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Ribosome stoichiometry: from form to function.

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

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

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Abstract
The existence of eukaryotic ribosomes with distinct ribosomal protein (RP) stoichiometry and regulatory roles in protein synthesis been speculated for over sixty years. Recent advances in mass spectrometry and high throughput analysis have begun to identify and characterize distinct ribosome stoichiometry in yeast or mammalian systems. In addition to RP stoichiometry, ribosomes play host to a vast array of protein modifications, effectively expanding the number of human RPs from 80 to many thousands of distinct proteoforms. Is it possible 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'.

Ribosomes, the cellular machinery of protein synthesis, are present at up to ten million copies per cell in mammals. Despite their abundance and the wide array of known modifications to both ***ribosomal proteins*** (RPs) 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**, color-coded by the type of evidence.

For many years, the dominant **'abundance' model** of translational regulation by the ribosome suggested a limited role for ribosomes in translation regulation [2]. In this model, if ribosomes have different initiation affinity 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 [4], relies on the non-linear dependence of translation initiation on free ribosomes and applies quite generally. Indeed, recent research from the Sankaran lab suggested this mechanisms could explain the failure of erythroid lineage commitment seen with Diamond-Blackfan anaemia [5]. However, other experiments performed in yeast suggested this model is insufficient in wild-type cells [6]. Translational regulation

by the abundance model is limited in magnitude by the changes of total ribosomal content and in flexibility since it provides unidirectional regulation for all proteins.

The concept of ribosome specialization

In the **'specialized' ribosome model**, ribosomes do not possess constant structure or composition (**Figure 2A, B**), and instead exhibit altered stoichiometry of what were previously thought to represent 'core' ribosomal proteins (**Figure 1**) [7,8]. In this model, different ribosomal compositions are functional and have specific roles in translation. Specialized ribosomes could co-exist within cells, or between different cells or tissues [9,10]. **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.

A partial parallel for the specialization model is found with epigenetics and the '***histone code***', where different ***post-translational modification*** (PTMs) states of the histone proteins can drive activation or repression of transcription [11–14]. 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 which can recognize the modified histones, providing further functionality and regulation. These modifications are proposed to function combinatorically with these modified proteins or ***proteoforms*** massively expanding the level of control 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, driving either the recruitment of accessory factors [15], 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 the 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. Whilst the authors of this review have focused on the roles of RPs and RP modifications, distinct rRNA transcripts and modifications may also contribute to ribosome specialization [8,9,16,17]. Regulation by specialized ribosomes can provide unique advantages for the cell – such as direct integration between cytoplasmic metabolites, and translational regulation [18], 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 conceptual models. We will not focus on the molecular aspects of previous work that have been extensively reviewed [8,19–22]. Future studies of ribosome specialization can benefit from well formulated hypotheses about the degree of mRNA-specificity, the time-scale of regulation, and the potential regulatory benefits to the host cell of ribosome specialization. Thus, we highlight previous research with a focus on formulating hypotheses that can guide future experiments.

Evidence for ribosome specialization

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Wildtype cells make ribosomes with altered stoichiometry [23,24], and this is illustrated in **Figure 2**. Genetic perturbation of RPs have highly specific phenotypes [25–27] (**Figure 1**).Yet it remains possible that such specific phenotypes may be mediated by extra-ribosomal 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 wildtype 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 40S fractions of bacterial ribosomes purified from sucrose gradients [28,29]. These fractions also contained immature ribosome biogenesis particles [30–32] that complicated the interpretation of measured stoichiometries; furthermore, difficulties of protein identification made it hard to distinguish between core RPs and auxiliary factors [28,29]. More recently, quantitative mass-spectrometry (MS) has begun to provide direct evidence for differential RP synthesis [33] and differential RP stoichiometry in isolated ribosomes [23]. In addition, advances in cryo-EM make it feasible to identify missing RPs [34].

Regulating gene expression

There is substantial evidence that modified ribosomes can specifically alter the translation of particular classes, or even individual mRNAs (**Figure 3A**). Much of this evidence is in the context of mutation or knockout settings rather than in wild-type cells [5,35,36]. Conceivably, each ribosomal structure – characterized by its rRNA and protein composition, and their modifications – might be specific to a single mRNA transcript, or even a single transcript isoform. In favor of broader specificity, Zhen Shi and colleagues recently demonstrated this can occur in wild-type cells with ribosomes enriched in RPL10A preferentially translating a subset of mRNAs containing ***IRES*** elements [24]. Recent work in yeast also identified RPS26-deficient ribosomes that preferentially bind mRNAs involved in select stress response pathways by selecting mRNAs with deviations in their Kozak sequence at the -4 position [37]. Horos *et al.*, 2012 [38] reported that a single RP, S19, 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 [24,39]. RPs are routinely dysregulated in the context of cancer [36,40], and adjusted throughout cell growth and metabolic cycles [41]. Our measurements of differential RP stoichiometries of ribosomes isolated via sucrose gradients also suggest broader mRNA specificity [23].

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 [39,42]. Loss of RPS25 also resulted in inhibition of viral IRES-based translation, though 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].

Many of these data on the mRNA-specificity of specialized ribosomes were obtained using sucrose gradient fractionation or immune enrichment, and thus these data reflect population averages over all ribosomal structures in each sucrose fraction, and likely 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 microRNAs or other translational regulators such as RNA binding proteins (RBPs) [48]. Another mechanism may involve proteins interacting with specialized ribosomes and exerting direct feedback on the translation of their mRNAs and thus buffer the inevitable transcriptional noise. Good candidates for this mechanism of noise reduction are the RPs themselves.

RP levels correlate very poorly to their corresponding mRNA levels [49]. This poor correlation may reflect many posttranscriptional mechanisms, such as protein degradation. Indeed, when RPL3 mRNAs is transcribed 7.5 times as much as in wildtype cells, the RPL3 level increases by less than 20% [50–53]. A particularly intriguing mechanism could be that some RPs, when incorporated into ribosomes, inhibit the translation of their own mRNAs, thus providing an efficient functional feedback-loop that buffers mRNA variability.

Speed vs. Accuracy: The elongation and error rates of the ribosome

Whilst altered RP stoichiometry may influence which mRNAs a specific ribosome may bind, it could also allow for modulation of how the ribosome behaves 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*** [54,55] (**Figure 3D**). Elongation rates have been understood to be variable for decades [56], with cellular tRNA pools impacting the relative and local elongation rates [57,58]. Similarly, the ***error rate*** of the ribosome could be adjusted.

Data presented in Jovanovic *et al.*, 2015 [33] showed that translation rates of hundreds of proteins were altered in response to lipopolysaccharide treatment of monocytes. This was especially true for ***housekeeping proteins*** which are generally highly expressed and understood to be more translationally robust [59,60]. Indeed, 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 permit 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 a species. For example, we have demonstrated differences in RP stoichiometry in ribosomes purified from wildtype cells [23], though the functional specificity is implied by a correlation, not shown by direct measurement. Even the prominent example suggesting ribosome specialization, RPL38 regulating HOX genes [39,42], 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 wildtype cells is assumed, not measured. However, more recent data from the Barna lab identified distinct mRNA subsets exhibiting enriched or diminished ribosome association with subsets of ribosomes enriched for RPL10a [24], and Ferretti & colleagues demonstrated a specific role for RPS26-containing ribosomes [37]. These data had the advantage that the ribosomes and their mRNA specificity were identified from transgenic cells, rather than knockouts or a disease state. A rigorous experimental proof should demonstrate functional specialization of distinct ribosomal structures/compositions found in wildtype cells. Dynamic settings, such as a time course or differentiation protocol, offer the most straightforward means of inducing heterogeneity within a well-controlled framework, thus minimizing the potential for introducing 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 has the key advantage that 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. Mass spectrometry approaches using isobaric or metabolic labeling can be applied to these fractions to yield data on the relative abundance of core and ribosome-associated proteins. We successfully applied this approach in Slavov *et al.*, 2015, 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**). While sucrose gradients allow fractionation of ribosomes, the method has limited resolution for separating ribosome populations and the underlying populations do not represent pure ribosomal populations, and instead represent different levels of individual ribosome sub-populations. For example, every mRNA must be present in the monosome fraction at some point during its translation as there must always be a first ribosome to bind the mRNA.

Different variations on this approach include the application of targeted proteomic assays to yield data on the absolute, rather than relative abundance of individual RPs, though the more time-intensive nature of this method has to date limited it to a subset of core ribosomal components [24]. A difficulty with mass spectrometry-based analysis of ribosomes taken from sucrose fractions, is that the ribosome obtained represent mixed populations. As such, the observed alterations to RP stoichiometry are usually slight and at the limit of the methods available for detection and quantification. Whilst isobaric tagging approaches such as ***TMT*** allow for greater multiplexing, they suffer from quantification errors due to co-isolation with other peptides during MS/MS [61]. Ways to mitigate these issues include repetition of MS analysis with an alternate protease, thereby producing different peptides and changing the patterns of co-isolation during MS/MS [23]. Alternatively, repeating the experiments, or a select subset with ***SILAC***-based analysis [62] allows for quantification at the MS1 level. SILAC-based quantification has its own weaknesses but since they are independent

from those of TMT approaches, it can serve as a rigorous cross validation of TMT data, albeit at reduced multiplexing capabilities.

Subcellular compartmentalization of specific ribosome populations is also an emerging area for research. Advances in methods for detecting ongoing translation in cells such as puromycylation have helped underline subcellular variation in translation [47]. Whilst ribosomes are generally taught as being 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. Localisation of organelle proteins by isotope tagging (LOPIT) could therefore be applied to distinguish ribosome populations of interest [63]. 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 mass spectrometry on average-sized mammalian cells [64]. Indeed, the data suggested altered RP stoichiometry between the ribosomes from the two cell lines under study. However, in both the case of single cell, and subcellular localization-based approaches, it is uncertain whether the RPs demonstrating altered abundance are incorporated into fully assembled ribosomes or not, and therefore follow-up experiments would be required to determine whether changes determined in whole cell lysates are representative of assembled ribosomes.

Once RPs exhibiting altered stoichiometry are known, specific isolation of more homogenous ribosome populations can be attempted. Methods for this include affinity purification either by use of epitope tagged RPs, or using an antibody directed against the endogenous RP (**Figure 4B**). A caveat with this approach is that a 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 RP. Whilst this has the potential to yield useful data of its own, a ‘gold standard’ 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**). Quantification could then be performed by either of the relative or absolute quantitative methods described above.

RPs are host to a huge array of PTMs [65], with over 2500 modifications of core human RPs listed in Phosphositeplus [66] as of February 2018 (**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 mass spectrometry by enriching for individual PTMs such as phosphorylation, methylation and acetylation which represent a majority of currently known RP PTMs. Sample preparation approaches such as SEPTM (illustrated in **Figure 4D**) allow for serial enrichment of different PTMs and thus a fuller view of the ribosomal PTM state [67]. One additional consideration when investigating RP PTMs is the case where the addition of a PTM induces the loss of ribosome association of 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]. As such, the inclusion of either whole cell lysate or soluble cytoplasmic extracts prepared from the same cells used for sucrose gradient

fractionation would allow comparison and determination of whether PTM status is affecting 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 [68]. Functional validation of the impact of PTMs could be determined using inhibitors, knock-out 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 ribosome. A key task in demonstrating the functional divergence of specialized ribosomes, is in the identification of outputs which 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 non-radioactive versions of this approach using stable isotope labeling are an established method in proteomics. These approaches allow for the investigation of the turnover and degradation rates of thousands of proteins, with a recent study combining SILAC and TMT labeling characterizing the dynamics of over 6000 proteins [69,70] However, protein synthesis rate per mRNA can change not only because of ribosome remodeling but also because of translation factors affecting translation 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, two ribosomal attributes stand out for investigation because they relate directly to ribosome activity, though can still be influenced by trans-factors [71]. These are the elongation and error rates of the ribosome (**Figure 5C, 5D**). 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 [72], reduced ribosome density on a given mRNA at extending intervals after the addition of the inhibitor 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 [73], though 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 investigating ribosome specialization. Typically, such assays are low-throughput, and rely on stop codon readthrough or frameshift/coding errors to generate a detectable signal, typically by a luciferase or a fluorescent reporter. These systems have been used for a number of years, however these methods are very context-dependent and may therefore miss trends in error rates outside their specific context. A higher throughput means of detecting error rates is therefore highly desirable. A recent preprint has suggested a possible mass spectrometry-based approach [74], by exploiting the Maxquant software's [75] ability to perform a blind peptide modification

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search. While the sensitivity of the approach may limit it to study of the more abundant mis-translation error products, the authors data included altered error rates following perturbations such as amino acid starvation and the addition of an antibiotic known to effect ribosomal proofreading function, suggesting the method holds promise as a high-throughput means of investigating ribosomal error rates.

Complementary approaches

Mass spectrometry represents a powerful tool for investigating RP stoichiometry, though 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 from Ming Sun and colleagues [34] highlighted how mass spectrometry measurements could be combined with cryo-EM to map the changing proportions of yeast ribosomes containing the non-functionally interchangeable paralogs RPL8A and RPL8B, or lacking RPL10 and RPS1A/B following changes to the growth media. However, in this example the proteins identified by mass spectrometry and cryo-EM differed. Alternative or complementary 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 as used. Alternatively, top-down mass spectrometry approaches, where intact proteins or complexes can be analyzed to determine structural and conformational information have also begun to identify altered ribosome compositions [76]. This ability to precisely define specific, individual ribosome conformations, will prove invaluable for proving true RP heterogeneity within single cells.

Finally, whilst 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 [77], to methods requiring extensive fractionation [78–82]. 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.

Ribosome specialization: more than just RPs

We have focused on the impact of RP stoichiometry for ribosome function. However, equally important and interesting are modifications of the rRNAs that may also confer ribosome specificity as discussed by Mauro and Matsuda, 2016 [8]. Indeed, rRNA isoforms are expressed in tissue-specific patterns [9], complementing observations of cell-specific RP transcripts [83,84]. rRNA's exhibit extensive and pervasive variation at the level of rDNA between individuals [9], and rRNA modifications were identified at sub-stoichiometric amounts recent studies [16,17]. Technological advances such as the ability to directly sequence full-length RNA molecules and identify modifications through the use of nanopore sequencing [85], could be combined with the above proteomic approaches to investigate rRNA heterogeneity and function in order to obtain a more complete perspective on the constellation of features that distinguish individual ribosomes and their function.

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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.

Elongation rate: The rate at which the ribosome is able to extend the growing polypeptide chain as it proceeds along a mRNA.

Error rate: The rate at which the incorrect amino acid is mis-incorporated into elongating polypeptide chain by the ribosome.

Extra-ribosomal: Some RPs are proposed to have roles within the cell separate to their role in forming part of the ribosome. When these proteins are found outside the ribosome they are termed extra-ribosomal.

Heterogenous ribosomes: Ribosomes possessing variation in their RP, rRNA or PTM stoichiometry. Specialized ribosomes are a subset of heterogenous ribosomes where the heterogeneity has been linked to specific functions.

Histone code: The 'histone code' is the hypothesis that post-translational modifications of histone proteins function combinatorically to provide highly customizable control of transcription.

Housekeeping protein(s): Proteins required for the basic functioning of the cell. Constitutively expressed, often to high levels.

IRES: Internal ribosome entry site. A 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.

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 as such monosome populations can not be assumed to be fully translationally active.

Polysome: Multiple ribosomes present on a single mRNA. Polysome fractions from cells are studied as unlike monosome fractions, the presence of multiple ribosomes on a mRNA indicates active translation.

Proteoform: A term that describes different modification states of a single protein. For example unmodified RPS6, and RPS6 phosphorylated at

Serine 148 represent different proteoforms of the same protein, and potentially possess distinct functions or behavior.

PTM: Post-translational modification. Common post-translational modifications of proteins include phosphorylation, acetylation and methylation. The addition or removal of a PTM can cause changes to a proteins structure, binding partners or function.

Ribosome code: The hypothesis that modifications to ribosome stoichiometry or 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.

RP: Ribosomal protein. In humans there are approximately 80 ribosomal proteins.

SILAC: Stable isotope labelling of amino acids in cell culture. A metabolic-labelling technique permitting relative quantification of proteins in a sample by mass spectrometry at the MS1 level.

TMT: Tandem Mass Tags. An isobaric labelling method allowing multiplexing and quantification of multiple samples by mass spectrometry. Unlike SILAC-quantification, quantification occurs at the MS2 level.

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Figure legends:

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

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Fig. 1: Timeline

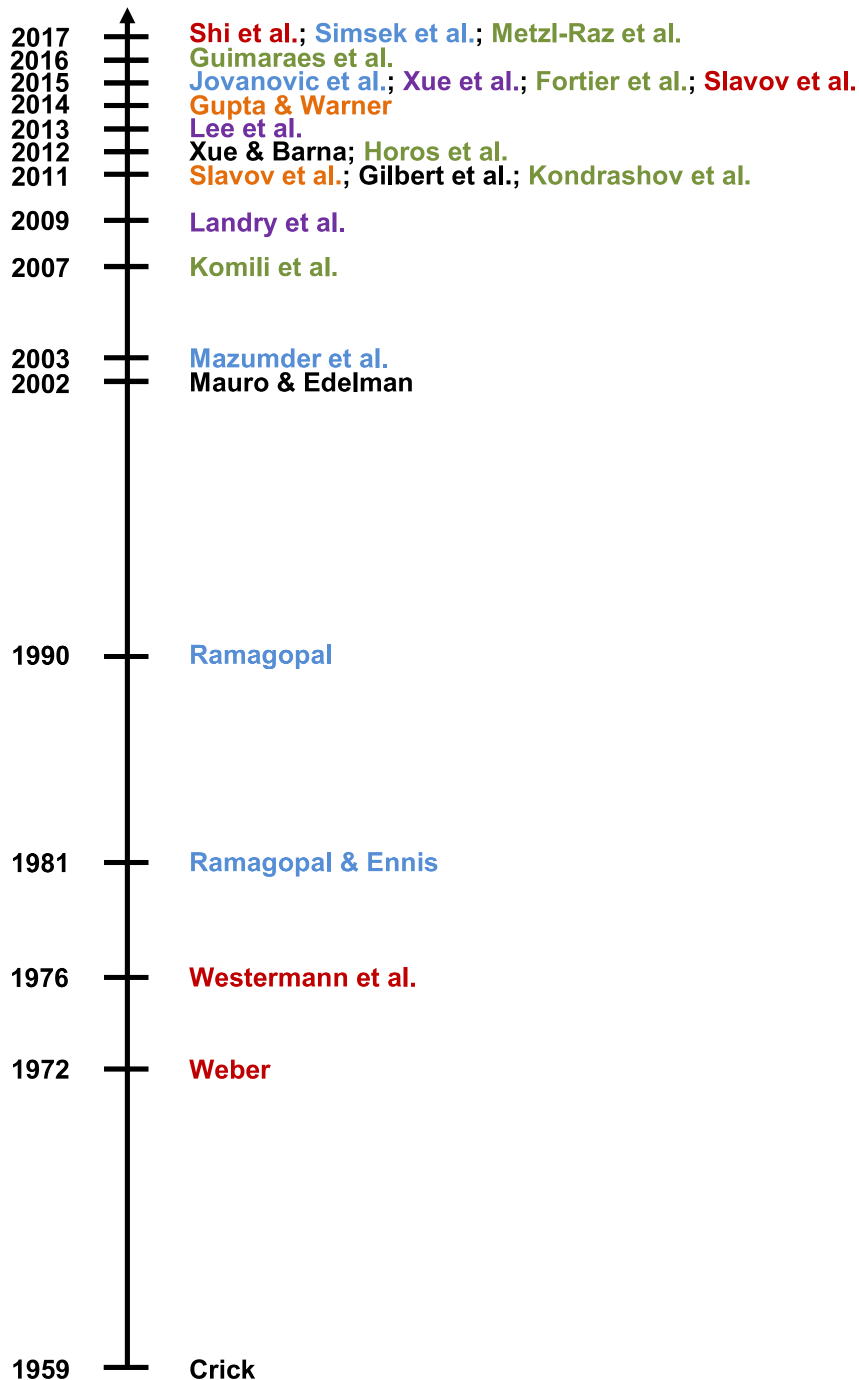

Figure 2. Heterogenous Ribosomes and their PTMs*. A) Ribosomes can be divided into the small (40S, shown in gold) and large (60S, shown in bronze) subunits, which in humans comprises 4 rRNAs (shown in blue) and 80 ribosomal proteins. B) Mass spectrometry analysis of human ribosomes reveals RPs are not all present at stoichiometric levels (Levels in monosomes compared to 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* [86] *in the UCSF ChimeraX software.*

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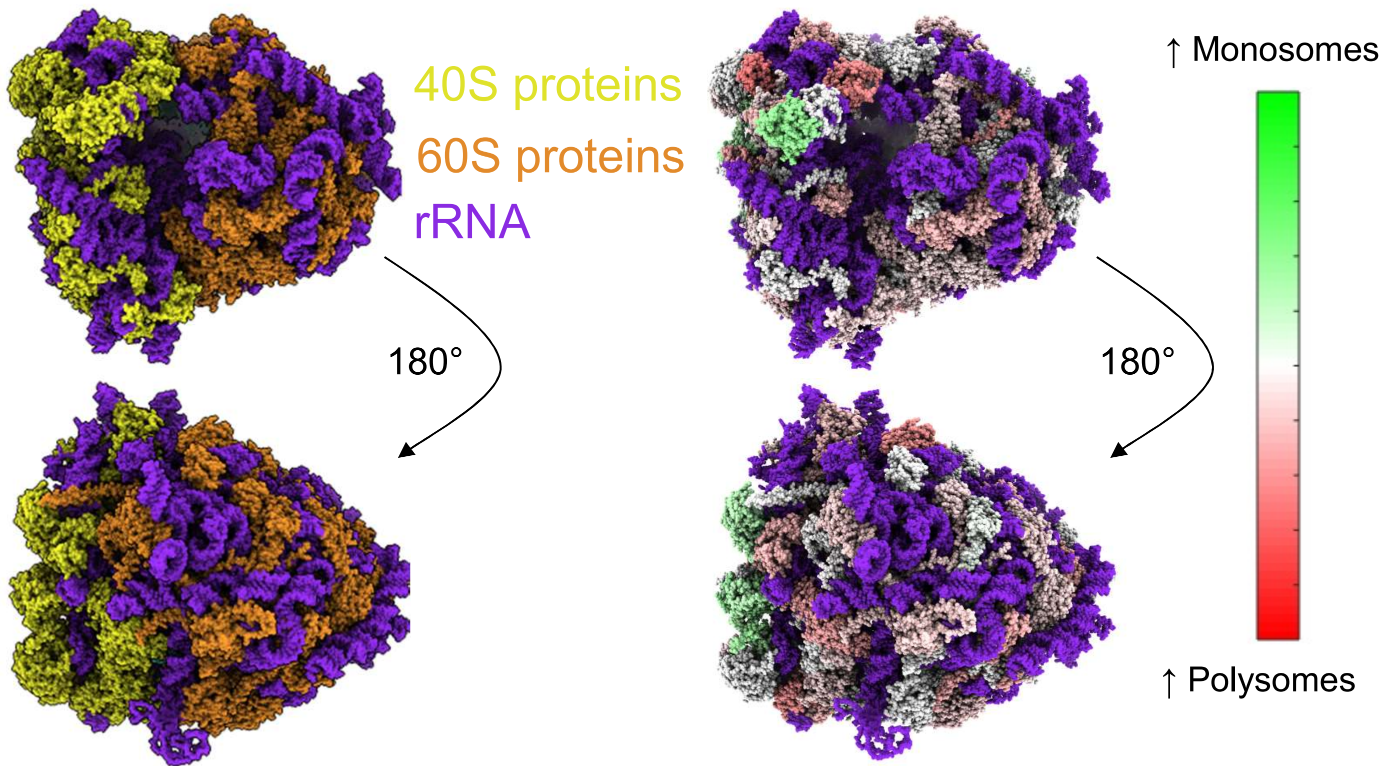
Fig. 2: Heterogenous ribosomes and their PTMs



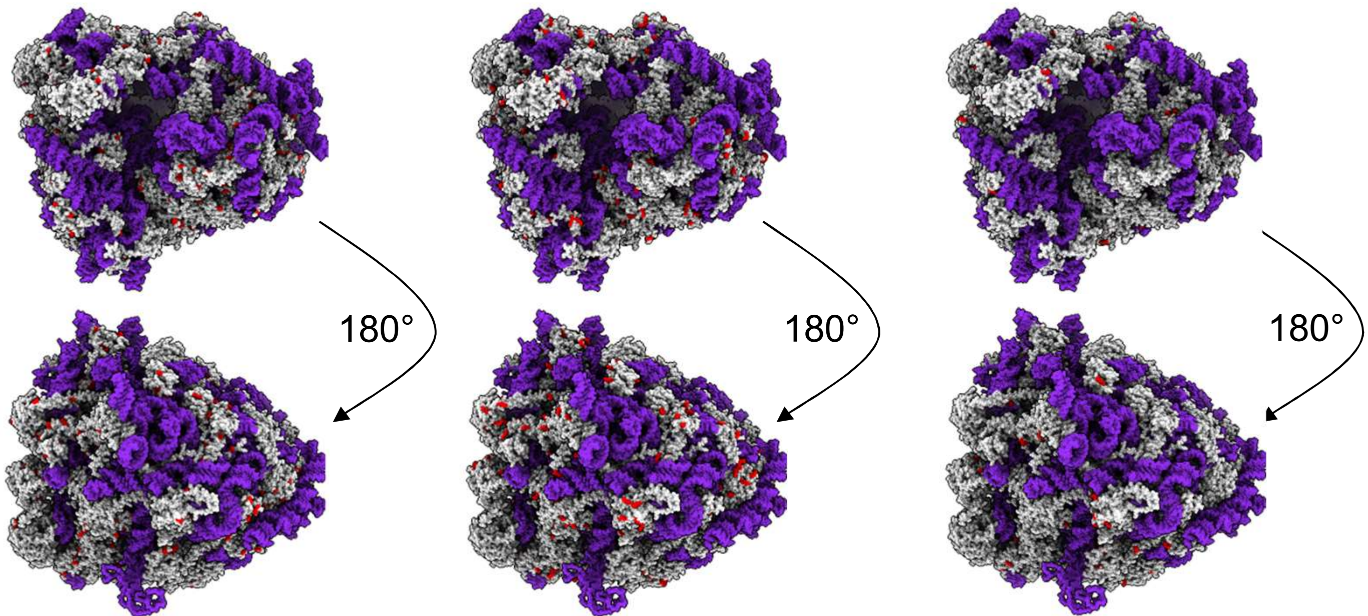


Figure 3. Ribosome specialization. *If populations of ribosomes exhibit distinct phenotypes there are multiple ways in which these functional differences could exist. A) Distinct ribosome subpopulations could have a range of specificities for their mRNAs. These could be from the individual mRNA level to global translation regulation. B) The timescale at which changes to RP stoichiometry or PTMs could exert effects on translation can potentially range from the 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.*

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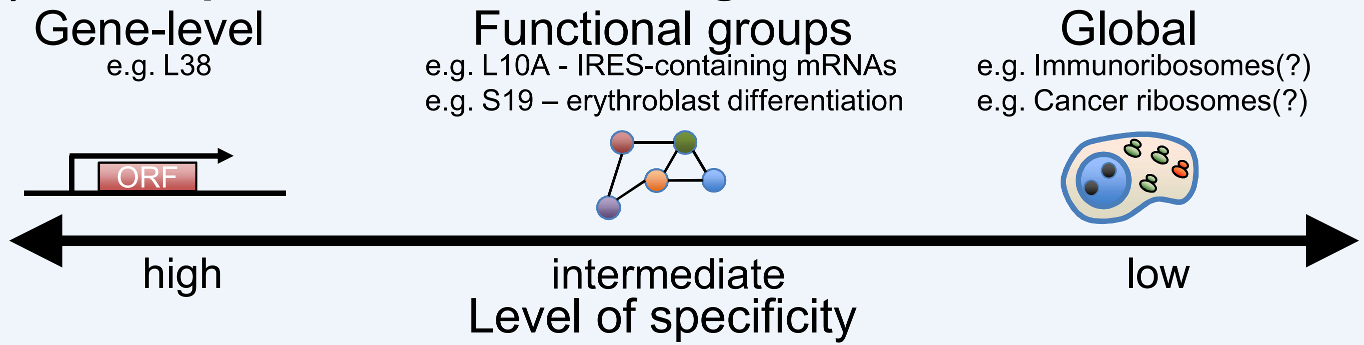
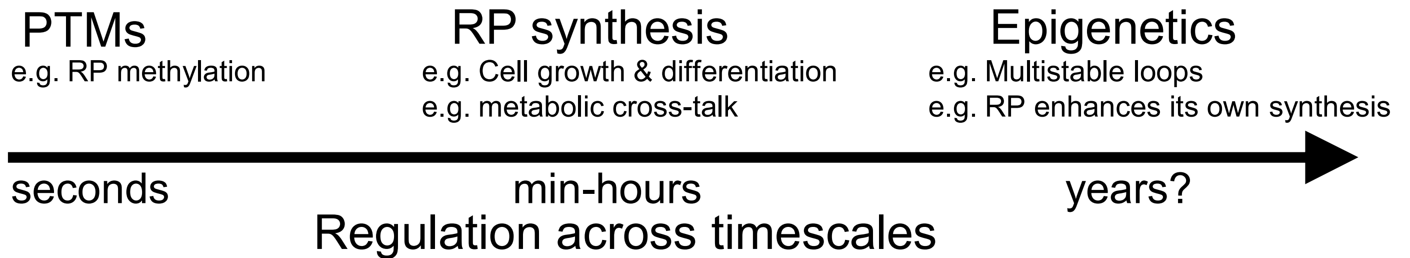
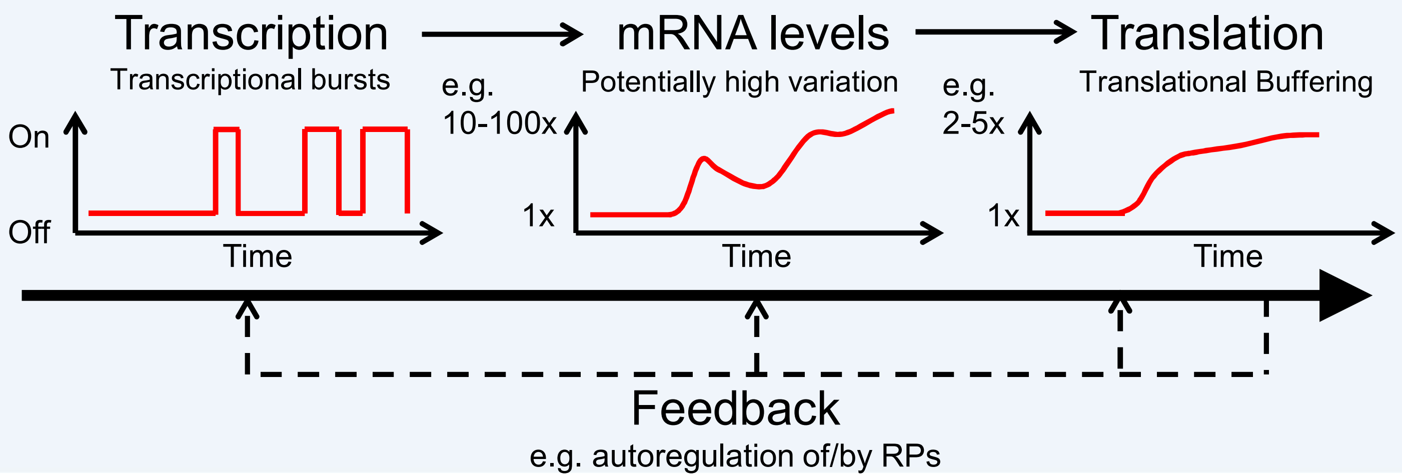
Fig. 3: Ribosome specialization







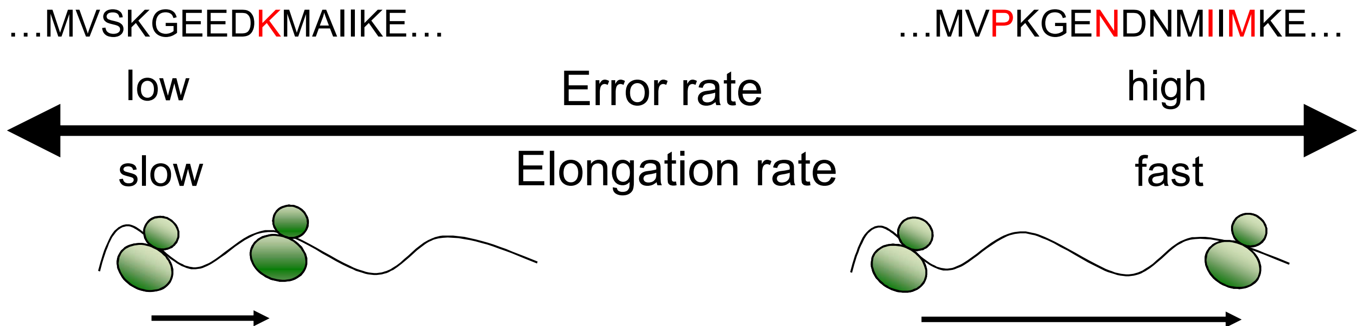


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Figure 4. Identifying altered RP stoichiometry and PTMs. *A) For decades, the gold standard approach for isolating RPs in the context of intact, functional, ribosomes has been sucrose gradient centrifugation. B) Affinity purification is a powerful means of identifying differential RP association with complexes, however it cannot definitively say whether an RP is resident in a ribosome or represents an extra-ribosomal 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 amongst ribosomal protein modifications is a promising new area of research, and the methods required to explore this are an extension of those for identifying changes to 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.*

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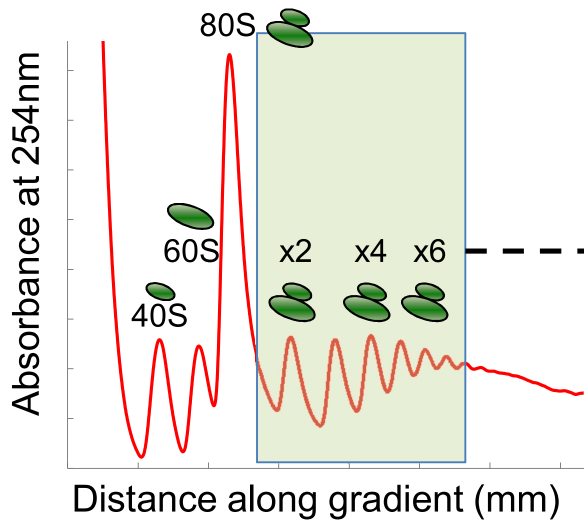
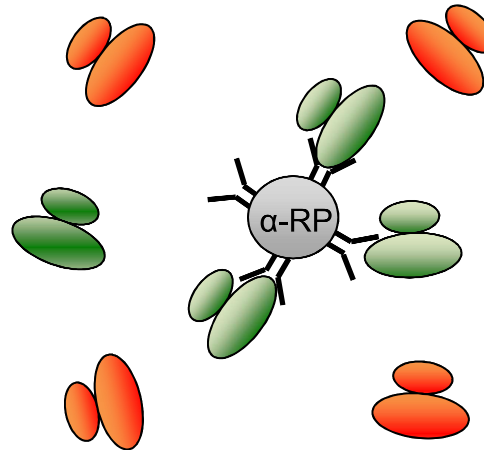
Fig. 4: Identifying altered RP stoichiometry and PTMs

a) Sucrose gradient isolation

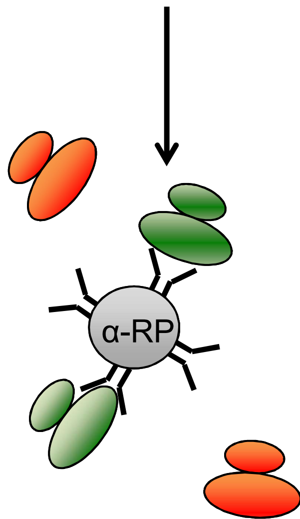


b) Affinity purification



c) Combined approaches

Affinity purification from isolated polysomes



d) Identifying functional PTMs on RPs

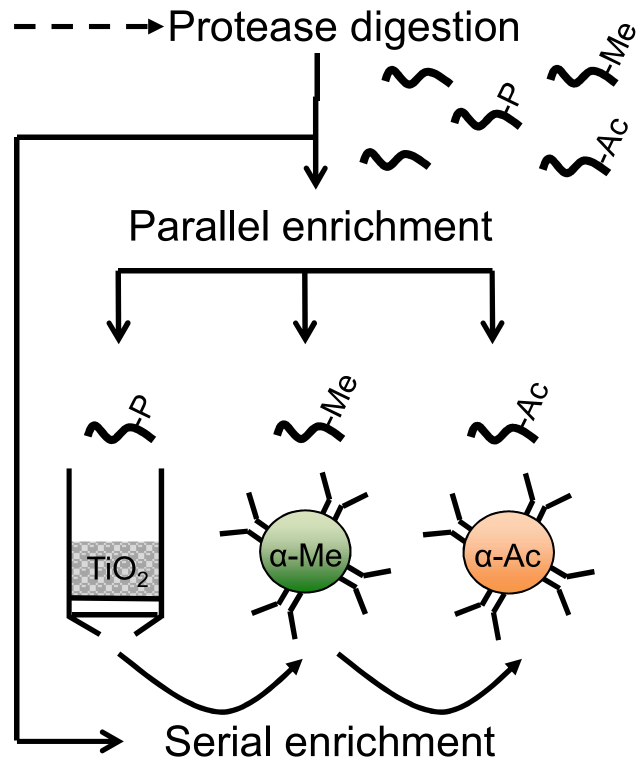


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Figure 5. Testing for functional specialization. *A) To experimentally prove ribosome specialization, several outputs for measurement stand out. These are the mRNA binding specificity, elongation rates and error rates. A conclusive demonstration of functional ribosome specialization will likely employ several or all of these. B) If specific mRNAs are favored by individual ribosome conformations then 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.*

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Fig. 5: Testing for functional specialization

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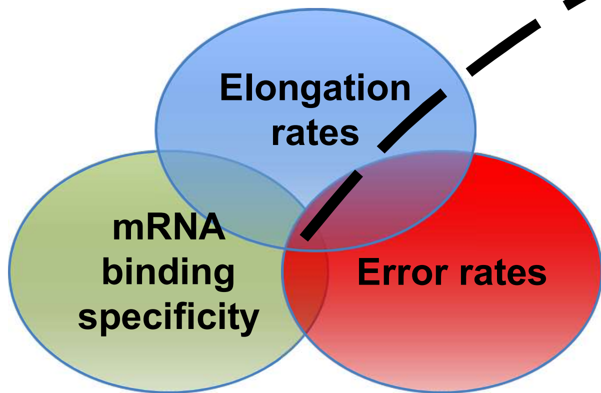
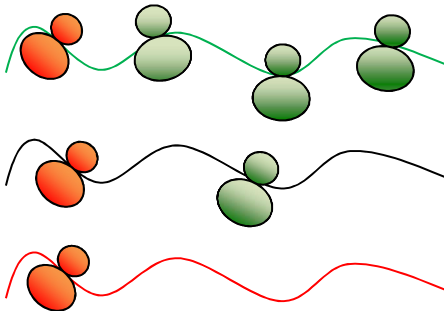
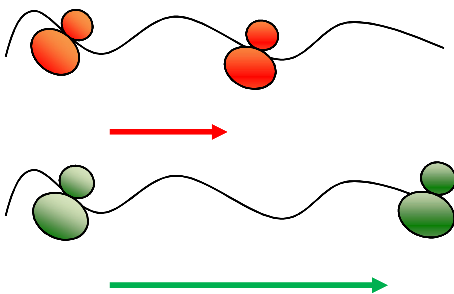
a) Correlating RPs to features of mRNA translation



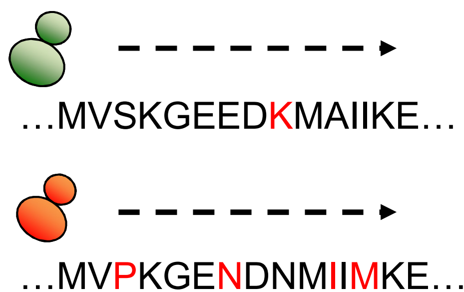
b) mRNA specificity



c) Elongation rates



d) Error rates



e) *In vitro* reconstitution