Guarding the ‘translation apparatus’: defective ribosome biogenesis and the p53 signaling pathway

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Ribosomes, the molecular factories that carry out protein synthesis, are essential for every living cell. Ribosome biogenesis, the process of ribosome synthesis, is highly complex and energy consuming. Over the last decade, many exciting and novel findings have linked various aspects of ribosome biogenesis to cell growth and cell cycle control. Defects in ribosome biogenesis have also been linked to human diseases. It is now clear that disruption of ribosome biogenesis causes nucleolar stress that triggers a p53 signaling pathway, thus providing cells with a surveillance mechanism for monitoring ribosomal integrity. Although the exact mechanisms of p53 induction in response to nucleolar stress are still unknown, several ribosomal proteins have been identified as key players in this ribosome–p53 signaling pathway. Recent studies of human ribosomal pathologies in a variety of animal models have also highlighted the role of this pathway in the pathophysiology of these diseases. However, it remains to be understood why the effect of ribosomal malfunction is not a universal response in all cell types but is restricted to particular tissues, causing the specific phenotypes seen in ribosomal diseases. A challenge for future studies will be to identify additional players in this signaling pathway and to elucidate the underlying molecular mechanisms that link defective ribosome synthesis to p53.

INTRODUCTION

The translation of messenger RNAs (mRNAs) into proteins is catalyzed by ribosomes, the most complex macromolecules of the cell. In eukaryotes, a mature ribosome (80S) has two subunits, large (60S) and small (40S), that together contain four ribosomal RNA (rRNA) species and 79 different ribosomal proteins (RPs). Although eukaryotic ribosomes possess more rRNAs and RPs than those in eubacteria, ribosomal components have been conserved to a substantial degree throughout evolution. Ribosome biogenesis is a multistep process that begins with the synthesis of preribosomal particles in the nucleolus and ends with the assembly of two mature subunits in the cytoplasm. The synthesis of the ribosome is tightly regulated and uses a substantial amount of cellular energy. All three types of RNA polymerases and several hundred accessory factors (ribosome-associated proteins and noncoding RNAs), in addition to the RPs and the rRNAs themselves, participate in this massive process that occurs throughout the cell. rRNA genes are transcribed by RNA polymerase I (Pol I) into a single rRNA precursor within the nucleolus, which is subsequently cleaved and modified by several accessory factors to yield 18S, 5.8S, and 28S rRNA. The 5S rRNA gene is transcribed separately in the nucleoplasm by RNA polymerase III (Pol III). The RP genes are transcribed in the nucleoplasm by RNA polymerase II (Pol II) and these transcripts are exported to the cytoplasm for translation. RPs and 5S RNA are imported to the nucleolus, where they assemble with rRNAs to form the small (40S) and the large (60S) subunits. These preassembled subunits are then exported to the cytoplasm, where they
undergo additional maturation to form the mature (80S) ribosome.\textsuperscript{11}

Cell growth and cell proliferation, two intricately connected events, are associated with changes in ribosome production.\textsuperscript{12} Upon the detection of favorable stimuli (growth factors or nutrients), cells induce ribosome synthesis and protein synthesis to ensure accurate growth and proliferation. In contrast, in stress situations, cells must downregulate ribosome activity to reduce protein synthesis and halt proliferation. A landmark study by Volarević et al.\textsuperscript{13} identified a direct link between ribosome biogenesis and the cell cycle. This study showed that the conditional deletion of \textit{RPS6} in mouse liver cells results in the complete loss of regenerative capacity of liver cells following partial hepatectomy due to interruption of the cell cycle at the G1 phase. Thus, the control of ribosome biogenesis is critical for cell cycle progression, and the loss of this control may result in an altered cell cycle. This study showed that the conditional deletion of \textit{RPS6} in mouse liver cells results in the complete loss of regenerative capacity of liver cells following partial hepatectomy due to interruption of the cell cycle at the G1 phase. Thus, the control of ribosome biogenesis is critical for cell cycle progression, and the loss of this control may result in an altered cell cycle and deregulated cell growth, such as that seen in cancer.\textsuperscript{14,15} Indeed, many human diseases have been linked to defects in ribosome biogenesis, and, interestingly, the majority of these diseases appear to be associated with increased cancer susceptibility.\textsuperscript{16–18}

How do cells control the fidelity of ribosome biogenesis? Do the cells possess a surveillance mechanism for monitoring ribosomal integrity? What are the pathways that link ribosome biogenesis to the cell cycle? Although our knowledge about the exact mechanisms is still in its infancy, we do know that several RPs, presumably through extraribosomal functions,\textsuperscript{19} participate in a surveillance mechanism that senses defects in ribosome biogenesis. Not surprisingly, this involves the p53 tumor suppressor protein, the master guardian of the cell.\textsuperscript{20} The ability of some RPs to interact with murine double minute 2 (MDM2) (HDM2 in humans), the negative regulator of p53, has been shown to be critical for p53 activation (discussed later in this review).

Recent evidence seems to suggest that the pathways linking defective ribosome biogenesis and p53 signaling are very complex, and several additional factors and regulatory loops, which remain to be identified, may be involved in this network in addition to the MDM2-binding RPs. For instance, human ribosomal disorders or RP mutations in mice produce distinct tissue-specific phenotypes; the p53 pathway appears to be critical for the clinical manifestations, but it is not clear why the effects of ribosomal dysfunction are not universal but rather are restricted to particular cell types, such as red blood cells, neural cells, or skin cells. Here, we focus on recent advances toward understanding this ribosomal stress–p53 signaling pathway, with an emphasis on \textit{in vivo} studies of ribosomal anomalies in various mammalian and nonmammalian model organisms.

HELPING THE MASTER GUARDIAN: RPs IN p53 ACTIVITY AND REGULATION

The p53 protein orchestrates a myriad of signaling pathways that prevent a damaged or abnormal cell from undergoing malignant transformation (Figure 1). A variety of cellular stresses (DNA damage, oncogenic activation, and hypoxia) activate p53\textsuperscript{,21} which then undergoes many posttranslational modifications\textsuperscript{22,23} and transactivates several genes critical for cell growth inhibition. The four major outcomes of an activated p53 response are cell cycle arrest, apoptosis, DNA repair, and senescence. These ultimately contribute to tumor suppression. The importance of p53 in tumor prevention can be gauged by the fact that almost half of human tumors carry mutations in this gene\textsuperscript{24}; in the remaining tumors, the p53 network is functionally inoperative.\textsuperscript{25} Although cancer prevention remains the most important function of p53, it is becoming increasingly clear that p53 also functions in diverse cellular processes such as metabolism, reproduction, stem cell renewal, microRNA processing, and development.\textsuperscript{26–28} Interestingly, these functions do not require stress-induced activation, but rather depend on basal levels of p53. Thus, the control and maintenance of p53 levels (both transcriptional and translational) are critical for normal homeostasis. Usually, MDM2, an E3 ubiquitin ligase, controls the p53 protein level through ubiquitin-mediated proteolysis;\textsuperscript{29} whereas MDMX (an MDM2 homolog, also known as MDM4) controls the transcriptional activity of p53.\textsuperscript{30} MDM2 expression is stimulated by increased levels of p53 because \textit{MDM2} is a transcriptional target of p53.\textsuperscript{31} MDM2 mediates the attachment of ubiquitin to p53; the ubiquitin is recognized by proteasomes, resulting in p53 degradation. Decreased levels of p53, in turn, reduce the transcription of MDM2, thereby providing an autoregulatory feedback loop.\textsuperscript{32} Therefore, the disruption of this MDM2–p53 regulatory loop is critical for activation and stabilization of p53. Depending on the type of stress signal, several distinct pathways modify p53 or MDM2 to block their interaction and stabilize p53.\textsuperscript{33} Recently, it was shown that ribosomal stress can also suppress MDM2 activity through the extraribosomal functions of RPs.\textsuperscript{19}

At present, four RPs, RPL11,\textsuperscript{34,35} RPL23,\textsuperscript{36,37} RPL5,\textsuperscript{38,39} and RPS7,\textsuperscript{40} have been confirmed as MDM2 regulators. These RPs bind in similar yet unique patterns to the zinc finger domain within the central acidic region of MDM2.\textsuperscript{40–42} By doing so,
FIGURE 1 | The p53 signaling pathway. The tumor suppressor p53 protein plays the central role in coordinating a complex network of signaling pathways that prevent aberrant cell growth and proliferation. In normal conditions, p53 is maintained at low steady-state levels. Crucial for this regulation are two proteins, murine double minute 2 (MDM2) and MDMX. MDM2, through its E3 ligase function, mediates the attachment of ubiquitin (Ub) molecules to p53 and targets it for proteasomal degradation. MDM2 also binds p53 and influences its transcriptional activity. MDMX lacks the E3 ligase function and suppresses the transcriptional activity of p53, which is independent of MDM2. However, it also forms a heterodimeric complex with MDM2 and stimulates MDM2-mediated p53 degradation. The expression of MDM2 is controlled by p53 itself through a negative feedback loop (see text for details). MDMX levels are controlled by MDM2 through the Ub-mediated degradation. Deregulated functions of these p53 inhibitory proteins are critical for an activated p53 response in stress situations. For example, exposures to ionizing radiation, ultraviolet (UV) light, and many other DNA-damaging stressors activate several kinases [ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and other checkpoint kinases], which modify p53, MDMX, and MDM2. The modifications cause conformational changes in these proteins and block their interactions, resulting in p53 stabilization. Overexpression of oncogenes stimulates the production of alternative reading frame (ARF; p14ARF in human, p19ARF in mouse) that binds to MDM2 and stabilizes p53. An impaired ribosome biogenesis causes the release of several RPs, which bind to and suppress the E3 ligase activity of MDM2, resulting in p53 accumulation. An activated p53 protein subsequently transactivates several target gene expressions. Depending on the cell type and the stressors, the consequence could either be cell cycle arrest, DNA repair, apoptosis, or senescence. Basal levels of p53 (inactivated state) also contribute to many cellular processes in normal cells, which are independent of its gene transcription functions.

ey inhibit the E3 ligase activity of MDM2 and prevent MDM2-mediated p53 degradation, resulting in activation and stabilization of p53. Numerous studies have demonstrated that perturbation of ribosome biogenesis either by drugs that inhibit RNA Pol I activity or by growth inhibition enhances the interaction of these RPs with MDM2, whereas depletion of these RPs attenuates the stress-induced p53 stabilization (for detail, see review in Ref 43). Moreover, the interaction of these RPs with MDM2 appears to increase only in conditions of ribosomal stress, but not of DNA damage or oncogene overexpression.35,36 These RPs can also inhibit MDM2 more efficiently in combination, as seen for RPL5 and RPL11.44 These observations led to the widely accepted notion that inhibition of ribosome biogenesis causes nucleolar stress. As a result of this stress, several RPs enter the nucleoplasm, where they interact with and suppress the E3 ligase activity of the MDM2s to promote p53 stabilization.43 Indeed, disruption of the nucleolus is required for p53 activation and stabilization.45

However, recent evidence suggests that RPs functionally interact with MDM2 during other genotoxic stresses as well, such as those induced by DNA-damaging agents. The induction of p53 in response to various types of stress caused by different kinds
of drugs is attenuated in a similar manner when either RPS7 or RPL11 is downregulated. Hence, it appears that general nucleolar disruption, rather than a specific ribosomal biogenesis defect, can cause the interaction of these RPs with MDM2. Another issue is the role of the MDMX protein in the RP–MDM2 interaction. Activation of p53 by ribosomal stress requires degradation of MDMX, and RPL11, but not RPL5 or RPL23, stimulates MDMX polyubiquitination by MDM2. In contrast, RPS7 requires MDMX to inhibit the E3 ligase activity of MDM2. These results imply that each RP may have a unique way of inhibiting MDM2 function. Interestingly, MDM2 itself can target its RP-binding partners, suggesting an alternative means of p53 regulation. For instance, RPL26, which binds to p53 mRNA and promotes its translation, is ubiquitinated by MDM2, resulting in proteosomal degradation of RPL26 and decreased binding with p53 mRNA. RPS7 is also a substrate for MDM2, but it is not known if MDM2 targets it only for proteosomal degradation or also for other functions.

Recently, another RP, RPS3, was identified that physically interacts with both p53 and MDM2. Whereas the KH domain of RPS3 appears to be critical for this interaction, the central acidic domain of MDM2 is dispensable. Full-length MDM2 with a mutation in the acidic domain can still bind to RPS3. Furthermore, a reduction in RPS3 levels attenuates the oxidative stress-induced MDM2 inhibition and p53 activation. However, RPS3 depletion does not induce ribosomal stress because a 50% reduction in RPS3 levels has no effect on ribosomal stability and protein synthesis; only cytosolic, ribosome-free RPS3 is reduced, whereas ribosome-bound RPS3 remains unaffected.

Thus, it is clear that several RPs interact with MDM2 and suppress its function in response to a variety of nucleolar stresses, including genotoxic stresses other than ribosomal stress. It is also clear that these MDM2-binding RPs do not compensate for each other in reducing MDM2 function. For example, the induction of p53 in response to various stress conditions is attenuated when RPL11 or RPS7 is depleted, although one would expect other RPs, such as RPL5 or RPL23, to bind to MDM2 and stabilize p53. In contrast, knockdown of RPL23 increases p53 levels even in unstressed conditions, but downregulation of RPL11 decreases p53 levels. Moreover, the response of a cell to reduced levels of an RP, e.g., RPS7, is not consistent in all cell types. In some cells, the ability to inhibit MDM2 and stabilize p53 is decreased due to RP57 depletion, but in other cells, ablation of RPS7 leads to p53-dependent and -independent cell cycle arrest, suggesting that other mechanisms are responsible for p53 induction independently of MDM2. Despite differences in cellular responses to the depletion of a single RP, it is conceivable that ribosomal biogenesis defects can lead to the accumulation of several RPs within the nucleus, resulting in p53 induction by RPs, either through inhibition of MDM2 function or through increased p53 translation (e.g., RPL26). However, knockdown of RPs decreases the abundance of other RPs belonging to the same subunit, probably due to the decreased stability of individual RPs because RPs are usually produced in excess and ribosome-free RPs are quickly degraded in the nucleoplasm. The mechanism by which these RPs are stabilized in the nucleoplasm so that they can interact with MDM2 in response to nucleolar stress is still unknown. Nevertheless, given that MDM2-binding RPs continue to be identified, it is likely that many RPs (if not all) have extraribosomal MDM2 modulatory functions and that the interacting RP varies depending on the type, duration, and intensity of the stress signal. The growth rate of the cells might also be important for relocalization or accumulation of specific RPs in the nucleoplasm. In an MDM2 peptide pull-down assay, RPS20, in addition to RPS3, coprecipitated as an MDM2-interacting protein, and unpublished data in a recent review indicate that RPS27L (RPS27-like protein) could be yet another MDM2-binding RP.

**RIBOSOMAL STRESS AND p53: THE ABERRANCY OF RIBOSOME BIOGENESIS FACTORS**

As mentioned earlier, the synthesis of the ribosome requires active participation of several hundred accessory factors, commonly known as Ribi (ribosome biogenesis) factors. These include many nucleolar proteins and small nucleolar RNAs (snoRNAs) that play critical roles in pre-RNA processing and preribosomal assembly within the nucleolus, the organelle where the majority of ribosome biogenesis takes place.

Over the last 10 years, several interesting findings have demonstrated that the control of ribosome biogenesis within the nucleolus is rate limiting for cell proliferation. Many oncoproteins and tumor suppressors regulate ribosome biogenesis within the nucleolus, and this regulation can be lost in cancer cells, which require increased protein synthesis and increased ribosome production. Recently, the oncoprotein c-Myc was identified as a direct regulator of ribosome biogenesis and protein synthesis due to its ability to enhance Pol I-mediated transcription of rRNA genes, the Pol II-mediated transcription...
of RPs and other ribosome-associated genes, and the Pol III-mediated transcription of transfer RNA, 5S rRNA, and other small RNA genes. Deregulated c-Myc activity leads to tumorigenesis and overexpression of c-Myc has been observed in many human cancers. Tumor suppressors such as retinoblastoma protein (RB) and p53 inhibit rRNA synthesis by repressing Pol I and Pol III transcription activity in the nucleolus. Indeed, mutations in p53 and RB cause an increase in rRNA synthesis that results in upregulation of ribosome biogenesis, and p53 and RB are frequently mutated in human tumors. The most compelling evidence that demonstrates a direct link between nucleolar ribosome biogenesis and the control of cell proliferation comes from the study of Pestov et al. In this pioneering study, it was demonstrated that expression of an aberrant dominant-negative form of a nucleolar protein involved in rRNA processing and ribosome assembly, Bop1, causes nucleolar stress that leads to cell cycle arrest in a p53-dependent manner. Subsequently, several studies in cell lines and animal models have shown that alterations in many other ribosome-associated nucleolar proteins elicit a similar response. For instance, overexpression of nucleophosmin activates p53 through direct binding of these proteins to MDM2. On the other hand, the deficiency of nucleolar proteins such as WDR12, WAP, WDR55, hUTP18, and WDR3 also leads to p53 induction, which in some cases (hUTP18 and WDR3 deficiency) depends on RPL11. Intriguingly, both overexpression and reduction of nucleostemin activate p53. Overexpressed nucleostemin stabilizes p53 and induces p53-dependent cell cycle arrest by directly binding to MDM2, whereas knockdown of nucleostemin enhances the interaction of RPL5 and RPL11 with MDM2, resulting in an activated p53 response.

Collectively, these findings indicate that the aberrant expression of nucleolar proteins affects ribosome biogenesis, and the resultant nucleolar stress activates p53. Given that many of these nucleolar proteins can bind to MDM2, the activated p53 response could also be due to interaction of these proteins with MDM2, rather than with the RPs alone. In fact, we have observed an upregulation of several nucleolar proteins, including those that bind MDM2, in RPL11-deficient zebrafish (unpublished data). Interestingly, besides regulating the MDM2–p53 feedback loop, RPL11 also acts as a feedback regulator of c-Myc transcriptional activity and a reduction of RPL11 enhances c-Myc-mediated transcription of ribosome-associated genes. Thus, it is likely that RPL11 regulates ribosome biogenesis by controlling c-Myc function and the loss of RPL11 activates a checkpoint response to prevent aberrant ribosome biogenesis due to deregulated c-Myc activity. Although RPL11 is an MDM2-binding RP, our results showed that a deficiency of this protein activates the p53 pathway in zebrafish, supporting the above hypothesis.

**RIBOSOMAL STRESS AND p53: THE DEFICIENCY OF RIBOSOMAL PROTEINS**

Insufficient RP production can also trigger a ribosomal stress response leading to p53 accumulation and stabilization. To date, several reports of RP deficiency in various in vivo and in vitro models have been described (Table 1). The results of these studies have brought up several interesting issues. First, the activation of p53 seems to be a general response of the cell to RP insufficiency. As mentioned earlier, several RPs (RPL5, RPL11, RPL23, and RPS7) have been identified as positive regulators of p53. However, we have shown that in vivo knockdown of RPL11 activates p53 in zebrafish, a response similar to in vitro knockdown of RPL23 in human cell lines. Thus, the deficiency of any RP, regardless of its role in p53 regulation, may activate p53.

Second, the phenotypes associated with an RP deficiency in vertebrates are quite heterogeneous. In the invertebrate *Drosophila*, haploinsufficiency of many RPs results in similar-looking mutants, known as *Minutes*, which show complex phenotypes such as slow development, short bristles, and impaired fertility. These phenotypes are usually thought to arise from insufficient ribosome production because RPs are considered to be ‘housekeeping’ genes, essential for all cell types. However, unlike *Drosophila*, RP depletion in mammalian and nonmammalian vertebrates causes variable phenotypes. Depending on the type of RP, the effect can either be lethal or pleiotropic, with a specific defect in a particular tissue and additional phenotypes, or it may not have any effect at all. It is not clear whether *Dmp53* (the *Drosophila* p53 homolog) contributes to *Minute* phenotype in *Drosophila* as there is no known *Drosophila* homolog of MDM2 and developmental defects due to impaired ribosome synthesis [loss of transcription initiation factor IA (Tif-IA)] are not rescued in a *Dmp53* null background. However, the abnormalities due to RP deficiency in vertebrates mostly involve the p53 pathway. Heterozygous mutations in *RPS6* in mice result in a severe phenotype leading to embryonic lethality, whereas a conditional deletion in the liver results in the complete loss of hepatocyte proliferative capacity following partial hepatectomy due to cell cycle arrest at G1 phase. Mutations in many RP genes result in...
TABLE 1  | Ribosomal Protein (RP) Deficiency and the p53 Pathway

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similar phenotypes in zebrafish: homozygous mutants show deformities in the head and gut,\textsuperscript{90} similar to what we have seen for RPL11 and other RP-deficient zebrafish,\textsuperscript{79,91} whereas heterozygous mutants show developmental delay and growth impairment.\textsuperscript{92} Interestingly, 17 of these heterozygous mutants develop a rare kind of malignant tumor.\textsuperscript{93} Mutations in RPS19 and RPS20 result in dark-skinned mice with a reduced body size and mild anemia.\textsuperscript{85} Our morpholino-induced knockdown of RPS19 in zebrafish, however, shows a more drastic erythropoiesis defect with severe anemia, in addition to other developmental defects.\textsuperscript{94} Surprisingly, RPL22-deficient mice show specific defects only in T-cell development,\textsuperscript{86} whereas haploinsufficiency of RPL24 in mice results in a kinked tail, white hind feet, and a ventral midline spot [belly spot and tail (Bst) mutants].\textsuperscript{95} Intriguingly, the loss of RPL29 does not cause any phenotypic effects except for general growth impairment.\textsuperscript{96}

Third, disruption of the nucleolus and defects in rRNA processing are not a prerequisite for p53 induction in response to an RP deficiency. For example, the loss of RPS6 and RPL24 in human A549 cells leads to an activated p53 response without any effect on the integrity of the nucleolus. Although RPS6-deficient cells show impaired rRNA processing, RPL24-silenced cells show no defects.\textsuperscript{80,87} Similarly, silencing of RPS9 in U2OS cells affects 18S rRNA production and causes an upregulation of the p53 pathway, but the nucleoli remain relatively intact.\textsuperscript{83}

Fourth, the deficiency of RPs and the resultant p53 induction are not dependent on ribosomal stress alone. For instance, the depletion of RPS9 not only reduces the synthesis of 18S rRNA (ribosome stress) but it also causes DNA replication stress.\textsuperscript{83} Similarly, downregulation of RPS7 (cell-specific) and RPL11 attenuates stress-induced MDM2 inhibition and p53 stabilization not only in ribosomally stressed cells (ActD-treated cells) but also in cells treated with various kinds of DNA-damaging agents.\textsuperscript{46}

Finally, p53 seems to have contrasting roles in determining survival versus death in response to RP deficiency. The p53 pathway is responsible for the embryonic lethality of many RP-deficient embryos (RPS6, RPS19, RPL11; see Table 1), and coinhibition of p53 rescues the phenotypes. However, the activation of p53 during embryonic stages promotes the survival of Bst mutants (RPL24-deficient mice).
In these mice, induction of p53 delays the cell cycle during embryonic development (a p21-dependent process), and the p21-independent apoptotic consequence of p53 induction causes the Bst phenotypes in the adults.87

**RIBOSOMES, HUMAN DISEASES, AND p53**

As discussed above, ribosomes are ubiquitous organelles essential for all types of cells. Any perturbation in ribosomal components would therefore be expected to have serious consequences for cell survival. However, this is not the case. It is now universally accepted that ribosomal dysfunction due to haploinsufficiency of RP genes or mutations in genes essential for ribosome biogenesis can lead to specific disease conditions in humans called ribosomopathies, a collection of rare genetic disorders associated with an increased risk of developing cancer.16,17 However, it should be noted that the type and occurrence rate of cancers vary considerably in these diseases. Intriguingly, increased ribosome activity is also a common feature in many malignancies; in fact, overexpression of several RPs is seen in cancer cell lines and primary tumors,15,97 although an elevated ribosomal function in cancers could simply be a result of increased cellular proliferation. It is also not known whether any extraribosomal function of RPs is responsible for cellular transformation. Hence, our discussion in this section is restricted to diseases caused by decreased ribosome activity, particularly those involving the p53 pathway (Table 2).

**X-Linked Dyskeratosis Congenita (OMIM 305000)**

X-linked dyskeratosis congenita (X-DC) is a rare genetic disorder characterized by multiple pathological features, including bone marrow failure, skin abnormalities, and high risk of cancer.113 X-DC is caused by a mutation in the *DKC1* gene, which encodes dyskerin.114 Dyskerin is a nucleolar protein that associates with boxH/ACA (Box H: AnAnnA/Box ACA: ACA) snoRNPs to catalyze a posttranscriptional modification (pseudouridylation) of rRNA.115 Dyskerin is also a component of the telomerase complex that maintains telomere length.116 Hypomorphic *Dkc1* mutant mice (*Dkc1<sup>−/−</sup]*) recapitulate many clinical features of X-DC and display impaired rRNA modification.113 Mouse cells harboring a knock-in of a *DKC1* human deletion, *Dkc1<sup>Δ155</sup>* show increased p53 levels, independent of telomere length.98 Surprisingly, both *Dkc1<sup>−/−</sup>* cells99 and *DKC1*-depleted human breast cancer cells117 show a significant reduction in p53 mRNA translation mediated by an internal ribosome entry site (IRES). This impaired p53 expression allows these cells to bypass p53-mediated responses, such as oncogenic or genotoxic stress responses, leading to increased cell proliferation.99,117 X-DC patients also show similar defects in p53 IRES-dependent translation and display reduced p53 protein expression99, suggesting a basis for the increased cancer susceptibility in X-DC patients.99,117

**Treacher Collins Syndrome (OMIM 154500)**

Autosomal dominant mutations in *TCOF1* cause Treacher Collins syndrome (TCS), a disease characterized by numerous anomalies in the face, head, and neck.101 Tumors or other forms of malignancy have not been reported in TCS. *TCOF1* encodes Treacle, a nucleolar phosphoprotein involved in transcription of ribosomal DNA into rRNA118 and methylation of 18S rRNA.119 In a mouse model of TCS, haploinsufficiency of *Tcof1* results in diminished production of ribosomes, which correlates with decreased proliferation of neural ectoderm and neural crest cells.120 An activated p53 pathway is responsible for the clinical manifestation of this disease, and inhibition of p53 (either genetically or pharmacologically) rescues the craniofacial defects in *Tcof1* haploinsufficient mice.102

**Diamond-Blackfan Anemia (OMIM 105650)**

Diamond-Blackfan anemia (DBA) is an inherited genetic disease of infants characterized by bone marrow failure, congenital anomalies, and a severe erythrocytosis defect.121 The patients are also predisposed to an increased risk of cancer, although, according to the latest statistics from the DBA registry, malignancy has been observed only in a small percentage of patients.16 A subset of patients (∼30–50%) also show other physical anomalies, such as short stature, craniofacial abnormalities, upper limb malformations, and kidney dysfunction.122 The most commonly affected gene in DBA is *RPS19* (∼25%).103 However, some patients show mutations in other RPs, including *RPL5* (∼6.6%),104 *RPS10* (∼6.4%),105 *RPL11* (∼4.8%),104 *RPL35A* (∼3%),106 *RPS26* (∼2.6%),105 *RPS24* (2%),107 *RPS7* (∼1%),104 and *RPS17* (1%).108 Together, these nine genes account for approximately 53% of DBA cases.105 In addition, possible mutations in *RPL9*, *RPL36*, *RPS15*, and *RPS27A* have been reported.104,105 Physical abnormalities are more predominant in patients with *RPL5* and *RPL11*.
mutations; the cleft lip and/or palate phenotype is associated only with RPL5 mutations, except for one patient with a RPS26 mutation,\textsuperscript{104,105} whereas isolated thumb abnormalities are predominant in patients with mutations in RPL11.\textsuperscript{104} Despite considerable research on DBA, the exact molecular mechanism underlying this disease is not fully understood. Although several interesting hypotheses have been proposed for the DBA pathophysiology (see recent reviews in Refs 16, 121, 123), we are still far from unraveling this disease completely. Although the ‘ribosomal stress and p53’ hypothesis appears to be the most widely accepted, the presence of RPL5 and RPL11 mutations in DBA patients challenges this hypothesis, as both of these RPs are clearly essential (in mammalian cells) for p53 activation under conditions of ribosomal stress (discussed above). Cells with siRNA-mediated downregulation of DBA-associated RPs such as RPS19,\textsuperscript{124} RPS24,\textsuperscript{125} RPL5 and RPL11,\textsuperscript{104} or RPS10 and RPS26\textsuperscript{105} exhibit defects in the rRNA maturation pathway. Similarly, CD34\textsuperscript{+} cells from DBA patients and RPS19-deficient erythroid cells show defects in rRNA processing.\textsuperscript{126} These cells also undergo cell cycle arrest\textsuperscript{127} and increased apoptosis,\textsuperscript{128} suggesting the involvement of the p53 pathway in DBA. RPS19 deficiency in zebrafish faithfully recapitulates the hematopoietic and developmental phenotype of DBA.\textsuperscript{94} Using a similar strategy, Danilova et al.\textsuperscript{84} demonstrated that an activated p53 pathway causes the erythroid defects, and mutations in p53 or drug-induced inhibition of p53 function can improve the DBA phenotype of zebrafish. An initial attempt to model DBA in mice was unsuccessful because Rps19 heterozygous mice displayed no abnormalities in the hematopoietic system, whereas Rps19 null mice were embryonic lethal.\textsuperscript{129} Subsequently, another model of DBA has been developed in mice that harbor a heterozygous missense mutation in Rps19 [Dark skin 3 (Dsk3) mutant].\textsuperscript{85} However, unlike DBA patients, the Dsk3 mutants show only mild anemia and a more prominent pigmented phenotype (dark skin) without any physical deformities.\textsuperscript{85} The role of p53 in mediating DBA phenotype was further confirmed in this mouse model, which showed the complete rescue of all the phenotypes, including erythrocytic hypoplasia, when crossed with p53 knockout mice.\textsuperscript{85}

The 5q\textsuperscript{−} Syndrome (OMIM 153550)
The 5q\textsuperscript{−} syndrome is a type of myelodysplastic syndrome associated with hematological abnormalities and is caused by a deletion of the long arm of chromosome 5.\textsuperscript{109} The patients typically have macrocytic anemia, normal/elevated platelets counts, and

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<th>Disease</th>
<th>Mutated Gene</th>
<th>Clinical Features</th>
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<td>X-linked dyskeratosis congenita (X-DC)</td>
<td>DKC1</td>
<td>Bone marrow failure Skin hyperpigmentation Nail dystrophy Oral leukoplakia</td>
<td>High risk of leukemia, solid tumors, and pulmonary fibrosis</td>
<td>Controversial; both upregulated and downregulated p53 activity observed in mouse models and patients</td>
<td>98–100</td>
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<td>Treacher Collins syndrome (TCS)</td>
<td>TCOF1</td>
<td>Craniofacial abnormalities</td>
<td>Not known</td>
<td>Upregulated p53 activity; coinhibition leads to phenotypic rescue in a mouse model</td>
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<td>Diamond-Blackfan anemia (DBA)</td>
<td>RPS19, RPL5, RPS10, RPL11, RPL35A, RPS26, RPS24, RPS17, RPS7, RPL9, RPL36, RPS15, RPS27A</td>
<td>Anemia Bone marrow failure Retarded growth Thumb abnormalities Craniofacial anomalies Kidney dysfunction</td>
<td>Debatable; can progress to acute myeloid leukemia (AML), osteosarcoma</td>
<td>Upregulated p53 activity; proapoptotic erythroid precursors Suppression of p53 leads to phenotypic rescue in mouse and zebrafish models</td>
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<tr>
<td>5q\textsuperscript{−} Syndrome</td>
<td>RPS14</td>
<td>Macrocytic anemia Normal or high platelet count Hypolobulated megakaryocytes</td>
<td>Low risk of AML in drug-responsive patients High risk of AML in nonresponsive patients</td>
<td>Upregulated p53 activity; coinhibition leads to phenotypic rescue in a mouse model</td>
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nonlobulated megakaryocytes in the bone marrow. Patients who respond well to lenalidomide (an immunomodulatory drug) treatment have a low risk for acute myeloid leukemia (AML) but in nonresponders the risk is significantly higher. The 5q− common deleted region contains 40 genes. Deficiency of RPS14 probably accounts for the erythroid defects, whereas its contribution to the other phenotypes remains unclear. Knockdown of RPS14 in normal hematopoietic progenitor cells impairs erythroid differentiation, blocks the processing of 18S rRNA, and results in 40S subunit deficiency. Cells from patients with 5q− syndrome also show a pre-rRNA processing defect, and overexpression of RPS14 rescues the erythroid defects in these patients. Recently, it was shown that deletion of miR-145 and miR-146a, which are transcribed from the common deleted region, is responsible for the clinical manifestation of many ribosomal diseases of humans, which often present with a prominent tissue-specific phenotype, such as the anemia in DBA and the 5q− syndrome. Interestingly, associated anomalies such as short stature, craniofacial abnormalities, and physical deformities are also common in ribosomal diseases. Thus, it appears that the activation of the p53 pathway is a common response in all cells, but the consequences of this response vary among different cell types, which may explain why we have such heterogeneous pathological outcomes in these diseases. Indeed, coinhibition of p53 in animal models of ribosomal pathologies completely rescues not only the tissue-specific erythroid defects but also the associated pleiotropic phenotypes of growth retardation, craniofacial abnormalities, and hyperpigmented skin. The factors that make p53 target only the erythroid cells in diseases like DBA and the 5q− syndrome, while allowing other cells to grow, remain to be defined. It has been suggested that rapidly developing tissues, such as the bone marrow, would require increased ribosome activity, and hence would be more susceptible to a ribosomal deficiency. However, in a developing embryo, bone marrow is not the only rapidly dividing tissue and, most importantly, myeloid differentiation is almost normal in these diseases, with only the erythroid precursors affected.

A deregulated translational readout due to ribosomal or RP insufficiency may lead to an increase or a decrease in the translation of specific mRNAs, which interacts with the p53 regulatory system, causing the upregulation and stabilization of p53. Indeed, disruption of the 40S ribosomal subunit due to RPS6 deficiency leads to the upregulated translation of 5’-terminal oligopyrimidine tract (TOP) mRNAs, including that of RPL11. This increases the levels of ribosome-free RPL11 protein, making it available for interactions with MDM2 and subsequent p53 induction. Consistent with this finding, an activated p53 response due to RPS9 deficiency involves the upregulation of RPL11. These observations raise the possibility that in diseases like DBA and the 5q− syndrome that show predominant mutations in 40S subunit proteins, targeting the signaling pathways contributing to RPL11 upregulation, rather than suppressing p53, could be a potential therapeutic strategy that would not increase the risk of cancer development. However, to make matters more complex, RPL11 is also mutated in DBA, along with other 60S subunit RPs (RPL5, RPL35A, RPL36). Depletion of an RP causes a decrease in the levels of the other RPs belonging to the same subunit but, surprisingly, the loss of RPL24 (one of the 60S proteins) results in the accumulation of ribosome-free RPL11 without any effect on ribosome biogenesis or pre-rRNA processing.

How do the cells maintain this increased level of...
In normal cells (upper panel), the biogenesis of the ribosome begins with the transcription of ribosomal DNA into pre-ribosomal RNA (rRNA) precursors by RNA polymerase I in the nucleolus (gray). This pre-RNA cluster then undergoes a series of modifications and cleavage by several hundred small nucleolar RNAs (snoRNAs) and ribosome-associated nucleolar proteins [accessory factors, shown as filled green rhombi; synthesized in the cytoplasm (white)] to form rRNA intermediates and finally the mature rRNAs (18S, 5.8S, and 28S). Seventy-nine ribosomal proteins (RPs, shown as purple circles; produced in the cytoplasm) and 5S rRNA [synthesized in the nucleoplasm (sky blue)] are imported into the nucleolus, where they associate with other rRNAs to form the small (40S) and large (60S) subunits, which are then assembled and exported to the cytoplasm to form the mature ribosome for the initiation of protein synthesis. Under these conditions, p53 is maintained at low levels in the nucleoplasm by murine double minute 2 (MDM2). In ribosomally stressed cells (lower panel), deficiencies of RPs (shown as dotted purple circles) or accessory factors (shown as partially filled green rhombi) cause defects at various stages of rRNA processing, leading to defective 60S and 40S biogenesis, evoking a nucleolar stress response. This results in the translocation of ribosome-free RPs or accumulated (unutilized) accessory factors to the nucleoplasm, where they bind and inhibit MDM2, leading to the activation and stabilization of p53. Impaired 40S biogenesis can also lead to translational upregulation of 5′-terminal oligopyrimidine tract (5′ TOP) messenger RNAs (mRNAs), such as RPL11 and possibly other MDM2-binding RPs, which can enter the nucleoplasm and intercept the MDM2-mediated p53 degradation. Depending on the cell type, the consequence of the activated p53 response could either be cell cycle arrest or apoptosis.

RPL11, when excess RPs are quickly degraded in the nucleus, is possibly some nonribosomal components that remain to be identified are important for synthesis and stabilization of RPL11 and possibly other MDM2-binding RPs.

There may be another twist to this story: a p53-independent pathway might be responsible for the erythropoietic failure in DBA. We have seen that simultaneous knockdown of p53 in RPS19-depleted zebrafish rescues the morphological phenotypes, but not the erythroid defects (unpublished data). Indeed, two reports presented in a recent scientific conference (51st ASH Annual Meeting) showed that both RPS19-depleted zebrafish and RPL35A-deficient human bone marrow progenitors exhibit erythroid defects even on a p53 mutant background, further supporting our data. More recently, Iadevaia et al. showed...
that alterations in ribosome synthesis within erythroid cell lines, either by knocking down RPs (RPS19, RPS6, and RPL7a) or by inhibiting RNA Pol I activity, cause a drastic reduction in pim-1 oncogene (PIM1), a serine/threonine kinase involved in cell cycle regulation. A decrease in PIM1 level induces an increase in p27kip1 (a CDK inhibitor and PIM1 target), which causes cell cycle arrest and blocks cell proliferation even in the absence of p53. This suggests that ribosomal stress can also trigger a p53-independent response.\textsuperscript{136}

Finally, the ribosomal stress–p53 pathway has been suggested as a cellular surveillance mechanism for cancer prevention,\textsuperscript{43} and we know that this pathway is functional in many ribosome-associated diseases of humans (discussed above). Yet, many ribosomal diseases confer a high propensity to the development of cancers, including leukemia, sarcomas, and other solid organ tumors. Given the variable outcome of ribosomal defects in different tissues, several studies have tried to answer the fundamental question of how different cells can respond differently to the same stress. The exciting findings made it clear that the pathways linking defective ribosomes and p53 signaling are more complex than previously imagined, and it is most likely that several players in addition to these MDM2-binding RPs are involved. A challenge for future studies will be to identify the additional players in this signaling network and to elucidate the underlying molecular mechanisms that link defective ribosome synthesis to the p53 signaling pathway.

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REFERENCES


53. Lam YW, Lamond AI, Mann M, Andersen JS. Analysis of nucleolar protein dynamics reveals the nuclear


