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Voices

The promises and pitfalls of specialized ribosomes

The concept of specialized ribosomes has garnered equal amounts of interest and skepticism since it was first introduced. We ask researchers in the field to provide their perspective on the topic and weigh in on the evidence (or lack thereof) and what the future may bring.

Maria Barna
Stanford University, USA

Fine-tuned for specific functions

Ribosome heterogeneity and specialization is an underappreciated field but perhaps one of the most exciting in our understanding of gene regulation. In the past few years, there has been undeniable evidence that ribosomes can be heterogeneous at all levels from ribosomal proteins (RPs), RP paralogs, ribosome-associated factors, rRNA, and rRNA modifications. These are profound observations that have the potential to change our perception of how the genetic code can be translated. Given that there are upwards of ten million ribosomes per mammalian cell, this variation in ribosome composition may translate into hundreds of thousands of ribosomes that can be distinct and perhaps serve to fine-tune how proteins are produced in subcellular space and across different cell types. At the same time, there has also been a divide between a reductionist approach to the study of ribosomes (for example, biochemistry in a test tube) to more omics or biologically slanted research (for example, genetics and organismal biology). I strongly suspect that for some the realization that ribosomes can be different is a nuisance, one that distracts from a purely foundational approach to understanding these molecular machines, while for others this represents fertile ground for new biology and discoveries. To advance the field at this critical juncture, I believe that both sides need to meet; that ribosome biology encompasses a multidisciplinary approach where one can easily go from structure to organismal biology (where ribosome variation might have the most biological meaning) and back. I believe that this is the key for unlocking the meaning of ribosome heterogeneity in the next decade.

While we know that ribosomes can be different, there are several outstanding questions. (1) How pervasive is ribosome heterogeneity, and how is composition regulated? (2) How do changes in ribosome composition change the biochemical properties of ribosomes? (3) What is the biological meaning of having ribosome variation, and how does it impact organismal biology? For some, there can be skepticism as to whether and how heterogeneity translates into specialization. That is, the notion that ribosomes are tuned to a specific biological activity, such as the translation of a specific network of mRNAs. I believe that the most compelling examples are ones in which different populations of ribosomes are tagged and pulled down for ribosome profiling. This has led to the appreciation that different subsets of transcripts are functionally being translated by different types of ribosomes. Yet, we lack real molecular insight into how this is achieved. Here, the field would benefit from detailed biochemical insight and the convergence of scientists from different disciplines. Mouse genetics may extend observations of ribosome specialization *in vivo*. For example, heterogeneous RPs might have distinct, tissue-level phenotypes, yet how this is biochemically achieved remains of profound importance.

In the coming years, it is perhaps the greatest challenge to understand the biochemical properties of ribosomes of distinct compositions. Here, a convergence on structural biology—for example, examining how specific transcript-ribosome interactions are influenced by ribosome heterogeneity—is needed. Also, measuring differences in ribosome composition is challenging, requiring advanced mass spectrometry and, in cell tagging, approaches to isolate ribosomes of distinct compositions. Finding new ways of making these measurements tangible to a broader scientific community would be of importance. Lastly, the field has grown enough to warrant its own scientific community, and I welcome the opportunity for having dedicated meetings focused

on ribosome heterogeneity and specialization where diverse scientist can meet to potentially crack this ribosome "code."

Katrin Karbstein
UF Scripps Biomedical Research, USA

The cost of ribosome heterogeneity

While there is ample evidence for ribosome heterogeneity, the lack of functional data to support specific roles for most ribosome subpopulations is one of the reasons the topic remains controversial. In addition, there are also conceptual problems with the proposal of different ribosome populations modulating gene expression. First, cells go to great lengths to ensure the uniform composition and functional integrity of the ribosome, exactly the opposite of what the concept suggests. Moreover, ribosomes lacking individual RPs or rRNA modifications are associated with a number of different diseases. Together, these considerations suggest that ribosome heterogeneity has dangerous negative consequences that cells carefully avoid. Second, two (or more) ribosome populations with different elongation speeds, or propensities to slow at different sequences, will lead to ribosome collisions as the faster type catches up with the slower one. These collisions result in decay of the bound mRNA and the offending ribosome, thus purifying the cell of the heterogeneity. Third, implicit in the proposal of ribosome heterogeneity contributing to regulated gene expression is the idea that ribosome populations change. However, ribosomes are exceedingly stable and turned over only via cell division. Moreover, ribosome assembly is very energetically costly and turned off under any stress—the exact condition when one would want to change gene expression.

These considerations do not mean that heterogeneous ribosomes do not play functional roles in physiological processes. Instead, they suggest that functional heterogeneity is likely limited to certain circumstances, where the above considerations no longer hold. For example, cell-type-specific ribosomes could contribute to cell-type-specific translational profiles, although, in that case, each cell type has a homogeneous ribosome population. Also, during development, rapid cellular division and the resulting requirement for massive ribosome synthesis could lead to a (relatively) rapid change in ribosome populations. If these are distributed asymmetrically during cell division, this could further lead to rapid separation of the two ribosome pools. Ribosome populations could also change rapidly and reversibly via reversible post-translational modifications or via reversible release of RPs, as we have recently shown for Rps26. Both mechanisms also allow for regulation, important in avoiding overproduction of RP-deficient ribosomes with pathogenic potential. Ribosome collisions can be avoided if two ribosome populations have selectivity for different mRNAs during initiation, e.g., by contributing to Kozak sequence recognition, as we have shown for Rps26, or by binding uncapped viral mRNAs. Moreover, cellular localization of mRNAs and ribosomes might also contribute to demixing of ribosome populations. Ribosomes that are defective in the response to collisions could also escape their purifying selection. Because the interface of the collided ribosomes involves specific RP-dependent contacts, it is likely that ribosomes lacking those RPs would be defective in responding to collisions, as shown for Asc1-deficient yeast. Finally, if heterogeneity is combined with reduced ribosome numbers, collisions might also be avoided, especially if combined with defects in collision clearance. These examples suggest that while ribosome heterogeneity is problematic for cells, and dealt with swiftly, there are niches where alternative ribosomes might contribute to regulated gene expression.

David Tollervey
University of Edinburgh, Scotland

Ribosomes are not all equal

Two fundamental types of specialization can be envisaged. (1) There are established cases, in several systems, of cell-type-specific differential expression of variants in RPs, correlated with altered global translation patterns. (2) More controversially, functionally distinct ribosome subpopulations could be present within single cells or perhaps regions of polarized cells, e.g., axons or epithelial cells.

Certainly, ribosomes are not all equal, with a bewildering array of potential distinctions: some of the 81 RPs can be sub-stoichiometric (i.e., absent from some ribosomes) or have distinct isoforms, and RPs are subject to huge numbers of post-translational modifications. Mammalian rRNAs have primary sequence variants and more than 200 sites of rRNA post-transcriptional modification, many of which can be sub-stoichiometric during development. Modification of the rRNA seems a likely source of functional variants. Specific functions have not been attributed to most individual modifications, but many are conserved in evolution—so clearly have significant functional relevance. In yeast, at least, all rRNA modifications are individually dispensable, suggesting that the conserved function might indeed be regulatory.

So, substantial ribosome heterogeneity will exist in most or all cell types. Some fraction of these variations will undoubtedly generate functional differences that would characterize a "specialized ribosome." However, defining these remains challenging: effects of specialized ribosomes on overall translation are probably modest, while the sheer complexity of the system makes it hard to unambiguously identify key drivers of translation regulation in any given cell type.

Functional analyses have revealed correlations between translational engagement and ribosomal variants. Advances in ribosome fractionation, perhaps to include specific rRNA or protein modifications, will extend such correlative approaches. Selective translational profiling, combined with translation inhibitors, might indicate whether variant ribosomes alter initiation or elongation rates. However, the detailed structure/function studies needed to transition from correlations to mechanisms will require a large commitment of resources for each ribosomal variant tested. Paradigmatic examples will need to be selected for detailed analysis.

Overall, the heterogeneity in ribosomes across cell types and developmental states will surely include many examples of specialized ribosomes, but identifying the functional variants and mechanisms remains a major challenge.

Davide Ruggero
University of California San Francisco, USA

Decorating rRNA depending on the party

It is interesting how a few words, "ribosome heterogeneity" and "specialization," have shocked the ribosome community. We have been working on ribosome modifications for years without calling this ribosome specialization. Yet, without a doubt, these were very early examples of ribosome heterogeneity. The ribosome is decorated with hundreds of rRNA modifications, including 2′-*O*-methylation (2′-*O*-me) and pseudouridylation (Ψ). The number of these modifications change across evolution, with a greater number in mammals compared to yeast and bacteria. Often, the roles of these individual rRNA modifications remain elusive. They have been thought to stabilize RNA-RNA interactions as well as protein-RNA interactions. Yet, it remains an outstanding question how a specific pattern of modifications or modification of a single nucleotide modulate ribosome activity during different steps of translation. More intriguing is how these modifications can change the translation of a specific network of mRNAs. It is clear now that that some of these modifications are sub-stoichiometric at steady state or can be changed by specific growth stimuli, nutrient availability, or differentiation signals. For example, downstream of RAS activation, we showed that rRNA modifications can dynamically change. Therefore, ribosomes can become decorated differently depending on different activities of a cell or cellular "parties." Yet, many of these modifications are inferred, for example, by measuring the levels of non-coding RNAs that guide them. However, there is tremendous excitement for new technologies that directly analyze 2′-*O*-me and Ψ on rRNA at a global scale. What is the fraction of ribosomes within a cell that has a different pattern of modifications, and how do these

translate to specific cellular phenotypes such as whether a cell decides to grow, proliferate, or differentiate? How can we isolate different subtypes of ribosomes?

Years ago, we showed that changing rRNA modifications can affect IRES (internal ribosome entry site)-dependent translation and translation fidelity. We were also studying these changes in the context of human disease and cancer. This led to the thought-provoking possibility that cancer ribosomes may have a different constellation of modifications compared to normal cells. The effect of changes in rRNA modification has been observed in different contexts from yeast to mammals. However, we still do not understand the dynamics by which changes in rRNA modifications affect the initiation step of translation. It has been difficult to go in depth into precise biochemical and biophysical mechanisms. To crack the code of which modifications do what requires new technologies. One major breakthrough has been cryo-electron microscopy (cryoEM). which has enabled the visualization of rRNA modifications at low Å resolution. For example, cryoEM structures of Ψ free ribosomes suggests effects of chemical modifications on ribosome conformations. Single-molecule fluorescence resonance energy transfer analysis of purified ribosomes to monitor translation kinetics can be an additional and powerful approach to decipher the role of specific nucleotide modifications in ribosome activity. For example, by employing this technology, it has been shown that ribosomes lacking only two specific modifications have a defect in binding to distinct tRNAs associated with impairments in translation fidelity. Therefore, while it has been well demonstrated that changes in rRNA modifications can establish ribosome heterogeneity, productive skepticism should be centered on the functional roles of these modifications, which is still in its infancy of being discovered but reflects an exciting new frontier. Another outstanding challenge is to understand the possible convergence of multiple levels of ribosome heterogeneity. For example, how do changes in rRNA modification influence the binding of ribosome proteins or ribosome-associated proteins to combinatorial impact on a "ribosome code."

Gloria Brar
University of California Berkeley, USA

Conceptual appeal, experimental challenges

It is appealing to think that the ribosome could be specialized to modulate translation differently in different conditions. Such a strategy seems useful and achievable, given that the ribosome contains many ribosomal proteins (RPs), some of which are encoded by two different genes, and many of which are targets of post-translational modification. Modularity can, after all, enable efficiency, and differences in ribosome composition have been seen. Many researchers have thus gone in search of functional evidence for specialized ribosomes, but the topic remains controversial, in part due to two features that complicate analysis of RP loss-of-function data: (1) direct gene expression changes from general translation deficiency and (2) secondary gene expression changes from poor cellular growth and adaptation to it.

Ze Cheng led an approach to define these effects in mitotic yeast cells using strains that were each deficient in an individual RP and displayed a range of translation levels (and thus growth rates). In parallel, he measured mRNA abundance, translation, and protein levels globally. Strains with severe translation defects were unstable, frequently becoming aneuploid, a feature that is not typically queried in RP-deficiency studies. Even in euploid strains, the dominant changes to translation patterns, in general, did not reflect RP specificity and were rather dose dependent with a degree of translation deficiency and small- or large-subunit identity. Furthermore, secondary effects dominated these patterns, presumably from cellular adaptation to translation deficiency.

Disentangling specific and direct effects from consequences of general translation deficiency continues to be an important and often unmet challenge of many studies pursuing the specialized ribosome hypothesis. Another is semantic. How do we decide what is a core ribosomal subunit versus an accessory factor? It would not be surprising if an accessory factor modulated translation in a condition-specific manner, but the line between them and core RPs is not as sharp as it may seem. Thus far, this distinction is physical. Core RPs showed roughly stoichiometric signal on a 2D gel following ribosome isolation under specific conditions. It is worth noting that RPs studied most

heavily, to date, for specialized roles and non-constitutive ribosome association are not required for robust cell growth, a functional feature of only 8 of ~80 RPs. The type of case that I find most compelling is exemplified by Rpl26, which is essential and can be reversibly released from the ribosome under stress conditions, allowing rapid regulation of the ribosome in response to changing environmental conditions. Determining the pervasiveness of this type of regulation is an exciting front in specialized ribosome research that may benefit from approaches to identify RPs that are differentially important to cells under different conditions.

Eric Lieberman Greer
Boston Children's Hospital and Harvard Medical School , USA

What's next for ribosome heterogeneity

Historically, it was believed that what proteins were translated was solely regulated by the genome and which transcripts were available. However, recent work has suggested that specialized ribosomes can translate unique sets of transcripts under stress conditions. Work from the past four decades has provided increasing evidence that indeed specialized ribosomes do exist. Because of the multifaceted nature of the ribosome (4 ribosomal RNAs and ~80 RPs), it is perhaps unsurprising that each portion of this complex can be modified to specialize the ribosome. The first crack in the theory of a homogeneous ribosome came with the demonstration that in different cell types, the ribosome was made up of different ribosomal proteins. Later, immunoprecipitation of different RPs and sequencing of transcripts demonstrated that different subunits helped to specify the ribosome to subsets of transcripts. The realization that rRNAs can be post-transcriptionally modified in a sub-stoichiometric manner suggests that unique ribosomal complexes can be generated by both the incorporation of these distinctly modified rRNAs and different RPs, each of which may also be post-translationally modified. Most recently, we and others have demonstrated that rRNA modifications can also contribute to this ribosomal specialization. The tools have now been refined for a thorough characterization of the makeup of the ribosome, tools including ribosome sequencing, ultra-high-performance liquid chromatography coupled with mass spectrometry for quantifying modifications to rRNAs, and sensitive tagging and mass spectrometry techniques. With these tools in hand, the next frontier for ribosome heterogeneity remains the identification of what biological contexts ribosome specialization helps to regulate.

To help solidify the scientific community's confidence in ribosome heterogeneity, it will be critical to develop orthogonal techniques to validate ribosome heterogeneity and to determine what processes these specialized ribosomes regulate. Recent work has demonstrated that under stress conditions, the ribosome preferentially bind to stress resistance transcripts, providing evidence for rapid adaptability through this layer of regulation of protein translation. Ribosome heterogeneity has been proposed to control which proteins are translated in contexts ranging from development to stress resistance to aging to cancer. Our group has recently discovered that some specialized rRNAs are transmitted from parents to their children in response to an altered parental environment and that this transmission may help to allow the subsequent generation to prepare for harsh conditions with an altered production of proteins. The involvement of ribosome heterogeneity in regulating epigenetic inheritance adds to a rapidly expanding list of biological processes regulated by ribosomal specialization. Understanding ribosome heterogeneity will add to a complex and critical regulation of the proteome in both physiological and pathological conditions. All in all, this is an exciting era for ribosome heterogeneity and for uncovering the involvement of this additional layer of regulation of which proteins are produced in new biological roles.

Jonathan D. Dinman
University of Maryland, USA

Hunting for specialized ribosomes

The discovery of oocyte and somatic versions of 5S rRNAs in *Xenopus* in the early 1970s was the first of many pieces of evidence for ribosome heterogeneity. Subsequently, heterogeneity has been demonstrated to affect all aspects of the ribosome: multiple rRNA alleles, expansion sequence differences, differences in post-transcriptional modification of rRNA and post-translational RPs, and cell-type-specific differences in RP homolog and paralog expression. Aside from obviously specialized ribosomes, e.g., in mitochondria, chloroplasts, extremophiles and various parasites, the evidence for specialization in mammalian cells remains elusive. Following historical precedent, genetics approaches have been in the vanguard, demonstrating such phenomena as 5S rRNA allele-specific differences in translational fidelity, cell-type-specific differences in RP utilization, and preferences of distinct ribosomes for specific classes of mRNAs. However, such observations are all indirect evidence. Where are the best places to look and methods to use?

Regarding places to look, much interest has been focused on potential roles for specialized ribosomes during development, leading to the discovery of ribosomes lacking specific RPs arising during later phases of development of specific cell lineages. Although this has been interpreted as evidence of specialization, it is not the only explanation. Defective ribosomes and conditions that cause cytoplasmic ribosome disassembly can trigger the integrated stress response, often resulting in apoptosis. Thus, ribosomes may be programmed to shed specific RPs at specific points during development to trigger terminal differentiation and apoptotic trimming. Furthermore, during embryogenesis, the need of rapidly proliferating cells for large amounts of new proteins may place an emphasis on bulk ribosome synthesis as opposed to production of highly specialized ribosomes. And indeed, this may similarly complicate efforts using the types of rapidly growing cells typically employed in cell culture. Rather, I suggest that fully differentiated organs might be better places to look for functional ribosome specialization: they are functionally specialized and express limited protein repertoires. Further, a class of RP paralogs called "ribosomal protein-like" proteins may be the most promising candidates. In contrast to uncontrollably stochastic rRNA post-transcriptional modification, current genetic methods can enable production of cells expressing ribosomes harboring only one paralog or its cognate RP. This is critical because biochemical/biophysical characterization, likely using single-molecule approaches and other emerging technologies, will be required to definitively demonstrate specialization. Paralogs to examine include the testis-specific paralog of ribosomal large-subunit protein Rpl39, called Rpl39-like (Rpl39L), which is essential for mouse spermatogenesis: perhaps this protein optimizes protein synthesis at lower temperatures. The Rpl3 paralog Rpl3L is preferentially expressed in mature muscle cells, and its overexpression impairs muscle repair. Might Rpl3L enable ribosomes to trade accuracy for speed, enabling them to accurately synthesize titan, the extremely long, muscle-specific protein? Other candidates include but are not limited to RPL12L, RPl22L, RPL23L, RPL35AL, RPL39L, and RPS27L. Specialization may also be conferred by subcellular localization, an area of research that is being facilitated by new technologies in microscopy. While much has been learned since the ribosome was first discovered, the hallmark of good science is that it leaves more questions than answers. The next generation of ribosome scientists have their work cut out for them.