individuals [12] and rRNA modifications were identified at substoichiometric amounts in recent studies [19,20]. Technological advances such as the ability to directly sequence full-length RNA molecules and identify modifications through the use of nanopore sequencing [84] could be combined with the above proteomic approaches to investigate rRNA heterogeneity and function to obtain a more complete perspective on the constellation of features that distinguish individual ribosomes and their function.

Outstanding Questions

To what degree do specialized ribosomes contribute to the regulation of protein synthesis in wild-type cells? Is this a common or niche regulatory mechanism?

How mRNA specific are specialized ribosomes? Are there ribosomes for specific isoforms or splice variants of an mRNA?

Do ribosomes exist in wild-type cells with distinct elongation or error rates? Are these regulated (or dysregulated) in response to stress or stimuli?

How much of a role do tissue-specific specialized ribosomes play in differentiation and cell-specific translation?

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