Role of ribosomal protein mutations in tumor development (Review)

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Abstract. Ribosomes are cellular machines essential for protein synthesis. The biogenesis of ribosomes is a highly complex and energy consuming process that initiates in the nucleolus. Recently, a series of studies applying whole-exome or whole-genome sequencing techniques have led to the discovery of ribosomal protein gene mutations in different cancer types. Mutations in ribosomal protein genes have for example been found in endometrial cancer (RPL22), T-cell acute lymphoblastic leukemia (RPL10, RPL5 and RPL11), chronic lymphocytic leukemia (RPS15), colorectal cancer (RPS20), and glioma (RPL5). Moreover, patients suffering from Diamond-Blackfan anemia, a bone marrow failure syndrome caused by mutant ribosomal proteins are also at higher risk for developing leukemia, or solid tumors. Different experimental models indicate potential mechanisms whereby ribosomal proteins may initiate cancer development. In particular, deregulation of the p53 tumor suppressor network and altered mRNA translation are mechanisms likely to be involved. We envisage that changes in expression and the occurrence of ribosomal protein gene mutations play important roles in cancer development. Ribosome biology constitutes a re-emerging vital area of basic and translational cancer research.

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1. Introduction

Cancer cells display a number of abnormal properties in order to maintain their unrestrained growth and proliferation (1). Ribosome biogenesis and protein synthesis are in this context critical cellular processes necessary for sustained cancer cell growth. Historically, ribosomes were considered to be relatively stable entities. However, with the discoveries of mutations affecting ribosomal protein (RP) genes in the Diamond-Blackfan anemia (DBA) syndrome it became evident that mutant RPs may cause complex, variable, and viable phenotypes (2). Of note, DBA and other syndromes involving mutant ribosomal or nucleolar proteins are often associated with an increased life time risk of cancer (3). Recently, a number of studies using next generation sequencing technologies describe RP gene mutations also in cancers without a previous known history of bone marrow failure disorder. By applying whole-exome sequencing, RNA seq, or whole-genome sequencing, RP gene mutations have been detected in the genome of cancer cells, including from endometrial cancer, T-cell acute lymphoblastic leukemia (T-ALL), chronic lymphocytic leukemia (CLL), colorectal carcinomas, and high grade gliomas (4-7). The mechanisms underlying cancer development in the setting of a ribosome biogenesis defect remain poorly understood. In this review, the most recent studies are summarized and possible mechanisms by which mutant ribosomal proteins are linked to cancer development are discussed.

2. The ribosome at a glance

The basis of protein synthesis is the translation of messenger RNA (mRNA) to an amino acid sequence. Translation of mRNA is carried out by the ribosome, transfer RNA (tRNA), with the assistance of an army of different helper proteins. The intrinsic catalytic activity of ribosomes is thought to be dependent on ribosomal RNA (rRNA), that is involved in mRNA

decoding and formation of peptide bonds. Certain chemical modifications on rRNA, including pseudo-uridylation and ribose methylation, are critical for maintaining proper rRNA structure and modulate the interactions between rRNA and proteins (8). The ribosome consists of two subunits, each of which is made up of ribosomal RNA (rRNA) and many RPs. Eukaryotes have 80S ribosomes, consisting of the small (40S) and the large (60S) subunit. The large 60S subunit is composed of a 5S rRNA, a 28S rRNA, a 5.8S subunit, and ~46 RPs. The small 40S subunit has an 18S rRNA and ~33 RPs. Note that the 5S rRNA is transcribed by RNA polymerase III, while 28S, 5.8S, and 18S rRNAs are processed from a long precursor (pre-) rRNA transcribed by RNA polymerase I (9). The maturation of pre-rRNA occurs in the nucleolus involving both endo- and exonucleases that remove external and internal transcribed sequences. In the nucleolus the 45S pre-RNA associates with RPs, ribonucleases, RNA helicases, small nucleolar RNPs and other accessory factors, to form 90S pre-ribosomes. During the maturation process, the 90S pre-ribosome is separated into pre-40S and pre-60S subunits that are exported to the cytoplasm where maturation is completed (9). It should be noted that RPs are synthesized by pre-existing ribosomes in the cytoplasm and imported into the nucleus where majority of the RPs home into the nucleolus to assemble with rRNA, and majority of RPs are essential in ribosome biogenesis (10-12). Strikingly, RPs have high isoelectric points allowing them to interact with rRNAs, mRNAs, and tRNAs (13). The names of the RPs belonging to the large subunit include the prefix L and the names of the RPs of the smaller subunit include the prefix S. A new universal nomenclature has been launched and we will provide both names at their first mentioning in the text (14).

Ribosome heterogeneity. Some cells have the potential to produce ribosomes with a different composition of RPs, and post-translational modifications, in response to changing extracellular demands. These adaptations have mostly been studied in bacteria, plants, and yeast but recently also in mammalian cells (15). There are a number of potential mechanisms leading to ribosome heterogeneity (16), although the nature of the heterogeneity is variable, from subtle changes in post-translational modification patterns to the loss of an RP. Duplicated RP genes exist in the genomes of some species such as plants. These extra RP genes are sometimes encoding for a variant protein (paralog) that may differ in amino acid sequence (17). Paralogs might have specific functional roles. For example, *Rpl22*$^{-/-}$ mice have only subtle phenotypes with no significant translation defects because in these mice there is a compensatory increase in Rpl22-like1 *(Rpl22l1)* expression and incorporation into ribosomes (17). Importantly, knockdown of Rpl22l1 impairs growth of cells lacking Rpl22 (17). Post-translational modifications of RPs (e.g. ubiquitination and phosphorylation) have been described and these may alter the functional properties of ribosomes (18). Another layer of ribosome heterogeneity may stem from differences in modification of the rRNA itself (8). RP genes also generate a large number of processed pseudogenes that are dispersed throughout the genome (13,19). While the pseudogenes have been considered to be inactive there are studies indicating that they have the potential to produce functional coding RNA and protein (20). Finally, it should be added that long non-coding RNAs are involved in regulating mRNA translation, a number of long non-coding RNAs associates with cytoplasmic ribosomes, and if we also include these regulatory levels, the complexity becomes even higher (21,22). Taken together, there are a number of potential different mechanisms contributing to ribosome heterogeneity, and these are probably functionally relevant to both normal and cancer cells. One may suspect that certain mechanisms are dominant in cancer cells when compared to normal cells. It will be important to identify these differences as it might open up novel avenues for anti-cancer treatment.

A critical issue to keep in mind concerns the fate of pre-ribosomes in the context of an RP mutation or deletion (23). It is known that the synthesis of ribosomes is a process regulated and balanced at multiple levels (24), and that RPs produced in excess are rapidly degraded in the nucleus (25,26). Depletion of an individual RP in normal cultured cells often, but not always, results in a decrease in the total level of the other RPs belonging to the same ribosomal subunit, thus creating an unbalanced ribosome assembly pathway (27,28). In the setting of an RP loss by deletion or an early truncating mutation one may therefore expect reduced numbers of ribosomes to be a common outcome. Normal and cancer cells may try to compensate a ribosome deficit by activation of pathways that boost ribosome production, *e.g.* the mTOR pathway (29). This situation may create a pressure to mutate components in the cell that normally restrains the pathway activity in question.

3. Mutations and altered expression of ribosomal proteins in cancer

Animal models with mutations in ribosomal protein genes increase cancer risk. Genes encoding RPs have been found mutated in some organisms including *Drosophila* and Zebrafish. Mutations in RP genes have also been found in humans. *Minutes* is a class of *Drosophila* mutants known for their short slender bristles (stiff hair) on the body, overall reduced body size and delayed metamorphosis (30). *Minute* genes often encode RPs thereby explaining certain aspects of the *Minute* phenotype, for example reduced body size. Paradoxically, decreased levels of a subset of *Drosophila* RPs result in overgrowth of specific tissues for example hypertrophied hematopoietic organs and melanotic tumors. The lymph glands are overgrown in *Rps6 (eS6)* mutant larvae, due to increased growth and proliferation of the lymph gland cells indicating that *Rps6* has a tumor suppressive function (31,32). Decreased levels of Rps6 in the prothoracic gland reduce the steroid hormone ecdysone delaying development, but tissues or organs continue to grow abnormally (33). As another prelude to what is now an emerging research field in cancer biology serves the finding of heterozygous loss-of-function mutations in several RPs that cause development of malignant peripheral nerve sheet tumors (MPNSTs) in zebrafish (34,35). MPNSTs are sarcomas which emerge from peripheral nerves or from cells associated with the nerve sheath. Zebrafish carrying heterozygous mutations for 17 different RP genes are prone to MPNSTs. Noteworthy, MPNSTs also arise in zebrafish that have lost wild-type p53 function, and in line with this, p53 was not detected in cells derived from the tumors in the RP mutant

Table I. Examples of ribosomal protein gene mutations in human tumors.

RP	Tumor type	Mutation type	Examples	Ref.
RPL5 (uL18)	GBM, T-ALL, lung-adenocarcinoma	Missense, insertions, deletions	p.Arg58LysfsX55, p.Asp59fs, p.Gln63Arg, p.Arg179X, p.Asn57fsX12, p.Arg54Cys, p.Glu82Lys, p.Met212fs	(4,5)
RPL10 (uL16)	T-ALL	Missense	p.Arg98Ser, p.Arg98Cys	(4)
RPL11(uL5)	T-ALL	Missense	p.Arg18Pro, p.Gly30fs	(59)
RPL22 (eL22)	Gastric cancer, T-ALL, endometrial, colorectal cancer	Insertions, deletions	p.Lys15ArgfsX5, p.Lys16GlufsX9	(60-63)
RPS15(uS19)	CLL	Missense, nonsense	p.Gly105Ser, p.Ser111Phe p.Pro131Ser, p.Gly132Ala	(57,58)
RPS20 (uS10)	Colorectal cancer	Insertion	p.Val50SerfsX23	(6)

CLL, chronic lymphocytic leukemia; GBM, glioblastoma; RP, ribosomal protein; Ref, reference; T-ALL, T-cell acute lymphoblastic leukemia; fs, frameshift; X, stop.

fish (36). In contrast to *Drosophila* and Zebrafish, there are not many reports of increased tumor incidence in mice carrying mutations or deletions in RPs (for example Rps19, Rpl24 and Rps6). Although it is known that loss of a single Rpl22 allele accelerates development of thymic lymphoma in a mouse model of T-cell malignancy (37), and heterozygous *Rpl11* mice are more prone to radiation-induced lymphomagenesis (38). A recent study describes an increased incidence of soft tissue sarcomas in mice lacking one allele of *Rpl5* or *Rps24* (39).

Ribosomopathies and cancer risk in humans. Congenital diseases found in humans that are linked to genetic defects in RPs or ribosome biogenesis factors are collectively known as the ribosomopathies (40-42). These include Dyskeratosis congenita (DKC), Diamond-Blackfan anemia (DBA), and Shwachman-Diamond syndrome (SDS) that constitute major inherited bone marrow failure syndromes (41). The ribosomopathies are characterized by a number of abnormalities including birth defects and anemia (41). DBA is a dominant autosomal bone marrow failure syndrome associated with mutations in RP genes including *RPS19(eS19), RPS17(eS17), RPS24(eS24), RPL35A(eL33), RPS7(eS7), RPL5(uL18), RPL11(uL5), RPL26(uL24), RPL27(eL27), RPS10(eS10), RPS26(eS26), RPS27(eS27), RPL15(eL15), RPS28(eS28), RPL31(eL31) and RPS29(uS14)* (2,3,43-45). Patients with DBA experience a block in erythroid progenitor cell division in the bone marrow coupled to an increased apoptosis (46). DBA patients have a 5-fold higher lifetime risk of cancer than the general population, specifically a 28- to 36-fold higher risk of developing AML, osteosarcoma, or colon cancer (3). Although a somatic mosaic disorder, and not congenital, *RPS14(uS11)* heterozygous loss is associated with *5q-* syndrome and the development of anemia (47). Patients with 5q- syndrome or SDS are at higher risk of developing AML (48-50). DKC is a syndrome characterized by premature aging and increase in cancer susceptibility. X-linked DKC, which has a more severe phenotype compared with the autosomal dominant form of DKC, is caused by a mutation in *DKC1*, which encodes dyskerin (51). Dyskerin is in part a nucleolus located protein associated with the snoRNPs involved in rRNA modification (52,53). Patients with X-linked DKC are predisposed to AML, lymphoma, and a variety of solid tumors including squamous carcinoma (54). Note that both DKC and SDS have a higher risk of cancer development than DBA, especially the risk of leukemia, although some cohorts are rather small thus causing estimates with greater differences among the studies (3,48,49,54,55). It should be emphasized that the main problem in DBA patients is related to acute effects from bone marrow failure or complications due to chronic blood transfusions and not cancer *per se* (56).

Cancer associated mutations in ribosomal protein genes. Genome-wide sequencing indicates that RP gene mutations are relatively frequent in some cancer types. *RPS15(uS19)* mutations have been found in CLL and even more frequently in relapsed CLL (up to 19.5% of cases) (57, 58). Moreover, ~10% of children with T-ALL have mutations in RP genes including *RPL10(uL16), RPL5, RPL11,* and *RPL22* (4,59,60). In fact, 6.5% of T-ALL patients presented with an identical RPL10 Arg98Ser missense mutation (4) (Table I). A separate study in T-ALL patients identified a 10% incidence of heterozygous deletions in the region of chromosome 1p that harbors *RPL22* (60), and a number of T-ALL cell lines and relapse cases had point mutations in *RPL22* (60). In line with its potential role as tumor suppressor, *RPL22* is also mutated or have decreased expression in other cancers as well, including endometrial cancers, colorectal cancer, gastric cancer, breast carcinoma, and non-small cell lung carcinoma (7,61-63). Internal deletions and insertions resulting in early truncating frameshifts are most commonly seen, examples include RPL22 Lys15Arg and Lys16Glu (Table I). Truncating frameshift mutations in *RPL5* have been detected in glioblastoma (5) and *RPL5* (as well as *RPL22*) is identified as being mutated at a significant frequency in cancer (5). A closer look at TCGA

data using the cBioportal website suggests that *RPL10* and *RPL22* are deleted in cases of diffuse large B cell lymphoma, adrenocortical carcinoma, and sarcoma, and that *RPL5* is mutated in a few cases of human MPNSTs (64), and potentially in other cancer types including endometrial carcinoma and lung adenocarcinoma. Genetic linkage analysis and exome sequencing led to the identification of a truncating germline mutation in *RPS20(uS10)* predisposing to colorectal cancer, which is interesting given the association of DBA with colon cancer, but a previous history of DBA appeared unlikely (6). Deep sequencing uncovered the existence of *RPL39(eL39)* mutations in cells from breast cancer lung metastatic lesions (65). A more complete picture of the relevant RPs in cancer will emerge from additional sequencing projects and from functional studies. One must also recall that possibly not all relevant RP mutations are detected since the mutations may be present in a small subpopulation of cells (66). Especially solid cancers often exhibit cell heterogeneity that may prevent the identification of specific mutations. Cell sorting in combination with single cell genome sequencing and single cell RNA-seq may provide more detailed information in the future.

Comparison of RP mutations in cancer and DBA. Are the DBA associated RP gene mutations different from the mutations that have been found in cancer? A cross-comparison of TCGA data with associated recent publications and information available in the DBA database (67), indicates that the mutations described to date usually are different but a few are actually in common. As with regard to RPL5, mutations Lys5fs, Val6fs, Arg35fs, Asn57fs and Asp59fs have been found in cancer and also the RPL5 point mutants Glu82Lys and Arg54Cys (Table I). There are a large number of DBA associated RPL5 mutants including Met1Arg and Arg58Lys. Most interestingly, two of the RPL5 mutations seen in DBA were also found in T-ALL namely Arg179X and Arg58LysfsX55. The region in RPL5 between Arg54 to Asp59 appears to be a 'hot spot' in both DBA and cancer. RPS15 frequently mutated in CLL is rarely so in DBA and the Met70Val DBA mutant has to date not been found in CLL. Also, the few RPL11 mutations in T-ALL described have so far not been observed in DBA. It will be important to investigate whether cancer associated mutations in RPs occur in the setting of an underlying ribosome biogenesis disorder.

Alterations in RP gene expression patterns. Changes in the expression levels (mRNA) of RPs in cancer is common (68), although in most studies it remain unclear to what extent changes in RP expression is merely a necessity to sustain rapid cancer cell growth. For example, increased expression of *RPS2* was found in mouse hepatocellular carcinoma samples and in mouse livers after partial hepatectomy correlated with increased cell proliferation (69). Given the discovery of cancer associated genetic changes in RPs we must also consider changes in RP expression patterns as potentially relevant to cancer development. RPs have been found overexpressed in cancer, for example *RPL15(eL15)* and *RPL19(eL19)* in gastric cancer (70), and *RPL7A(eL8), RPL19(eL19), RPL37(eL37)*, in prostate cancer (71,72). RPs can also be expressed at reduced levels, e.g. *RPL27(eL27), RPL37A(eL43)* and *RPL41(eL41)* are downregulated in a subset of cell lines derived from nasopharyngeal carcinomas (73). Changes in the expression of RPs have in some cases been used to distinguish between normal and cancer cells, and these changes may even have prognostic or predictive values. For example, patients with prostate cancers that display low levels of *RPL19* have better survival (72). Increased levels of *RPS11(uS17)* and *RPS20(uS10)* predicted poor survival of primary glioblastomas (74). In contrast, RPL15 expression status may serve as a prognostic marker in pancreatic ductal adenocarcinoma in that decreased expression was significantly associated with poor overall survival. A potential explanation could be related to an increased invasive capacity of the pancreatic cancer cells with a reduction in *RPL15* (75). One study points out that levels of *RPL13(eL13)* correlated with clinical staging in gastric cancers (76). *RPL36(eL36)* has potential as a prognostic marker, its expression revealed better overall survival and was found to be an independent prognostic factor for overall survival in resected hepatocellular carcinoma (77). The aforementioned studies are just a few examples, and for more complete lists of cancer types with alterations in ribosomal proteins the reader is referred to recent reviews (68,78). High resolution comparative genomic hybridization, RNA-seq, and analysis of DNA methylation patterns in promoter regions on a global scale, will shed further light on RPs and their alterations in cancer.

4. Possible mechanisms whereby mutations in ribosomal proteins cause cancer

Checkpoint activation - lessons from the mouse. The mechanism(s) by which RP mutations increase the risk of developing cancer remains an important unanswered question and several hypotheses have been proposed (79,80). RP deficiency often causes complex phenotypes during development. These different phenotypes may arise from altered translation and/or from the effects of activation of cell stress responses including cell cycle arrest and apoptosis (81). This complexity is seen in a number of different mouse models. *Rpl24(eL24)*$^{+/-}$ mice display a size decrease of approximately 20%, white ventral midline spots, white hind feet, and kinked tails (82). *Rpl29 (eL2)*$^{+/-}$ mice suffer from a global growth deficiency and shortened lifespan. *Rpl38(eL38)*$^{+/-}$ mice present with tissue-specific patterning defects due to the perturbation of a subset of Homeobox mRNAs (83). Given these pleiotropic phenotypes several mechanisms could also be involved in cancer development.

The best known response to ribosome biogenetic defects involves the tumor suppressor p53 that induces cell cycle arrest, senescence, apoptosis, or differentiation (84,85). A number of mouse models confirm the involvement of p53 in mediating certain phenotypes. For example, deletion of only one allele of *Rps6* is enough to impair ribosome biogenesis, but the early embryonic lethality is due to activation of p53-dependent cell cycle arrest and apoptosis rather than to a general downregulation of protein synthesis (86). Furthermore, mutations in *Rps19(eS19)* and *Rps20(uS10)* in mice result in p53-dependent pigmentation defects (abnormal melanocyte proliferation), reduced body size, and anemia (87). Rpl22 deficient mice develop T lymphopenia by blocking αβ-T cell development in a p53-dependent manner (37,88). Supporting observations also came from studies on the 5q-

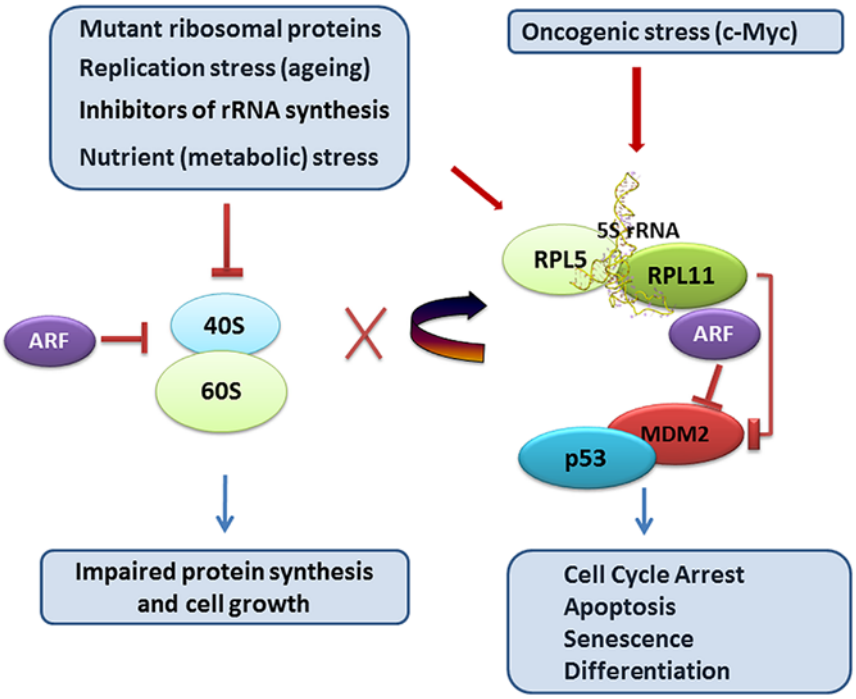

Figure 1. The 5S RNP complex (RPL5, RPL11 and 5S rRNA) regulates MDM2-p53 in response to cellular stress. Schematic overview showing the role of the 5S RNP in coupling disturbances in ribosome production that can be triggered by inhibitors of ribosomal RNA synthesis, mutations in essential ribosome components, nutrient stress, and replicative stress. Stabilization of p53 in response to illegitimate activation of oncogenes (c-Myc) relies partially on 5S RNP. Oncogenic stress also induces the ARF tumor suppressor that in turn inhibits ribosome biogenesis. Besides, ARF interacts with 5S RNP through RPL11/MDM2 association. Not shown in the figure is the DNA damage sensing pathway through ATM/ATR kinases that is connected to ARF and p53.

syndrome. The haplo-insufficiency of RPS14 has a critical role in the development of the anemia that characterizes 5q- syndrome (47). Bone marrow cells from a mouse model of 5q- syndrome shows elevated level of p53 and intercross with *Trp53*$^{-/-}$ mice rescued the macrocytic anemia and dysplasia phenotypes of the 5q- mouse (89). For a more exhaustive list of the different mouse models having mutations in ribosomal protein genes we refer the reader to an informative overview by Terzian and Box (90).

5S RNP-p53 control mechanism. Activation of checkpoints for quality control of ribosome biogenesis is contributing to the disease manifestations among the ribosomopathies (91,92). The hematopoietic phenotype in DBA patients is for example at least partially linked to the activation of p53 (93). What is the mechanism sensing ribosome dysfunction leading to p53 activation? It is now established that two RPs, namely RPL11 (uL5) and RPL5 (uL18), control p53-dependent cell cycle arrest, senescence or apoptosis in response to impaired ribosome biogenesis (91,94,95). Loss of RPL5 or RPL11 also impairs ribosome biogenesis and stalls cell proliferation similar to other essential RPs (27,95), but in the case of RPL11 or RPL5 there is no distinct cell cycle arrest (95). RPL11 and RPL5 regulate p53 as key components of the 5S ribonucleoprotein particle (5S RNP), in which the 5S rRNA is essential as well (96-98). When ribosome biogenesis is blocked, the 5S RNP pre-ribosomal complex is re-directed from assembly into 60S ribosomes to MDM2 E3 ligase inhibition (99-101) (Fig. 1). 5S RNP promotes cellular senescence in response to oncogenic or replicative stress, given that oncogenic stress accelerates rRNA transcription while replication stress delays rRNA processing both causing imbalances in ribosome production (102) (Fig. 1). The 5S RNP complex also act as a sensor responsible for stimulating fatty acid oxidation in response to nutrient depletion (103), and sets the level of p53 activation by ARF (p14ARF, p19Arf), a protein induced by oncogenes (97). The ARF and RP-MDM2 interactions are distinct regulatory pathways and function in non-redundant manner to boost the p53 response to oncogenic c-Myc yet to some extent they rely on each other (104). ARF is a joker in the game and there are now a number of unresolved issues regarding the functional interplay between ARF and 5S RNP. 5S RNP (RPL11/RPL5/5S rRNA and MDM2) has now with these findings emerged as a critical coordinator of signaling pathways at the interface of cell growth and proliferation control. Intuitively, p53 would then be influenced by a number of other factors regulating 5S RNP (96,97).

Role of 5S RNP-p53 activation in DBA models and links to cancer. What is the functional relevance of the 5S RNP-Mdm2-p53 pathway in DBA? Mice with reduced levels of Rps19, that display hallmarks of DBA and p53 activation, were crossed with Mdm2^{C305F} knock-in mice (105). The Mdm2^{C305F} mice have a disrupted 5S RNP-Mdm2 interaction (98) since the MDM2^{C305F} mutation causes a collapse of the MDM2 zinc finger, with subsequent loss of RPL5 and RPL11 binding (106-108). Upon induction of Rps19 deficiency, a disrupted 5S RNP-Mdm2 interaction by Mdm2^{C305F} was able to partially reverse the p53 response and improve the expansion of hematopoietic progenitors *in vitro*, and the anemia became less severe (105). One may then conclude that p53 activation through 5S RNP plays a role in DBA pathogenesis

although it is not the only mechanism involved. The role of p53 is debated, and the anemia seen in zebrafish models of DBA is sometimes not ameliorated by the concomitant inactivation of p53 (109-113). Discrepancies among the studies may in part be explained by the fact that a more complete knockdown of an RP often results in severe p53-independent phenotypes, whereas a milder reduction generates a milder p53-dependent phenotype. We must also keep in mind that p63 and p73 in some settings may serve as a back-up for p53 functions (109).

Deregulation of the 5S RNP-MDM2-p53 pathway may have a functional role in the evolution of 5q- syndrome and DBA into malignancy such as leukemia. It is not far-fetched to suggest that the chronic growth inhibition caused by p53 in turn could select for mutations that promote unrestricted growth and overcomes p53 function (for example in *RPL11, RPL5, MDM2* or *TP53*). Mutation in *TP53* is considered a critical step in the progression of the 5q- syndrome to AML (114,115). An unresolved issue at the moment relates to the involvement of *RPL11* and *RPL5* since they are frequently mutated in DBA to begin with, and thus raising questions about the role of 5S RNP checkpoint in these patients. Indeed, heterozygous conditional loss of *Rpl11* in adult mice triggered anemia similar to DBA patients (38), but the mice were more prone to radiation-induced lymphomagenesis, and also failed to induce p53 when treated with agents triggering ribosomal stress for example Actinomycin D (38). This is similar to $MDM2^{C305F}$ knock-in mice that fail to mount a p53 response upon treatment with Actinomycin D (98). Most studies that describe an increased association of RPL11/RPL5 with MDM2 rely on Actinomycin D treatment or a severe reduction of an RP. DBA, however, develops on a heterozygous ($RP^{+/-}$) background as a consequence of RP gene haploinsufficiency in hematopoiesis. Whether in the $Rpl11^{+/-}$ cells there is sufficient residual RPL11 and/or RPL5 for the checkpoint to operate is not clear. There is a need to better understand the dynamics of RPL5/L11 binding to MDM2 in the context of reduced levels of one component of the 5S RNP complex and explore 5S RNP-independent mechanisms. For example, one such mechanism potentially relevant to cancer development is related to the AKT pathway. RP mutations in zebrafish suppress activity of the AKT pathway resulting in proteasomal degradation of p53 and by re-activating the AKT pathway stabilization of p53 was restored (116,117). In support, RP-deficient zebrafish embryos (similar to RP haploinsufficient zebrafish tumor cells) exhibited normal p53 transcription, but reduced levels of p53 protein, and an impaired p53 response to DNA damage (36,116,117). The role of AKT pathway in RPL11 deficient cells should therefore be explored. In summary, accumulating evidence from cell culture, mouse models and DBA patients indicate the importance of maintaining a normal 5S RNP regulation of p53, although a number of unresolved issues remains (38). Encouraging for the future is that the molecular anatomy of the MDM2-RPL11 complex have been resolved in greater detail and this allows for efforts to design tailor-made drugs (118). Such compounds may then either enhance or block the p53 response with potential benefits to cancer and DBA patients, respectively.

Alternative modes of p53 regulation. In addition to the 5S RNP complex, other possible signaling molecules are thought to be activated in ribosome deficient cells and that may converge on p53 to increase its activity. ATR and ATM kinases are key components of the replication stress and DNA-damage checkpoints contributing to p53 activation. The ATR-Chk1 pathway was implicated in cell cycle arrest induced by inhibition of rRNA synthesis using Actinomycin D although in the absence of DNA damage (119), and was also found activated in RPS19-deficient human cells (120). Increased levels of DNA damage response markers including γH2AX were detected in U2OS cancer cells depleted of RPS9 (uS4) (27). Another potential mechanism could be related to maintenance of proper nucleolar structure and genome stability. The nucleolus plays an important role in the spatial organization of certain heterochromatin enriched chromosome domains (121). Disruption of the heterochromatin architecture surrounding nucleoli has been described in cells depleted of RPs indicating there is a fine balance between ribosome biogenesis and chromatin organization (122). Altered organization of heterochromatin including silent rDNA may predispose cells to genome instability and DNA damage (123).

Autophagy is probably a relatively common cellular response to loss of an RP. Autophagy could be dependent or independent of mTOR and p53 in a cell type-specific manner (117,124). There are other p53-independent effects seen in cells with defects in ribosome biogenesis for example directed degradation of the E2F-1 transcription factor. p53-independent ribosome biogenesis effects have been reviewed (84,125-127). In essence it is clear that activation of specific cell protective mechanisms appears as a common response to a shortage in ribosomes.

Alterations in mRNA translation. Other potential mechanisms that may play a role in cancers with RP mutations and in the ribosomopathies are related to the hypothesis that defective maturation of ribosomal subunits could delay translation of certain mRNAs or that malfunction of accumulated ribosomal precursors may cause aberrant translation (reduced fidelity). It may involve differential translation of specific mRNA transcripts or the use of alternative translation initiation sites. Both quantitative variations in actual ribosome numbers and qualitative alterations such as lack of rRNA modifications of the ribosomes have been reported. A first example is X-linked DKC, caused by a mutation in *DKC1*, which encodes dyskerin (51). Nucleolar dyskerin associates with a specific group of snoRNPs known as H/ACA, which function in the pseudo-uridylation of rRNAs, but mutant DKC1 alters the rRNA pseudo-uridylation pattern of ribosomes reducing translation of some mRNAs (53). A second example is fibrillarin, a nucleolar rRNA methyl-transferase (52). p53 represses fibrillarin by direct protein-protein interaction and high levels of fibrillarin are accompanied by abnormal rRNA methylation patterns and impaired translational fidelity (128). In this setting, p53 acts as a surveyor of protein synthesis by its ability to regulate ribosome activity (128). The translation fidelity model has gathered additional experimental evidence. The RPL10 Arg98Ser mutant, the most commonly identified ribosomal mutation in acute T-ALL, was functionally evaluated in yeast (129). The mutation leads to a failure to produce 60S followed by degradation of the defective ribosomes (129). The 60S subunit shortage puts pressure on cells to select

for suppressors of the ribosome biogenesis defect, allowing the yeast cells to boost ribosome production to sustain cell proliferation (129). However, the consequence of this bypass is synthesis of defective ribosomes that wreak havoc in the mRNA translation process (129). Whether similar mechanisms exist in humans and how they function remains to be investigated. It is interesting to note that some of the RPs mutated in cancer including RPL5, RPL10 and RPS20 are known to bind directly to mRNAs, moreover, two of them RPL5 and RPL10, have a preferential association with monosomes reflecting ribosome heterogeneity (15).

Another possibility to explain how defects in the synthesis or function of the ribosomes could affect the pattern of translated mRNAs and possibly lead to cell transformation involves changes in the mRNA translation patterns. A study in mice revealed a selective reduction in the translation of Hox mRNAs following deletion of *Rpl38* (83), and as another example serves the transcription factor GATA1 being critical for normal erythropoiesis. Its mRNA is inefficiently translated in DBA patients (130), while mutated in other DBA cases (131). In an interesting twist, GATA1 binding to RP gene promoters is important to sustain high levels of RPs in erythroid cells (132). A more specific hypothesis that has been discussed is that a ribosome deficit may impact on the translation patterns favoring the synthesis of oncogenic proteins by altering the ratio between translation initiation and elongation (133). Related to this is the hypothesis that a reduced number of ribosomes may cause a selective reduced translation of mRNAs that are difficult to translate while other mRNA could become increasingly translated. Indeed, a decrease in p53 mRNA translation has been suspected to be of relevance during tumor development (36). Reduced mRNA translation may also result in a shortage of DNA replication and repair factors as well as histones that in turn may result in genome instability. Ribosome profiling will in the contexts of pre-existing ribosome biogenesis or mature ribosome defects become an essential tool to study changes in translation patterns and finding novel targets for intervention (134).

Gain or loss of extra-ribosomal functions in cancer. RPs are often regulated in surprisingly sophisticated manner and several RPs possess extra-ribosomal functions. In addition to their roles in ribosome biogenesis and mature ribosome function, some RPs are involved in DNA repair, transcription, RNA processing and apoptosis (82,135-137). A few of these extra ribosomal functions are relevant to discuss in the context of cancer development. To begin with, a number of RPs may affect cell growth to promote cancer cell proliferation. For example, overexpression of RPS3A leads to the transformation of mouse NIH3T3 cells and the formation of tumors in nude mice (138). Another example is RPS13 (uS15) that promotes gastric cancer growth by decreasing levels of p27Kip1 (139). Upregulation of RPS13 accelerated the growth, enhanced *in vitro* colony formation and soft agar growth, and promoted *in vivo* tumor formation whereas downregulation of RPS13 in gastric cancer cells led to G_1 arrest (139). RPS13 as well as RPL23 (uL14) may also suppress drug-induced apoptosis of gastric cancer cells (140). Growth inhibitory functions of RPs have been described as well. The most obvious examples are perhaps RPL5 and RPL11 that when overexpressed inhibit MDM2 (141). Many other RPs including RPS15 also bind MDM2 and may impact on the p53 response (57,142). Decreased levels of RPL41 (eL41) led to anchorage-independent growth of NIH3T3 cells in soft agar and increased tumor growth in mice (143), while in contrast the enforced expression of RPL41 triggered cell cycle arrest and sensitized cancer cells to cisplatin (143). One must emphasize that cells are sensitive to enforced disturbances in the balance of RPs, and even that certain tags when fused to RPs including GFP, HA or FLAG may prevent or interfere with an RPs assembly into ribosomes (144). Therefore, anti-proliferative effects stemming from the ectopic overexpression of RPs may be indirect.

There are more elaborate mechanisms relevant to bring up in the context of the cancer-associated mutations occurring in RPL5, RPL22 and RPL10. For example, RPL10 has been linked to regulation of the oncogenic transcription factor JUN and other non-ribosome related proteins (145), and these functions could potentially be altered by the RPL10-Arg98Ser mutant with implications for cancer development. Another intriguing example is the inactivation of RPL22 that enhances transformation potential through induction of the Lin28B stemness factor (60). The mechanism whereby a deficiency in RPL22 induces Lin28B is not known. RPL22 is an RNA binding protein (146) but it also associates with chromatin and is involved in gene repression through complex formation with linker histone H1 (147). The possibility that RPL22 has specific functions in gene regulation on a transcriptional level must therefore be taken into consideration. This finding, together with the unusual mode of Rpl22 regulation in mice (17) and a number of links to p53 regulation (37,88,148,149) suggest that RPL22 is a very interesting candidate for use in diagnostic, prognostic and therapeutic applications related to cancer.

5. Ribosome biogenesis as a re-emerging target in the treatment of cancer

The rate of cell growth is often in proportion to the numbers of new ribosomes made (150,151). It may therefore not come as a surprise to learn that many anticancer drugs interfere with RNA pol I transcription or ribosomal RNA (rRNA) metabolism leading to preferential targeting of dividing cancer cells. Inhibition of ribosome biogenesis by chemotherapeutic drugs may contribute significantly to the efficacy of therapeutic regimens. Ribosome biogenesis has the potential to be more effectively exploited as a target in anticancer therapy given that it is one of the major biosynthetic activities in a cancer cell. RNA Pol I, the multiprotein complex that synthesizes rRNA, is very active in most cancer cells (152). Selective inhibitors of RNA Pol I may therefore offer a general therapeutic strategy to block cancer cell proliferation and small molecule compounds that specifically inhibit rDNA transcription have been developed by academic teams and biotech companies (153). One compound CX-3543, target rRNA synthesis by disrupting G-quadruplex DNA structures in the G-rich region of the rRNA repeat, thereby altering the binding of proteins required for rRNA transcription (154). A second compound, CX-5461 is an inhibitor of RNA pol I transcription that works by specifically impairing the binding of SL1/TIF-1B to the rDNA promoter thereby inhibiting the initiation of rRNA synthesis (155). This latter compound selectively inhibits

Pol I-driven transcription relative to Pol II-driven transcription, DNA replication, and translation. CX-5461 selectively kills B-lymphoma cells *in vivo* by induction of p53-dependent apoptotic signaling (156). The small molecule and acridine derivative, BMH-21 was found to have potent antitumorigenic activity (157). BMH-21 intercalates into GC-rich sequences in rDNA genes, and represses RNA Pol I transcription (158). A related compound, the acridine derivative CID-765471, inhibits rDNA transcription and activates p53 through 5S RNP also in the absence of detectable DNA damage (159). The mechanism involved in the case of CID-765471 is similar to BMH-21 in that there is a selective degradation of the RPA194 subunit of RNA polymerase I. Degradation of RPA194 could be a common event in the case of nucleolar disruption by non-genotoxic acridines, however it is not a general feature of all rDNA intercalating compounds (159). The type of anticancer activity and non-genotoxic activation of p53 represented by these different compounds mentioned holds great promise in future anticancer therapy, but whether selective targeting of ribosome biogenesis will be of broad clinical value in anti-cancer treatment remain to be seen.

One may of course also consider other targets in the ribosome biogenesis machinery including ribosomal proteins themselves. RPS2 (uS5) was reported to be a novel therapeutic target in prostate cancer whereas knock down of RPS2 expression had little effect on normal cells (160), in similar ways knock down of RPL19 (eL19) abrogated the aggressive phenotype of human prostate cancer (161). Depletion of the primary rRNA binding RPS9 (uS4) induced p53-dependent cell cycle arrest and differentiation in glioma cells (27). As an interesting example, RPL39 was found to be a protein that affects breast cancer stem cell self-renewal through a non-biased screening approach (65). Depletion of RPL39 reduced tumor growth and metastasis associated with fewer cancer stem cells with a potential link to the nitric oxide synthase pathway (65). Clearly, additional studies targeting ribosomal components in various *in vivo* cancer models are warranted. Finally, one may envisage that acquired ribosome defects, or 'cancer-specific' ribosomes, may become novel targets in anticancer therapy (162).

6. Conclusions and future perspective

From studies on the ribosomopathies it is clear that impaired ribosome biogenesis is to be considered a risk factor for cancer initiation. Remarkably, distinct and recurrent mutations in genes encoding for ribosomal proteins (RPs) have recently been implicated in cancer development in patients without a previous known history of a ribosomopathy. This has been a wake-up call in the tumor biology field and one may compare this with the parallel and equally remarkable discovery of histone H3.3 mutations in pediatric gliomas (163). The role of RPs in cancer is a complex issue and while some exert a direct effect on proto-oncogenes and tumor suppressor genes, *e.g.* p53, it is possible that mutations in other RPs may have general effects on mRNA translation. The trend evident from the assembled sequencing data suggests that RP mutations or changes in the expression patterns of RPs could be functionally relevant in a large number of cancer types and cases. A more complete picture of the relevant RPs in cancer is due to emerge from additional cancer genome analysis projects and functional studies.

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