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Probing the mechanisms underlying human diseases in making ribosomes

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Abstract

Ribosomes are essential, highly complex machines responsible for protein synthesis in all growing cells. Because of their importance, the process of building these machines is intricately regulated. While the proteins involved in regulating ribosome biogenesis are just beginning to be understood, especially in human cells, the consequences for dysregulating this process have been even less studied. Such interruptions in ribosome synthesis result in a collection of human disorders known as ribosomopathies. Ribosomopathies, which occur due to mutations in proteins involved in the global process of ribosome biogenesis, result in tissue-specific defects. The questions posed by this dichotomy and the steps taken to address these questions are therefore the focus of this review: How can tissue-specific disorders result from alterations in global processes? Could ribosome specialization account for this difference?

Introduction

Ribosomes are complex macromolecular machines that are responsible for translating all messenger RNAs (mRNAs) of the cell into their protein counterparts. These machines are essential for life and the process of making ribosomes is highly coordinated and regulated. Current knowledge of ribosome biogenesis in humans is only beginning to probe the intricacies of building ribosomes, and even less is known about the consequences when these processes go wrong. Several human disorders, collectively called ribosomopathies, are caused by defects in making ribosomes. While these disorders all alter the process of making ribosomes, only specific tissues are affected. Several possibilities have been brought forward to address this anomaly, including tissue-specific sensitivity to the pro-apoptotic protein p53. Additionally, the presence of ribosomopathies raises the question, could there be specialized ribosomes responsible for recognizing specific mRNAs depending on the cell or tissue type? This review seeks to address recent progress made on these outstanding questions in the field of human ribosome biogenesis: 1. How can mutations disrupting ubiquitous processes

create tissue-specific human disorders? 2. Can ribosomes, which are essential for translating all proteins, “specify” particular mRNAs?

1. How can mutations disrupting ubiquitous processes create tissue-specific human disorders?

Development of a properly functioning ribosome is crucial to life because without these machines the cell cannot produce the necessary proteins to grow and divide. The prevailing assumption for many years was that organisms bearing defects in making ribosomes would be inviable. This notion was turned upside down by the discovery of ribosomopathies, a group of disorders characterized by defects in ribosome biogenesis. Traditionally, for a disease to be considered a ribosomopathy, the signs and symptoms must be directly caused by defects in ribosome biogenesis¹. Recently, this definition has been questioned as several cancers, most notably T-cell acute lymphoblastic leukemia (T-ALL) have also been found to have defects in proteins required for making ribosomes^{2–4}. However, we do not yet know whether these ribosome biogenesis defects are the direct cause of cancer.

Ribosomopathies present with a wide range of distinct, tissue-specific phenotypes, often resulting from haploinsufficiency or point mutations. For example, ribosomal protein mutations and deficiencies cause Diamond-Blackfan anemia (DBA), resulting in anemia, low reticulocyte count, increased fetal hemoglobin, and increased adenosine deaminase activity^{5–6}. Patients may also have short stature or congenital malformations. In contrast, Treacher Collins syndrome is a ribosomopathy that manifests itself in the form of craniofacial defects and cleft palate⁷. Isolated congenital asplenia is a third ribosomopathy which occurs due to a mutation in the ribosomal protein uS2 (RPSA)⁸. For this ribosomopathy, afflicted patients are born without a spleen⁹. Still another ribosomopathy, North American Indian Childhood Cirrhosis, causes liver failure¹⁰. Furthermore, X-linked dyskeratosis congenita is a ribosomopathy occurring due to a mutation in the DKC1 gene¹¹. The resulting dyskerin protein is involved in modification of the ribosomal RNA, specifically through pseudouridylation¹². The primary cause of death is bone marrow failure^{13–14}. Each of these ribosomopathies are caused by a different protein involved in ribosome biogenesis (Fig 1), leading to the expectation then that the resulting phenotypes should all be the same and should result in an inviable organism. However, this expectation is incorrect as the disease signs and symptoms are drastically different, leading us to ask how mutation of a protein required for this ubiquitous process causes tissue-specific deficits. Also, the particular mechanism that governs which tissue is affected also remains unknown.

1.1 p53 stabilization contributes to the tissue-specific phenotypes of several ribosomopathies—For several ribosomopathies, the pro-apoptotic protein p53 has been implicated as an important player in the signs and symptoms of the disease. Both ribosome biogenesis and cell growth are intricately linked. The nucleolus, the sub-nuclear organelle where ribosomes are made, therefore acts as a stress sensor, activating certain pathways upon induction of cellular stress by heat shock, lack of nutrients, and more. During normal cell growth conditions, p53 is sequestered and targeted for degradation by the ubiquitin ligase, mouse double minute 2 (MDM2)^{15–17}. This inhibits the transcription of specific pro-apoptotic genes and allows the cell to proliferate. Upon induction of cellular stress, p53 is

released and is able to perform its necessary cellular functions to prevent cell growth, and, if needed, p53 will trigger apoptosis (reviewed in ¹⁸).

The first study to introduce the concept of a “nucleolar stress” response investigated the mechanism through which the loss of the nucleolar protein block of proliferation 1 (BOP1) induces cell cycle arrest ¹⁹. In NIH 3T3-derived LAP3 cells, expression of a dominant-negative BOP1 mutant protein caused alterations in pre-ribosomal RNA (pre-rRNA) processing that ultimately resulted in decreased synthesis of the 60S large ribosomal subunit (LSU) of the ribosome ²⁰. Interestingly, when the dominant-negative BOP1 was expressed and p53 was inhibited in LAP3 cells, the cell cycle arrest normally associated with mutant BOP1 expression was abrogated even though pre-rRNA processing remained impaired ¹⁹. Thus, Pestov, et al. suggested the “nucleolar stress response” in which interruptions in ribosome biogenesis caused by the BOP1 mutant caused the secondary consequence of cell cycle arrest, and this cell cycle arrest was mediated by the p53 protein ¹⁹. Thus, the nucleolar stress response, summarized in Figure 2, functions to trigger p53 stabilization upon interruptions in ribosome biogenesis.

Since the discovery of the nucleolar stress response, various scientists have endeavored to parse out the precise molecular mechanisms through which the nucleolus affects p53 levels during times of cellular stress. Rubbi and Milner showed that the nucleolus had to be disrupted in order to stabilize p53, making the functional nucleolus a key inhibitor of the p53 protein ²¹. Multiple ribosomal proteins have been identified as activators of p53 function. uL18 (RPL5) and uL5 (RPL11) are key factors that induce p53 stabilization when they accumulate outside of the functional ribosome due to cellular stresses ^{22–25}. Recently, it was shown that uL18 and uL5 work in a complex with the 5S rRNA to bind MDM2, the 5S ribonucleoprotein (RNP) complex ^{26–27}. p14^{ARF} promotes the formation of this complex, and binds MDM2 as well to promote p53 stabilization²⁶. This complex sequesters MDM2 and therefore stabilizes p53 levels ^{26–27}. Interestingly, scientists are continuing to discover new ribosomal proteins that bind MDM2. For example, uL4 (RPL4) was recently shown to bind to the central acidic domain of MDM2, helping promote the interaction between uL18 and uL5²⁸.

Much remains to be discovered about the p53 nucleolar stress response. For example, how can increased p53 stabilization and apoptosis manifest in different phenotypes (ie craniofacial defects versus defects in pancreatic function)? Also, what signals control whether the 5S RNP complex is incorporated into the large subunit of the ribosome instead of binding MDM2 ²⁹? In addition, multiple other methods of p53 regulation have been suggested that do not require uL18 or uL5. For example, under high levels of nucleostemin, nucleostemin will bind directly to MDM2, inhibiting its function and stabilizing p53 ³⁰. Interestingly, low levels of nucleostemin also stabilize p53, but through the uL18 and uL5-dependent mechanism listed above ³⁰. Additionally, MDM2 is also bound by p14^{ARF}. While recent studies have found that this stabilization is dependent on the expression of the 5S RNP complex ²⁶, p14^{ARF} also functions independently of this complex to inhibit pre-rRNA processing ^{31–33}. Also, the ataxia telangiectasia and Rad3 related (ATR) and ataxia telangiectasia mutated (ATM) kinases have been shown to play a role in p53 stabilization aside from the uL18/uL5 mechanism. ATM directly phosphorylates MDM2 ^{34–36}, or its

regulatory partner c-Abl^{37–38}, inhibiting MDM2's ability to ubiquitinate p53. Activated ATR works in a similar manner by phosphorylating MDM2³⁹. Interestingly, both ATM and ATR phosphorylate p53 as well, enhancing p53's ability to upregulate certain transcripts^{40–42}. While the role of ATM/ATR has been studied somewhat in zebrafish models of DBA⁴³, no studies have been conducted that systematically investigate the role of non-uL18/uL5 mechanisms of p53 stabilization in ribosomopathies.

p53 coordination and the nucleolar stress response have been implicated in the pathology of several ribosomopathies. Treacher Collins syndrome is one such ribosomopathy caused by mutations in one of several genes (*TCOF1*, *POLR1C*, or *POLR1D*), halting the transcription of the ribosomal DNA^{44–47}. While mutations in *Tcof1* in some murine strain backgrounds cause the craniofacial defects and cleft palate associated with Treacher Collins syndrome, Jones, et al. have shown that this phenotype can be rescued through either pharmacological or genetic inhibition of p53⁴⁸. The authors suggest that the cranial neural crest cells are particularly sensitive to cellular stresses that disrupt their rapid proliferation during embryogenesis⁴⁸. The sensitivity of this particular cell type to p53 upregulation could explain the connection between ribosomopathies and defects specific to craniofacial development. Additional support for the connection between cranial neural crest development and p53 upregulation comes from studies on the RNA polymerase I transcription and ribosome biogenesis factor, NOL11. Griffin, et al. demonstrated that morpholino knockdown of NOL11 in *Xenopus tropicalis* resulted in craniofacial defects for the developing embryos⁴⁹. These defects coincide with the upregulation of p53 specifically in cranial neural crest cells; the inhibition of p53 rescues this phenotype⁴⁹. In zebrafish, mutations in *Wdr43*, the ortholog of the yeast ribosome biogenesis factor Utp5, also cause p53-mediated defects in neural crest cell development⁵⁰. This provides evidence that the p53 sensitivity of cranial neural crest cells is a conserved response to nucleolar stress. The upregulation of p53 has also been shown to play a role in other ribosomopathies, such as dyskeratosis congenita^{51–53}, DBA^{54–55}, North American Indian Childhood Cirrhosis (NAIC)⁵⁶ and 5q- syndrome⁵⁷. The specific molecular mechanisms responsible for inducing the p53-mediated apoptosis responses in these ribosomopathies remain to be elucidated and will be an active area for future study.

While understanding p53's role in the pathology of these ribosomopathies has greatly increased our knowledge of the mechanism of the genetic disease, questions remain as to how p53 might be manipulated for therapy. p53 is a tumor suppressor known to play a key role in many cancers (reviewed in⁵⁸). Therefore, treatment of ribosomopathy patients with p53 inhibitors could lead to the subsequent development of cancer. Additionally, ribosomopathies, except for 5q- syndrome, are congenital, developmental disorders. Therefore, p53 inhibitors or other treatments may not be curative – therapeutics can only alleviate the signs and symptoms of such disorders.

1.2 Not all ribosomopathy phenotypes result from p53 induction—Not all signs and symptoms of ribosomopathies are so easily explained by p53 and the nucleolar stress response. For example, the ribosomopathy Shwachman-Diamond syndrome is characterized by exocrine pancreatic dysfunction, skeletal defects, and blood lineage cytopenia^{59–61}. Mutations in the SBDS protein result in dysregulated translation. SBDS and its interacting

partner elongation factor like GTPase 1 (EFL1), promote the dissociation of eukaryotic initiation factor 6 (EIF6) from the LSU. Multiple studies have sought to understand the complexity of the precise mechanism of action for SBDS^{62–65}. Recent studies in mice have shown that some signs of Shwachman-Diamond syndrome are dependent on p53 expression: ablation of p53 expression in conjunction with the SBDS mutation resulted in improved exocrine function, specifically in increased digestive enzyme synthesis and decreased acinar compartment hypoplasia, compared to the SBDS mutants alone⁶⁶. However, p53 removal did not improve 80S monosome levels, signifying that overall translation remained impaired in fetal liver cells⁶⁶. Overall growth and perinatal survival were unaffected by p53 ablation as well⁶⁶. These data support earlier studies conducted in zebrafish which showed that knockdown of p53 by morpholino oligo or p53 mutant alleles did not rescue the Sbds knockdown phenotype⁶⁷. While p53 knockdown did improve gross appearance of the zebrafish embryos, it was unable to rescue the pancreatic defects or cartilage defects (Fig 3)⁶⁷. Thus, the phenotypes seen in SBDS-deficient model organisms are likely to be due to a combination of issues arising from both p53-dependent and p53-independent affects. Therefore, while p53 and nucleolar stress can account for some tissue specific differences in ribosomopathies, it does not always account for all of them.

Further examples of p53-independent effects on ribosomopathies come from recent studies on DBA. RNA-seq studies in zebrafish have shown that p53-dependent pathways are significantly altered upon uL18 depletion⁶⁸. However, the same study showed that p53-independent pathways are also altered, including the pathway for aminoacyl-tRNA biosynthesis⁶⁸. This has important implications as other studies in zebrafish, mice, and humans have shown that addition of the amino acid L-leucine improves the signs and symptoms of DBA through a mechanism involving mTORC1 activation upon the binding of leucine to Sestrin2^{69–73}. Aspesi, et al. have also carried out transcriptome profiling of DBA mutations in human TF1 cells, which carry mutations on both p53 alleles, with the intent to find p53-independent pathways affected by these mutations⁷⁴. Interestingly, they found that the vast majority of transcripts differentially expressed upon ribosomal protein depletion were not transcriptional targets of p53 but reflected changes in pathways related to the oxidative stress response and amino acid and lipid metabolism⁷⁴. The effects of p53-independent pathways on the signs and symptoms of other ribosomopathies have yet to be examined.

2. Can ribosomes, which are essential for translating all proteins, “specify” certain mRNAs?

Contributing to the plethora of questions surrounding ribosomopathies is the idea of specialized ribosomes. Could the tissue-specificity of ribosomopathies be due to the selectivity of certain tissues or cell types for ribosomes with enhanced or specified functions? The presence of ribosomes with certain compositions conferring selectivity in translation is plausible: with over 80 ribosomal proteins, each with potential post-translational modifications and 212 known rRNA base modifications, the number of potentially varied ribosomes is unfathomable (summarized in Figure 3, reviewed in^{75–76}). Isolating these specialized ribosomes from a heterogeneous mixture, however, has proven a

challenging task. In addition, once a specialized ribosome has been isolated, it must be tested to determine whether it functions differently in mRNA translation.

A leap forward in addressing these questions came from the study of the ribosomal protein eL38 (RPL38). In mice, mutation of eL38 altered translation of a certain subset of mRNAs which are all characterized as Hox mRNAs⁷⁷. The Hox genes are largely responsible for the morphology of the axial skeleton (reviewed in⁷⁸). Interestingly, Maria Barna and her co-workers have shown that lack of functional eL38 results in defects in axial skeleton morphology while Hox mRNA levels remain largely unchanged⁷⁷. Instead, these Hox transcripts are translated far less frequently in the absence of functional eL38⁷⁷, indicating that eL38 plays a specific role in regulating the translation of these mRNAs. This study demonstrated a role for a specific ribosomal protein (eL38) in translating a specific subset of mRNAs. These mRNAs affect only specific tissues, namely the axial skeleton, further strengthening the argument for what may be considered specialized ribosomes.

The ribosomal protein eL40 (RPL40) has also been suggested to play a role in regulating the translation of certain viral transcripts⁷⁹. The Whelan lab showed that eL40 is necessary for the transcript-specific translation initiation of vesicular stomatitis virus (VSV) proteins upon VSV infection⁷⁹. Interestingly, the authors identify a number of candidate cellular transcripts whose translation is mediated through eL40 as well⁷⁹. Further studies are necessary to determine whether there are additional ribosomal proteins that translate particular mRNAs specifically.

2.1 mRNA sequence elements may cause differential translation—Specialized ribosomes may function in conjunction with specific elements of the mRNA sequence. For example, it was recently determined that both an internal ribosome entry site (IRES) and a translation inhibitory element (TIE) are present in the 5'UTRs of the HoxA mRNAs that are affected by eL38 depletion⁸⁰. Interactions between the IRES elements and eL38 seen in pull down experiments demonstrate that this element may be important in this protein's translation specificity⁸⁰. Additionally, the authors speculate that the TIE element may have evolved in humans as a way of inhibiting the cap dependent translation of these mRNAs and upregulating IRES dependent translation which can be specifically tailored due to the presence of eL38⁸⁰. Determining additional alterations in translation based on cis-acting mRNA sequence elements could therefore lead to an increased understanding of the mechanism of action of specialized ribosomes.

Future directions for understanding what parts of mRNAs could be regulating translation include the use of Transcript Isoforms in Polysomes sequencing (TriP-seq)⁸¹. This new technique enhances the current ribosome profiling technique, which sequences only the approximately 30 nucleotide-long mRNA fragments protected by actively translating ribosomes⁸², instead sequencing the entire transcript bound by the ribosome. Initial studies using this method in HEK293T cells have shown that the 3'UTR composition and length is especially important in determining the translation of the transcript⁸¹. Interestingly, translation of a 3'UTR reporter transcript varied depending on the cell type⁸¹. Further use of this method could connect translation of certain transcripts and the specifics of their translating ribosomes.

2.2 Modification of the ribosomal RNA (rRNA) may regulate translation—

One way to create a heterogeneous mixture of specialized ribosomes would be through the modification of the ribosomal RNAs (rRNAs) themselves. In humans, the four rRNAs have over 200 modified bases⁸³. The most common and well-studied modifications are pseudouridine and 2'-O-methylation. rRNA modifications in humans are highly conserved and exist primarily at important functional points of the ribosome: the small subunit SSU/LSU interface and the ribosomal core including near the decoding site and peptidyl transferase center, indicating the significant role of these modifications in the translation process. Additional evidence for the importance of these rRNA modifications comes from the fact that mutations in the enzymes responsible for making these modifications result in human disease (reviewed in⁸⁴ and⁸⁵). Such diseases include X-linked dyskeratosis congenita (see Introduction), as well as Bowen-Conradi syndrome, a neurodegenerative disorder with mutations in the EMG1/NEP1 gene encoding a methyltransferase⁸⁶, and Cri-du-chat syndrome, caused by deletions in of the short arm of chromosome p (including deletion of the NOP2 gene) and characterized by high-pitched crying, microcephaly, and mental retardation⁸⁷. Many hematologic and solid cancers have also been associated with changes in enzymes responsible for rRNA modification^{88–90}. Most recently, four rRNA base modifications have been proposed as important for translation because these modifications contact the eL41 protein which forms a bridge between the large and small ribosomal subunits⁸⁵. Thus, rRNA modifications could play a role in ribosomal function, but it remains to be shown that one specific rRNA modification results in measurable differences in translation.

Additionally, mRNAs have recently been shown to be pseudouridylated and 2'-O-methylated (reviewed in⁹¹). Current techniques such as pseudo-seq, piloted by Wendy Gilbert and coworkers,⁹² will allow for transcriptome-wide mapping of these sites in human cells. These modifications can affect ribosome function, as it has been shown that one pseudouridylation modification present in a stop codon allows for translational read-through by the ribosome⁹³. Single nucleotide modifications may be differentially translated by specialized ribosomes, although this has not yet been demonstrated.

2.3 The presence of some ribosomal proteins influences translation of specific mRNAs—

Finally, the ribosomal proteins themselves may perform specialized functions that result in varying degrees of functional importance depending on the tissue type. On such example is the ribosomal protein eL22 (Rpl22), which has recently been shown to have a specific role in early B cell development in the bone marrow of mice, and deficiencies in eL22 activate a p53-mediated stress response in these cells⁹⁴. This new role for eL22 in B cell development⁹⁴, along with its previously known role in T cell development⁹⁵, suggests a cell-type specific sensitivity to mutations in eL22. The precise mechanisms dictating why these cell types are sensitive to eL22 mutations are unknown. However, one possibility is that eL22 aids in the translation of certain mRNAs required specifically in those cells.

Additionally, studies have shown that different ribosomal proteins are expressed at different levels in different tissues, but the functional consequences of such changes are unknown^{77, 96}. This suggests that differential expression of ribosomal proteins could lead to

heterogeneous populations of ribosomes throughout an organism in which some tissues have a particular ribosomal protein while others do not.

Ribosome-associated factors, such as RACK1, could also contribute to ribosome heterogeneity and the function of specialized ribosomes. Recent studies by Wendy Gilbert's laboratory have shown that the RACK1 homolog in budding yeast, Asc1, enhances the translation of mRNAs with short open reading frames (ORFs), including mRNAs corresponding to mitochondrial ribosomal proteins⁹⁷. The authors hypothesize Asc1 stabilizes or helps to form "closed loop" structures of mRNAs with short ORFs which are then translated with increased efficiency. Thus, this ribosome-associated factor could be one of many that bind the ribosome upon certain cellular cues to enhance specificity of the ribosome towards certain transcripts. There are several human disorders that may be linked to Asc1/RACK1 loss or overexpression^{97–103}, but we do not yet know how its role in translation in a single-celled organism relates to human biochemistry and physiology.

Conclusions and Future Perspectives

Many questions remain in the study of human ribosome biogenesis. Much of our previous knowledge of ribosome biogenesis has focused on the budding yeast *Saccharomyces cerevisiae*. Humans, however, employ additional levels of cellular regulation not seen in yeast. Several recent studies have begun to fill in the gaps in our understanding of ribosome biogenesis in humans^{104–108}. Identifying the particular mechanisms of crosstalk between nucleolar function and cellular growth will be key to understanding ribosome biogenesis and function in human cells.

Disruption of nucleolar function has been shown to be the cause of several human diseases called ribosomopathies, in which alterations in a ubiquitous process present with tissue-specific defects. One possible explanation for such tissue specificity is that different tissues have varying sensitivity levels to p53, induced by the nucleolar stress response. p53-independent responses to disruptions in ribosome biogenesis are also being studied, but their mechanism of action is less clear.

It is also possible that ribosome specialization could account for differential responses of tissues to certain stresses (Fig. 3). Given that the ribosome is such a large, complex macromolecular machine that translates all cellular mRNAs to proteins, it is certainly possible that heterogeneity within the pool of ribosomes exists. Ribosomes may be optimized to recognize specific features of mRNAs, they may employ the use of highly-conserved rRNA modifications, or some cell types may produce different ribosomes in different tissues or even within the same cell to translate specific proteins due to cellular stimuli.

In all, continued efforts to understand the process of making a ribosome are needed. Although much progress has been made in laying the foundation to address these questions, future studies will enhance our understanding of human ribosome biogenesis and its relation to human genetic disease.

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Fig 1.

Ribosomopathies affect multiple steps in the ribosome biogenesis pathway. Figure adapted with permission from ¹⁰⁹ (additional iterations in ¹¹⁰⁻¹¹¹).

Fig 2.

The nucleolar stress response is activated when dysfunctions in ribosome biogenesis cause accumulation of free ribosomal proteins. Under normal cellular conditions, functional ribosomes are produced, leaving free MDM2 to ubiquitinate the protein p53. p53 is thus targeted for degradation, allowing for continued cellular growth and proliferation. Under conditions of cellular stress, free ribosomal proteins accumulate, such as the 5S RNP complex (uL18, uL5, and the 5S rRNA). Formation of the 5S RNP is promoted by p14^{ARF}. The 5S RNP complex, with or without p14^{ARF}, bind MDM2, resulting in p53 stabilization, cell cycle arrest, and apoptosis.

Fig 3.

Ribosome specialization may take place at multiple levels. Both large subunit (LSU) and small subunit (SSU) ribosomal proteins may contribute to compositional diversity of the ribosomal subunits. This could also be effected by differing post-translational modifications of the ribosomal proteins. Pseudo-uridylation and 2'-O-methylation of the ribosomal RNA or mRNA may confer additional specificity. Such heterogeneity of ribosomes may exist at the tissue, cellular, or sub-cellular level.