

The 5S RNP Couples p53 Homeostasis to Ribosome Biogenesis and Nucleolar Stress

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SUMMARY

Several proto-oncogenes and tumor suppressors regulate the production of ribosomes. Ribosome biogenesis is a major consumer of cellular energy, and defects result in p53 activation via repression of mouse double minute 2 (MDM2) homolog by the ribosomal proteins RPL5 and RPL11. Here, we report that RPL5 and RPL11 regulate p53 from the context of a ribosomal subcomplex, the 5S ribonucleoprotein particle (RNP). We provide evidence that the third component of this complex, the 5S rRNA, is critical for p53 regulation. In addition, we show that the 5S RNP is essential for the activation of p53 by p14^{ARF}, a protein that is activated by oncogene overexpression. Our data show that the abundance of the 5S RNP, and therefore p53 levels, is determined by factors regulating 5S complex formation and ribosome integration, including the tumor suppressor PICT1. The 5S RNP therefore emerges as the critical coordinator of signaling pathways that couple cell proliferation with ribosome production.

INTRODUCTION

The production of eukaryotic ribosomes is a major consumer of cellular energy and regulated by several tumor suppressors and proto-oncogenes (Stumpf and Ruggero, 2011). Indeed, ribosome biogenesis is upregulated by the oncogene *c-Myc*, downregulated by the tumor suppressor p14^{ARF}, and is linked to the regulation of the tumor suppressor p53 (Stumpf and Ruggero, 2011). Several genetic diseases, such as Diamond-Blackfan anemia, dyskeratosis congenita, and Treacher Collins syndrome, arise due to defects in ribosome production, and in a number of cases, this has been linked to the misregulation of p53 (Freed et al., 2010; Fumagalli and Thomas, 2011; Narla and Ebert, 2010). Surprisingly, several of these diseases, which are known as ribosomopathies, also predispose patients to a range of cancers.

The tumor suppressor p53 is activated by a wide range of cellular stresses, leading to either repair of the cellular damage,

cell-cycle arrest, apoptosis, or senescence. A key regulator of p53 is mouse double minute 2 homolog (MDM2), an E3 ubiquitin ligase that inhibits p53 activity through proteasome-mediated degradation. Several ribosomal proteins (RPs) bind to and inactivate MDM2, thereby activating p53 (Chakraborty et al., 2011), but recent work has shown that only RPL5 and RPL11 are essential for p53 activation in response to a block in ribosome biogenesis (Bursac et al., 2012; Fumagalli et al., 2012; Sun et al., 2010). MDM2 mutations found in several cancers, which disrupt the RPL11-MDM2 interaction, attenuate the p53-mediated response to nucleolar/ribotoxic stress and accelerate *c-Myc*-induced lymphomagenesis in a mouse model system (Macias et al., 2010; Pan et al., 2011). RPL11 also binds to and promotes the activity of the tumor suppressor p14^{ARF} (Dai et al., 2012), which interacts with and represses MDM2 and is activated by the overexpression of oncogenes such as *c-Myc*.

Although RPL5 and RPL11 inhibit MDM2 outside the ribosome, it is unlikely that they perform this function individually, as free ribosomal proteins are unstable in mammalian cells (Lam et al., 2007). RPL11, together with RPL5 and the 5S rRNA, comprise the 5S ribonucleoprotein particle (RNP), an essential subcomplex of the large ribosomal subunit. RPL5 binds the 5S rRNA and the 5S rRNA/RPL5 complex and then localizes to the nucleolus, where it binds RPL11 and is integrated into the ribosome (Chakraborty et al., 2011). RPL5 and RPL11 have been shown to be mutually dependent on one another for stability/accumulation when ribosome biogenesis is blocked (Bursac et al., 2012). Furthermore, it has been demonstrated that RPL11 activates p53 cooperatively with RPL5 and mutations, which are predicted to impede RPL11 interaction with the 5S rRNA, inhibit this induction (Horn and Vousden, 2008).

Proteins that regulate 5S RNP formation, localization, and integration into the ribosome are predicted to be central in regulating MDM2 activity and, therefore, p53 levels in the cell. PICT1 (GLTSCR2) has recently been identified as a novel tumor suppressor that induces p53 and activates the PTEN pathway/ATM checkpoint in response to DNA damage (Kim et al., 2011). Interestingly, PICT1 has also been shown to retain RPL11 in the nucleolus in normal cells. However, under ribotoxic stress conditions, RPL11 and PICT1 relocate to the nucleoplasm, where they activate p53 (Sasaki et al., 2011). Mechanistic details on how PICT1 performs this function are currently

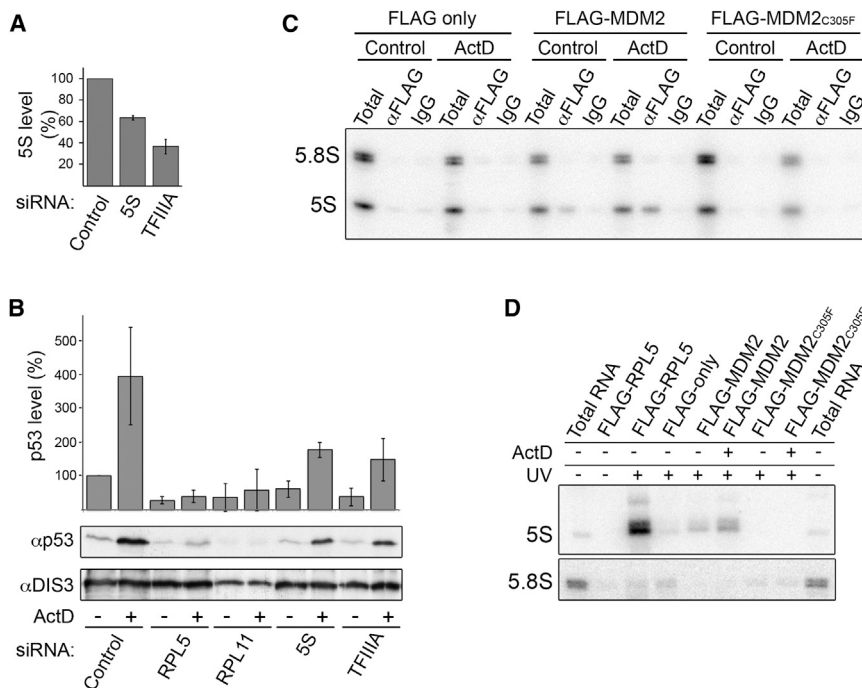


Figure 1. The 5S RNP Complex Is Required for p53 Induction through Direct Interaction with MDM2 in Response to Ribotoxic Stress

(A) The level of newly synthesized 5S rRNA in cells transfected with control siRNAs or those targeting TFIIIA or the 5S rRNA itself was determined by pulse-labeling followed by gel electrophoresis. Quantification is based on three independent experiments. Error bars indicate SD.

(B) U2OS cells were transfected with siRNAs against the core 5S RNP components and either untreated (–) or treated (+) with ActD for 10 hr. p53 levels were analyzed by western blotting. Quantification is based on three independent experiments. Error bars indicate SD.

(C) Anti-FLAG immunoprecipitations of extracts from HEK293 cells expressing FLAG-MDM2, -MDM2_{C305F}, or the FLAG-tag. Coprecipitated 5S and 5.8S rRNAs were detected by northern blotting. IgG, immunoglobulin G.

(D) Cells expressing FLAG-tagged proteins were UV crosslinked, and covalently linked RNA protein complexes were purified. Coprecipitated 5S and 5.8S rRNAs were detected by northern blotting.

See also Tables S1, S2, and S3 and Figure S1.

lacking, but because this protein is in fact homologous to the yeast ribosome biogenesis factor Nop53, we hypothesize that it may activate p53 through a role in ribosome biogenesis. Several other factors have been linked to the formation of the 5S RNP and its integration into the ribosome in yeast, making these good candidates for performing this role, but their human counterparts are yet to be characterized (Talkish et al., 2012; Zhang et al., 2007). Furthermore, how the function of RPL11 and RPL5 in p53 signaling relates to their role in ribosome production also remains unclear at present, but it is exciting to speculate that their dual function reflects a coordinated pathway coupling ribosome biogenesis to cell proliferation.

Given the importance of RPL11 and RPL5 as components of the p53 signaling pathway (Chakraborty et al., 2011), 5S RNP biology is an underinvestigated area of research that is relevant for understanding the basis of many human ribosomopathies. We show that the 5S rRNA, as part of an assembled 5S RNP complex, is essential for p53 homeostasis and p53 activation when p14^{ARF} is expressed. Our data indicate that the 5S RNP functions as a central regulator of p53, providing a means to directly relate ribosome biogenesis to cellular growth and cell-cycle regulation.

RESULTS

The 5S rRNA, Together with RPL11 and RPL5, Is Required for p53 Homeostasis and the Activation of p53 when Ribosome Biogenesis Is Inhibited

MDM2 is a critical negative regulator of p53 levels and the ribosomal proteins RPL5 and RPL11 have been shown to inhibit MDM2 activity when ribosome biogenesis is blocked, leading to p53 activation. During ribosome biogenesis, RPL5 and

RPL11 both associate with the 5S rRNA to form the 5S RNP, an essential subcomplex of the ribosome. To investigate the importance of the 5S rRNA in regulating MDM2/p53, two parallel approaches were taken to decrease the production of the 5S rRNA in U2OS cells and to monitor the effect on p53 levels: the 5S rRNA-specific transcription factor IIIA (TFIIIA) was depleted by RNAi and the 5S rRNA itself was directly targeted using siRNAs (Li and Gu, 2011). The effect of depleting RPL5 and RPL11 by RNAi on p53 levels was also analyzed for comparison (Figure 1 and Figure S1A). Decreasing TFIIIA to <5% of the normal level (Figure S1A) resulted in an approximately 3-fold reduction in 5S rRNA production in pulse-labeling experiments, whereas direct targeting of the 5S rRNA reduced 5S rRNA synthesis to about 60% of that seen in control cells (Figure 1A).

If components of the 5S RNP are required for p53 activation when ribosome production is blocked, depletion of these factors should diminish p53 induction by the chemotherapeutic agent Actinomycin D (ActD), which blocks rRNA synthesis by inhibiting RNA polymerase I. Interestingly, knockdown of RPL11, RPL5, TFIIIA, or the 5S rRNA all resulted in a reduction in the basal levels of p53 in untreated U2OS cells, suggesting that these 5S RNP components are required for maintaining cellular p53 levels (Figure 1B). In addition, treatment of cells with ActD caused the accumulation of p53 (approximately 4-fold), but reducing the levels of RPL5 or RPL11 eradicated this induction. Decreasing 5S rRNA levels also reduced but did not abolish ActD-mediated p53 activation (Figure 1B). This is consistent with the fact that 5S rRNA synthesis was only diminished and not eradicated (Figure 1A). Taken together, our data show that the 5S rRNA, together with RPL5 and RPL11, is required for ActD induction of p53 and p53 homeostasis in normal cells.

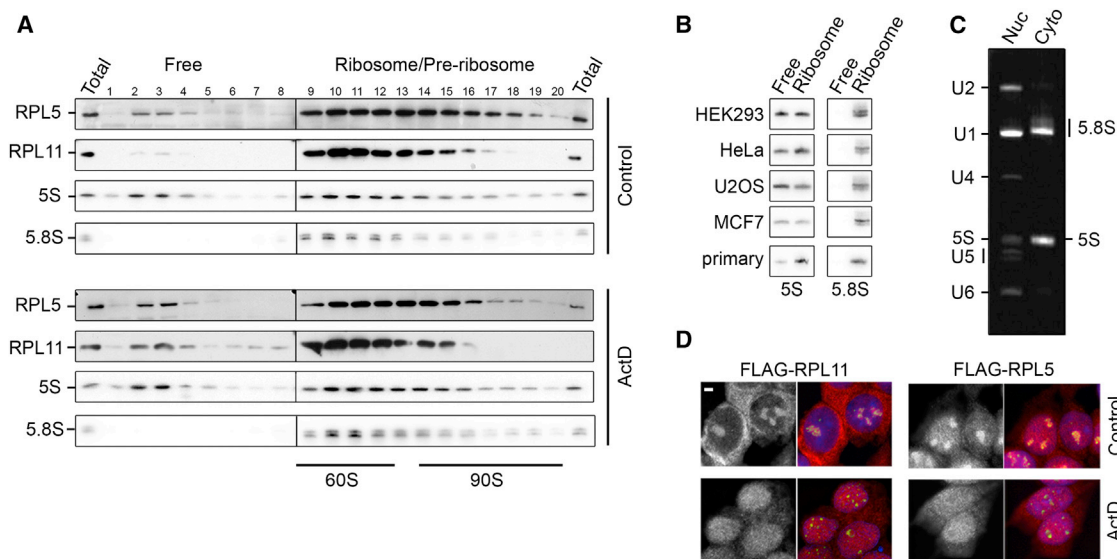


Figure 2. A 5S RNP Complex Containing 5S rRNA, RPL5, and RPL11 Only Accumulates in the Nucleoplasm when Ribosome Production Is Inhibited

(A) Extracts from HEK293 cells either treated (+) or untreated (–) with ActD were analyzed by glycerol gradient centrifugation followed by western and northern blotting. Antibodies or probes used are indicated to the left of each panel.

(B) Levels of 5S and 5.8S rRNAs in free (pooled fractions 1–7) and ribosomal (pooled fractions 9–20) complexes in various cell lines were analyzed by northern blotting. Primary, primary dermal fibroblasts.

(C) RNA from HeLa cell nuclear and cytoplasmic extracts was separated by polyacrylamide gel electrophoresis and detected using ethidium bromide staining.

(D) The localization of newly synthesized FLAG-RPL5 or -RPL11 in HEK293 stable cell lines was determined by immunofluorescence. Cells were grown in the presence (ActD) or absence (control) of ActD, and antibodies against the FLAG-tag (red/greyscale) and fibrillar (green; nucleolar marker) were used. Nuclear material was detected by DAPI staining (blue). The scale bar represents 1 μm .

See also Tables S2 and S3 and Figure S2.

MDM2 Directly Contacts the 5S rRNA

RPL11 and RPL5 activate p53 by binding to and inhibiting MDM2 (Chakraborty et al., 2011). We next asked whether the 5S rRNA is also specifically associated with MDM2. For this, human embryonic kidney 293 (HEK293) cells stably expressing inducible FLAG-MDM2 and an MDM2 mutant (C305F), which is not able to interact with RPL5 and RPL11 (Macias et al., 2010), were generated (Figure S1B). Immunoprecipitation experiments using whole cell extracts revealed that FLAG-MDM2 was associated with the 5S rRNA, but not the 5.8S rRNA (Figure 1C). Importantly, the amount of 5S rRNA coprecipitated from extracts was significantly higher after cells were treated with ActD. Neither 5S rRNA nor 5.8S rRNA coprecipitated with FLAG-MDM2_{C305F} indicating that the 5S rRNA-MDM2 association is dependent on the binding of RPL11 and RPL5 to MDM2. Importantly, the specific association of MDM2 with the 5S rRNA was also observed by immunoprecipitation using MDM2-specific antibodies from U2OS cell extracts (Figure S1C).

MDM2 has been demonstrated to bind both RPL11 and RPL5 raising the possibility that MDM2, which has previously been shown to bind RNA (Elenbaas et al., 1996), may also directly contact the 5S rRNA. To test this, cells expressing FLAG-tagged MDM2, MDM2_{C305F}, RPL5, or the FLAG tag alone (Figure S1B) were irradiated with UV light. Cells expressing FLAG-MDM2 or MDM2_{C305F} that had been treated with ActD were also included. The proteins, and any covalently bound RNAs, were then isolated from extracts derived from these cells using a denaturing two-step

purification procedure, and the associated RNAs were analyzed by northern blotting. The 5S rRNA was found to crosslink strongly to RPL5, as expected, but also specifically to MDM2 (Figure 1D). Consistent with our immunoprecipitation data, the amount of 5S rRNA crosslinked to MDM2 increased when cells were treated with ActD and the MDM2_{C305F} mutation abolished this interaction. These data demonstrate that, in addition to directly binding RPL11 and RPL5, MDM2 also directly contacts the 5S rRNA.

The 5S RNP, a Complex of RPL5, RPL11, and the 5S rRNA, Accumulates when Ribosome Biogenesis Is Blocked

Our data imply that, rather than functioning individually, the 5S rRNA, RPL11, and RPL5 act as a complex to activate p53. However, how these factors accumulate outside the ribosome in human cells, and how this is affected when ribosome biogenesis is blocked, is currently unknown. We used glycerol gradient centrifugation to define 5S RNP complexes that exist in normal cells and in cells in which ribosome production has been perturbed by treatment with ActD.

In untreated HEK293 cells, the 5S rRNA, RPL11, and RPL5 were present in complexes containing mature large ribosomal subunits (indicated by the position of the 5.8S rRNA; Figure 2A; fractions 9–18). These fractions also contained ribosome biogenesis factors, such as RBM28 (Figure S2A) and therefore pre-ribosomal complexes. A significant proportion of RPL5 and the 5S rRNA were found in fractions 2–4 (Figure 2A) demonstrating

that they are naturally present in nonribosomal fractions. The relatively high level of free 5S rRNA was not specific to HEK293 cells but also observed in HeLa, U2OS, MCF7, and primary human fibroblast cells (Figure 2B). Interestingly, RPL11 only significantly accumulated in this free pool after the cells were treated with ActD (Figure 2A). Consistent with our earlier data identifying interactions between 5S RNP components and MDM2 (Figures 1C and 1D), FLAG-MDM2 expressed in HEK293 cells also comigrated with RPL5, RPL11, and the 5S rRNA in the free fractions of a glycerol gradient, but not in the ribosomal fractions (Figure S2B). Immunoprecipitation using extracts from stable cell lines expressing FLAG-tagged RPL5/RPL11 was used to confirm that the comigrating RPL5, RPL11, and 5S rRNA in fractions 2–4 form a stable complex (Figures S1B and S2C). Interestingly, none of the ribosome biogenesis factors analyzed, including RRS1 and BXDC1, which are associated with a free 5S RNP when ribosome biogenesis is blocked in yeast (Zhang et al., 2007), were present in the free fractions under any of the conditions tested (Figure S2A).

Taken together, these data indicate that the 5S rRNA, RPL5, and RPL11 components, together with MDM2, are present as a complex in the free fractions of the gradient and that the amount of RPL11 in this complex increases when ribosome biogenesis is blocked.

The 5S RNP Accumulates in the Nucleoplasm when Ribosome Production Is Inhibited

Our data demonstrate that a free 5S RNP accumulates when ribosome biogenesis is blocked. This complex interacts with MDM2, but it is not clear where this interaction takes place. RPL11 and RPL5 were predicted to repress MDM2 in the nucleoplasm (Chakraborty et al., 2011), but a recent report suggested that the RPL5/RPL11/MDM2 complex accumulates in the nucleolus when ribosome biogenesis is blocked (Bursac et al., 2012). We investigated the subcellular distribution of the 5S RNP. Analysis of the RNA content of nuclear and cytoplasmic extracts revealed a high level of the 5S rRNA in the nucleoplasm (Figure 2C). These nuclear extracts do not contain nucleolar material (Watkins et al., 2004) indicating that the 5S rRNA is present in the nucleoplasm at levels higher than the spliceosomal U5 snRNA (~200,000 copies/cell; Reddy and Busch, 1988).

To determine the localization of newly synthesized RPL11 and RPL5, we used immunofluorescence to analyze HEK293 cells expressing tetracycline-regulated FLAG-tagged RPL11 and RPL5, in the presence or absence of ActD to block ribosome production. Importantly, the FLAG-tagged proteins were not overexpressed and showed the same profile on glycerol gradients as the endogenous proteins (Figures S1B and S2B). In control cells, RPL11 was predominantly present in the cytoplasm and nucleolus, whereas RPL5 was found throughout the cell with significant levels present in the nucleolus and nucleoplasm (Figure 2D). After treatment with ActD, there was a significant increase in RPL11 levels in the nucleoplasm and a major decrease in the levels of both RPL5 and RPL11 in the nucleolus (Figure 2D). This suggests that active ribosome biogenesis is required for the nucleolar localization/retention of both RPL5 and RPL11.

Taken together, our data indicate that the 5S/RPL5 complex is naturally abundant in the nucleoplasm but that the 5S RNP

(5S rRNA/RPL5/RPL11) accumulates in the nucleoplasm when ribosome biogenesis is blocked. Along with RPL5 and RPL11, the 5S rRNA interacts with MDM2, implying that the trimeric 5S RNP complex binds MDM2 in the nucleoplasm to affect p53 levels. Modulating the levels of the 5S RNP directly controls MDM2 activity. Consequently, the pathways that mediate 5S RNP formation, localization, and integration into the ribosome are central for regulating p53 levels and cellular proliferation.

Factors Linked to 5S RNP Biogenesis and p53 Activation through the RPL5/RPL11 Pathway Are Important for the Production of the Large Ribosomal Subunit

The nucleolar protein PICT1 has recently emerged as a key regulator of p53 and has been proposed to function by retaining RPL11 in the nucleolus (Sasaki et al., 2011). Similarly, the putative tumor suppressor PAK1IP1 is suggested to act through the ribosomal protein-MDM2 pathway, but it is currently unknown whether these factors function as regulators of the interaction of the 5S RNP with MDM2 or whether they perform these roles as components of the ribosome biogenesis machinery. We therefore investigated the role of several nucleolar proteins linked either to p53 regulation (PICT1 and PAK1IP1) or known to be important for 5S RNP integration into ribosomes in yeast (RRS1, BXDC1, and NOP2) in ribosome biogenesis in human cells. We also analyzed the importance of the 5S RNP components RPL5, RPL11, and the 5S rRNA for ribosome production. Individual proteins or the 5S rRNA were depleted using RNAi, and the production of newly synthesized ribosomes was determined by pulse-chase labeling of mature (18S, 28S, and 5.8S) and precursor (47S/45S and 32S) rRNAs (Figure 3A) followed by agarose-glyoxal or polyacrylamide gel electrophoresis (Figures 3B, S1A, and S3A).

Reducing the levels of RPL5, RPL11, PICT1, RRS1, or NOP2 resulted in a dramatic reduction in the production of the 28S and 5.8S rRNAs with minimal effects on 18S rRNA accumulation (Figure 3B). This is similar to the effect seen upon knockdown of the large ribosomal subunit biogenesis factor, BOP1 (Hölzel et al., 2005; Strezoska et al., 2000; Figure 3B). Knockdown of BXDC1, PAK1IP1, TFIIIA, or 5S rRNA also specifically decreased accumulation of the large subunit rRNAs, 28S and 5.8S, although to a lesser extent than with the other knockdowns. Interestingly, even though the siRNAs targeting TFIIIA and the 5S rRNA only had minimal effects on 5S rRNA synthesis (Figures 1A and 3B), this was sufficient to reduce 28S and 5.8S rRNA accumulation demonstrating that production/incorporation of the 5S RNP is required for large ribosomal subunit formation.

Whereas most of the knockdowns had a significant effect on 28S and 5.8S rRNA accumulation, their effect on 5S rRNA production was minimal implying that the accumulation of the 5S rRNA, which is transcribed by RNA pol III, occurs independently of the RNA pol I-transcribed rRNAs. Indeed, ActD inhibition of RNA pol I transcription or blocking pre-rRNA processing by treatment with 5-fluorouracil (5FU) had no notable effect on 5S rRNA accumulation (Figure 3C). Knockdown of RPL5, however, resulted in a significant reduction in 5S rRNA levels and the accumulation of a smear of longer 5S pre-rRNAs (Figure 3D). After transcription, the 5S rRNA undergoes 3' processing to remove a 2 to 3 nt extension (Ciganda and Williams, 2011), and our

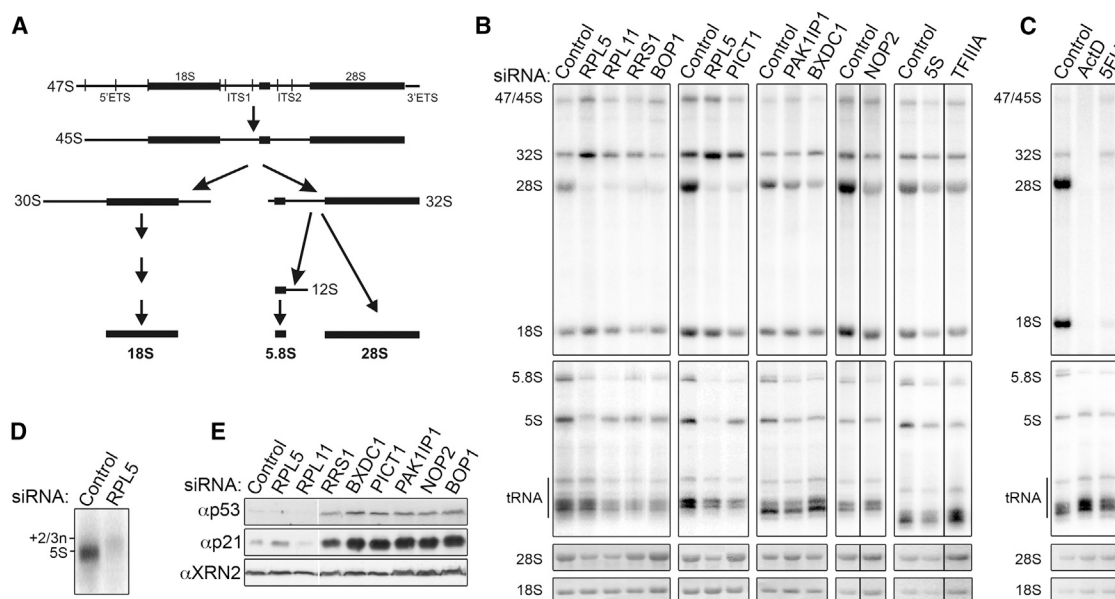


Figure 3. Putative 5S RNP Biogenesis Factors Are Required for Large Ribosomal Subunit Production and Their Depletion Activates p53

(A) Outline of the major pre-rRNA processing pathway in humans.

(B and C) HEK293 cells depleted of 5S RNP components or putative 5S biogenesis factors by RNAi, or treated with chemotherapeutics (ActD and 5FU), were pulse-labeled using ^{32}P orthophosphate. Labeled RNA was analyzed by agarose/acrylamide gel electrophoresis followed by phosphorimager analysis. Total RNA was visualized using ethidium bromide staining.

(D) Magnification of the pre-5S rRNA in cells depleted of RPL5 from (B).

(E) p53 levels in U2OS cells depleted of ribosome biogenesis factors were determined by western blotting. The antibodies used are indicated to the left of the panels.

See also [Tables S1](#) and [S3](#) and [Figure S3](#).

data indicate that RPL5 is essential for this maturation as well as for 5S rRNA accumulation.

Depletion of a ribosome biogenesis factor would block ribosome production, leading to increased accumulation of the 5S RNP and therefore cause activation of p53. However, if a ribosome biogenesis factor was also required for MDM2 regulation, depleting this protein would have no effect on p53 levels in the cell. Indeed, knockdown of RPL11 or RPL5 did not increase the levels of p53 or its downstream target, p21, even though these knockdowns efficiently blocked large ribosomal subunit production ([Figures 3B](#) and [3E](#)). In contrast, depleting BOP1, RRS1, BXDC1, PAK1IP1, PICT1, or NOP2 in U2OS cells resulted in significant increases in the levels of p53 and p21 ([Figure 3E](#)). Furthermore, p53 activation by the knockdown of factors such as PICT1 was nullified by the codepletion of RPL5 or RPL11 confirming that they function through the 5S RNP/MDM2 pathway ([Figure S3B](#)).

Taken together, our data demonstrate that knockdown of factors required for the synthesis of the large ribosomal subunit, other than components of the 5S RNP, results in the activation of p53.

RRS1 and BXDC1 Are Important for the Nucleolar Localization of the 5S RNP

Blocking ribosome biogenesis using ActD resulted in the accumulation of the 5S RNP in the nucleoplasm ([Figure 2D](#)). ActD causes a major change in the nucleolar structure, and we there-

fore wanted to test whether knockdown of individual ribosome biogenesis factors also leads to the relocalization of the 5S RNP. We therefore next examined whether any of the putative 5S RNP biogenesis factors that are essential for the production of the large ribosomal subunit are also required for the nucleolar localization of RPL5 and RPL11. To allow analysis of the localization of RPL5 and RPL11 produced only after the biogenesis factors were depleted, HEK293 cells expressing inducible FLAG-RPL5 or FLAG-RPL11 were transfected with siRNAs, and 36 hr later, expression of the tagged-protein was induced.

Consistent with our previous data ([Figure 2D](#)), RPL11 was predominantly found in the nucleolus ([Figure 4](#); marker: fibrillarin) and cytoplasm of control cells and did not significantly accumulate in the nucleoplasm, whereas RPL5 localized throughout the cell. Knockdown of either RRS1 or BXDC1 resulted in a major reduction in the amount of RPL5 in the nucleolus ([Figure 4](#)). In contrast, only knockdown of BXDC1 affected the nucleolar localization of RPL11. However, a common feature of depletion of either RRS1 or BXDC1 was an increase in the nucleoplasmic accumulation of both RPL5 and RPL11. The knockdown of PICT1, PAK1IP1, NOP2, or BOP1 did not significantly affect the distribution of RPL5 in the cell. In contrast, depletion of any of these proteins resulted in an increased relocation of RPL11 from the cytoplasm to the nucleoplasm but no significant change in the amount present in the nucleolus. Surprisingly, depletion of TFIIIA did not alter the localization of either RPL5 or RPL11, although this knockdown had a similar effect on rRNA biogenesis

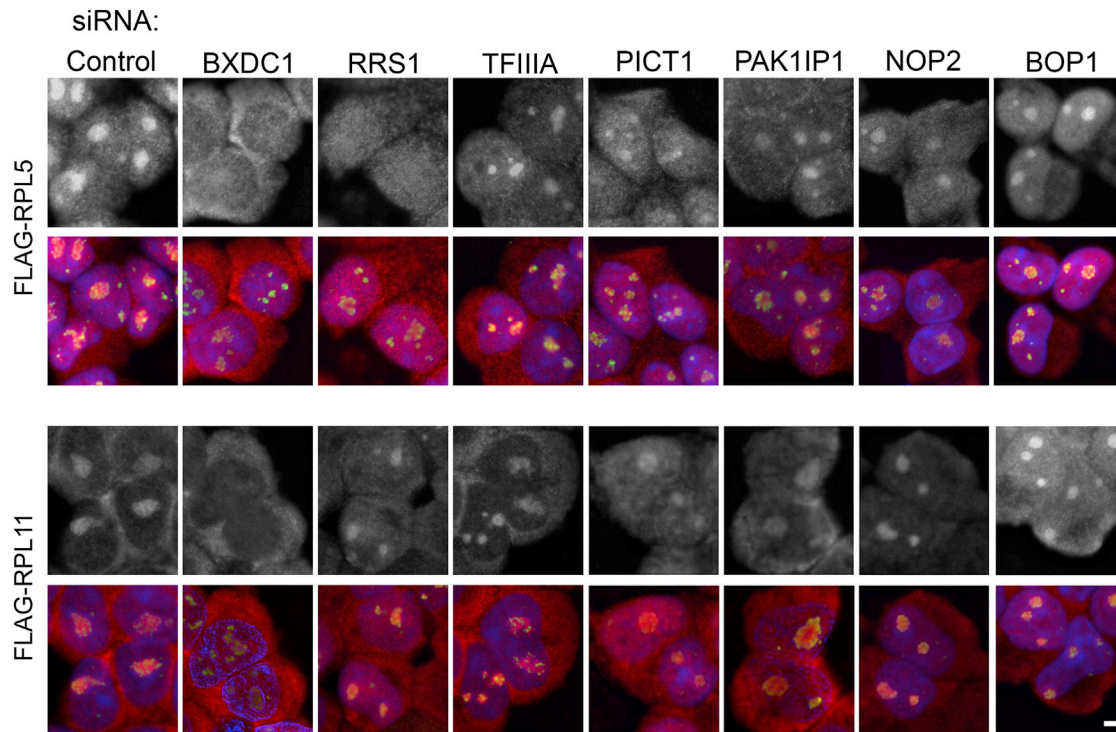


Figure 4. The Biogenesis Factors RRS1 and BXDC1 Are Required for Nucleolar Localization of the 5S RNP

Inducible HEK293 FLAG-RPL5 or FLAG-RPL11 cells were grown on coverslips and depleted of various 5S/ribosome biogenesis factors using RNAi. For the final 12 hr of the knockdown, tagged protein expression was induced and cells were fixed. Immunofluorescence was performed using antibodies to detect the FLAG-tag (red/greyscale) and fibrillarin (green). Nuclear material was visualized by DAPI staining (blue). The scale bar represents 1 μ m. See also [Table S1](#).

to depleting PAK11P1 ([Figures 3B and 4](#)). We conclude that, if the 5S rRNA is essential for RPL5 and RPL11 accumulation, it is likely that knockdown of TFIIIA results simply in reduced levels of the 5S RNP and does not cause its mislocalization.

Our data therefore indicate that, with the exception of depleting TFIIIA, blocking large ribosomal subunit production results in an increase in the nucleoplasmic levels of RPL11. Interestingly, RRS1 and BXDC1 are important for the nucleolar localization of RPL11 and RPL5, despite the fact that these proteins are not associated with a nonribosomal 5S RNP complex as they are in yeast ([Zhang et al., 2007](#)).

5S rRNA Integration into the Ribosome Is Slow

The amount of free 5S RNP in the cell must be strictly controlled in order to maintain appropriate levels of p53 and to regulate the rate of proliferation. The abundance of the free 5S RNP is influenced by the rate of both 5S RNP synthesis and its integration into the ribosome. Therefore, identification of factors that mediate 5S RNP integration into the ribosome will be critical for discovering key regulators of p53 in this pathway. We developed an assay with which 5S RNP recruitment into ribosomes could be monitored. For this, HEK293 cells were pulse-labeled with 32 P orthophosphate and then incubated with normal media for various time points over a 12 hr period. Extracts prepared from these cells were then analyzed by glycerol gradient centrifugation followed by gel electrophoresis ([Figures 5A and S4A](#)).

As observed for the steady-state 5S rRNA ([Figure 2A](#)), the newly synthesized 5S rRNA was found in two pools: the ribosomal and preribosomal complexes (fractions 9–20) and a free pool (fractions 1–7). The percentage of the 5S rRNA in the free and ribosomal/preribosomal complexes was then calculated and plotted relative to the processing of the 28S rRNA ([Figure 5B](#)).

Integration of the newly transcribed 5S rRNA into the ribosome fractions was slow, with more than 40% of the 5S rRNA found in the free pool after 6 hr and more than 30% still free after 12 hr. In contrast, processing of the RNA-pol-I-transcribed precursor-rRNAs into mature rRNAs, as measured by the accumulation of 28S rRNA, was 90% complete after 6 hr. The slow recruitment of newly synthesized 5S rRNA into the ribosome is probably due to the 5S rRNA being recruited from the large pool of free 5S rRNA naturally present in the nucleus (see [Figure 2](#)).

PICT1 and RPL11 Are Required for 5S RNP Integration into the Ribosome

Using this recruitment assay, we then established which of the potential 5S RNP biogenesis factors are required for 5S RNP integration into the ribosome. For these experiments, cells depleted of putative 5S RNP biogenesis factors were pulse-labeled followed by a 6 hr chase to allow significant integration of the newly synthesized 5S rRNA into ribosomes. We also analyzed 5S integration into preribosomes in cells treated with the chemotherapeutic agents ActD or 5FU, which block rRNA

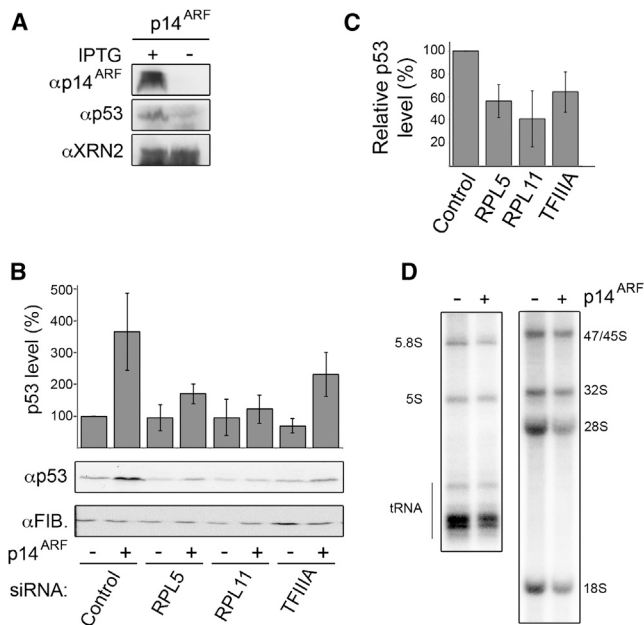


Figure 6. The 5S RNP Complex Is Required for p14^{ARF}-Mediated Activation of p53

(A) U2OS cells stably transfected with a plasmid expressing p14^{ARF} were untreated (–) or treated (+) with IPTG for 12 hr and then analyzed by western blotting.

(B) Expression of p14^{ARF} was induced (+) or not (–) in cells transfected with siRNAs targeting 5S RNP components. Levels of p53 in three replicate experiments were analyzed by western blotting and quantified. Error bars indicate SD.

(C) The relative difference between p53 induction in response to p14^{ARF} overexpression in cells transfected with control siRNAs and those targeting 5S RNP components was calculated based on the data shown in (B). Error bars indicate SD.

(D) Control U2OS cells or those expressing p14^{ARF} (as in [A]) were pulse-labeled using ³²P orthophosphate. RNA was analyzed by polyacrylamide and agarose-glyoxal gel electrophoresis and visualized using a phosphorimager. See also Tables S1 and S3.

of other steps upstream of 5S RNP biogenesis. It is highly likely, however, that factors that directly contact the 5S RNP are functionally involved in 5S RNP recruitment or biogenesis. We therefore used the UV crosslinking and complex purification method, described earlier for RPL5 and MDM2 (Figure 1D), to identify biogenesis factors that bind the 5S rRNA. HEK293 cells stably expressing FLAG-tagged RRS1, BXDC1, PICT1, NOP2, and TFIIIA (Figure S4C) were irradiated with UV light and the proteins, and any covalently bound RNAs, isolated using a denaturing, two-step purification procedure. Coprecipitated rRNA was then analyzed by northern blotting. The 5S rRNA was crosslinked to PICT1, TFIIIA, RRS1, and BXDC1 indicating that these proteins directly contact this RNA (Figure 5D). Little or no 5S rRNA was coprecipitated with either the FLAG-tag alone, FLAG-RCL1 (a small subunit ribosome biogenesis factor), or when UV crosslinking was omitted. NOP2 did not reliably precipitate 5S rRNA above background levels.

These crosslinking data therefore confirm that PICT1 plays a direct role in 5S RNP recruitment into the ribosome and that

RRS1 and BXDC1 regulate nucleolar localization of the 5S RNP by contacting the complex.

The 5S RNP Is Required for the Activation of p53 by the Tumor Suppressor p14^{ARF}

In addition to the 5S RNP-dependent pathway characterized above, p53 is also activated in response to many signals other than nucleolar stress. Interestingly, it has recently been shown that RPL11 is also important for the activation of p53 by the tumor suppressor p14^{ARF} following oncogenic stress (Dai et al., 2012), suggesting that the 5S RNP may coordinate p53 induction in response to multiple stresses. We therefore tested whether all the 5S RNP components are essential for the activity of p14^{ARF}. The levels of the 5S rRNA (via TFIIIA knockdown), RPL11, or RPL5 were reduced by RNAi in U2OS cells in which the expression of p14^{ARF} could be induced (Llanos et al., 2001), and the levels of p53 were then measured in the presence or absence of p14^{ARF} (Figures 6A and 6B).

In control cells, expression of p14^{ARF} resulted in increased levels of p53 as expected, but knockdown of RPL11 or RPL5 significantly reduced this response. The knockdown of TFIIIA, which reduced but did not abolish 5S rRNA transcription, also notably decreased p53 induction by p14^{ARF} overexpression suggesting that the 5S rRNA is also required for this response. Importantly, in each of three experiments performed, p53 induction was reduced relative to the control and appeared significant (Figure 6C). Our data therefore demonstrate that all the 5S RNP components are essential for the full activity of the tumor suppressor p14^{ARF}. Furthermore, expression of p14^{ARF} also inhibits ribosome biogenesis (Bertwistle et al., 2004; Itahana et al., 2003; Figure 6D). We propose that, in addition to directly inhibiting MDM2, p14^{ARF} also functions by increasing the amount of nonribosomal 5S RNP by blocking ribosome production. This highlights a central role for the 5S RNP in mediating crosstalk between different pathways of p53 activation.

DISCUSSION

We describe a mechanism by which ribosome biogenesis is directly coupled to cell proliferation, and we show that the abundance of the 5S RNP determines the basal levels of p53 in the cell. All three components of the 5S RNP, RPL5, RPL11, and the 5S rRNA, probably as a trimeric complex, are required for p53 activation in response to impaired ribosome production (Figure 7). After submission of our manuscript, the involvement of the 5S rRNA, as part of the 5S RNP, in the regulation of the MDM2-p53 checkpoint was also reported by the Thomas laboratory (Donati et al., 2013). Both papers come to the conclusion that the 5S RNP regulates p53 in response to changes in ribosome synthesis. Here, we also show that MDM2 directly binds the 5S rRNA in vivo and provide a detailed analysis of 5S RNP composition and dynamics. Furthermore, we define direct roles for PICT1 in 5S RNP integration into the ribosome and for RRS1 and BXDC1 in the nucleolar localization of 5S RNP components. Our data also indicate that p53 activation by the tumor suppressor p14^{ARF} involves the 5S RNP, suggesting crosstalk between the cellular responses to ribotoxic and oncogenic stress. Taken together, these data identify the 5S RNP as a key coordinator of

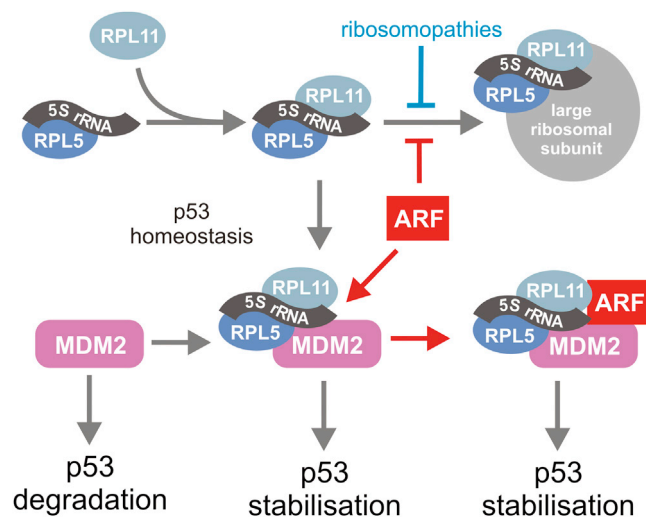


Figure 7. The 5S RNP Regulates p53 Homeostasis and Its Activation in Response to Nucleolar and p14^{ARF} Overexpression

Schematic overview showing the central role of the 5S RNP in coupling the pathway of ribosome biogenesis to cellular proliferation.

the cellular responses to both pro- and antiproliferative signals (Figure 7).

The 5S RNP Interacts with MDM2 and Regulates p53

The 5S RNP accumulates in the nucleoplasm when ribosome biogenesis is blocked, where it associates with MDM2. Our data show that the 5S rRNA directly contacts MDM2 and that this interaction is increased when ribosome biogenesis is inhibited. Furthermore, the high level of the 5S rRNA/RPL5 complex (>200,000 copies per cell) in the nucleoplasm of normal cells is far in excess of most basic transcription factors indicating that any interaction between the dimeric 5S rRNA/RPL5 complex and MDM2 is unlikely to affect MDM2 activity. Therefore, the trimeric 5S RNP, rather than its individual components or subcomplexes, is the central regulator of MDM2 and therefore p53, which is consistent with earlier reports that both RPL5 and RPL11 are required for p53 activation in response to ActD treatment (Bursac et al., 2012; Fumagalli et al., 2012; Sun et al., 2010) and the recent report that the 5S rRNA is also required for this regulation (Donati et al., 2013). A 5S rRNA, RPL5, MDM2, p53, and 5.8S rRNA complex was previously described (Marechal et al., 1994). We do not find the 5.8S rRNA in any of our “nonribosomal” 5S RNP complexes, and the presence of this rRNA, which is not normally found outside the ribosome, may also indicate coprecipitation of the large ribosomal subunit in the results reported by Marechal et al. (1994).

Our data demonstrate that RPL5 is required for the accumulation and 3' processing of the 5S rRNA, and it is likely that interaction with the RPL5/5S rRNA serves to stabilize RPL11. The abundance of the 5S rRNA/RPL5 complex in the cell, and the evidence that RPL11 is synthesized in excess (Lam et al., 2007), suggests that the binding of RPL11 to the 5S rRNA/RPL5 complex represents a key step in the p53 response and must be carefully regulated. In contrast to an earlier report

(Bursac et al., 2012), but consistent with other observations (Chakraborty et al., 2011), we see RPL11 and RPL5 accumulate in the nucleoplasm when ribosome biogenesis is blocked, which may, however, reflect a difference in cell types used in the different studies.

Using an MDM2 mutant that has previously been demonstrated to prevent interaction with both RPL5 and RPL11 (Macias et al., 2010), we show that the direct interaction of the 5S rRNA and MDM2 is dependent on the presence of RPL5 and RPL11. Interestingly, using a different approach involving RNAi-mediated depletion of 5S RNP components, Donati et al. (2013) also demonstrate a mutually dependent interaction of the complex components with MDM2. Thus, the findings of the two papers are complementary and both come to the conclusion that the assembled 5S RNP regulates MDM2 and thereby p53.

Factors Involved in Biogenesis of the Large Ribosomal Subunit Regulate 5S RNP-Mediated Modulation of p53

Integration of the 5S RNP into the ribosome is essential for 28S and 5.8S rRNA processing, meaning that production of the large ribosomal subunit is closely coupled to the amount of free 5S RNP in the cell and consequently to p53 levels. We find that defects in ribosome production cause an increase in RPL11 levels in the nucleoplasm and p53 activation in a 5S RNP-dependent manner. Interestingly, our data demonstrate that the tumor suppressor PICT1, which directly binds to the 5S rRNA in vivo in our UV crosslinking experiment, is an essential ribosome biogenesis factor that is directly responsible for 5S RNP integration into the ribosome. This explains earlier observations that PICT1 directly binds RPL11 and RPL5 in vitro and is essential for retaining RPL11 in the nucleolus (Sasaki et al., 2011). NOP2 or BOP1 were also important for 5S RNP integration into the ribosome but to a lesser extent. These factors could directly modulate 5S RNP integration, potentially by bridging interactions between the 5S RNP and the preribosome, or alternatively be essential for upstream steps in the process.

Much to our surprise, and in contrast to the situation in yeast (Zhang et al., 2007), we found that neither RRS1 nor BXDC1 was associated with the 5S RNP outside the preribosome. Furthermore, depletion of either of these proteins had little or no impact on 5S RNP recruitment into the ribosome. It is, however, clear that these two proteins, which directly contact the 5S rRNA, have an important function in the nucleolar localization of RPL5 or both RPL5 and RPL11 in humans. Surprisingly, this reduction of RPL5 and/or RPL11 levels in the nucleolus has only a minor effect on 5S RNP integration into the ribosome.

The 5S RNP Is Essential for the Cellular Response to p14^{ARF} Overexpression

RPL11 has been shown to bind p14^{ARF} and form an RPL11/p14^{ARF}/MDM2/p53 complex that is required for complete p14^{ARF}-dependent suppression of MDM2 and activation of p53 (Dai et al., 2012). We show here that RPL5 and the 5S rRNA, probably as part of the assembled 5S RNP complex, are also important for this process (Figure 7). This supports results where a mutation (C305F) in the zinc finger of MDM2, which cannot bind RPL11 and RPL5, accelerates Myc-induced lymphomagenesis in mice (Macias et al., 2010). It is well established that p14^{ARF}

induction inhibits ribosome biogenesis, primarily targeting the production of the large ribosomal subunit (Figure 6). These data imply that p14^{ARF} predominantly functions by impeding ribosome biogenesis, causing an increase in the levels of nonribosomal 5S RNP (Figure 7). The 5S RNP, together with p14^{ARF}, would then inhibit MDM2 leading to p53 activation.

Regulation of p53 Homeostasis and Cellular Proliferation by the 5S RNP

We have clearly shown that the amount of the nonribosomal 5S RNP in the cell determines the basal level of the tumor suppressor p53. This provides a means by which the rate of ribosome biogenesis is coupled to p53 homeostasis and therefore to cellular proliferation (Figure 7). However, Donati et al. (2013) observe only a slight reduction in p53 levels upon depletion of 5S RNP components. This may reflect differences in the efficiency or duration of siRNA-mediated knockdown of the various components or the different siRNA treatment times. Differences between transfection efficiencies may also explain why we observed a clear defect on 5S rRNA levels and pre-rRNA processing using the 5S rRNA-specific siRNA that was not observed in the other publication (Donati et al., 2013). The 5S RNP is a combined product of RNA pol II and RNA pol III, and the levels of free 5S RNP in the cell directly relate to the processing of the RNA-pol-I-transcribed rRNAs. The 5S RNP is therefore an ideal marker to coordinate gene expression from all three RNA polymerases and the rate of ribosome biogenesis, itself an essential aspect of cellular growth. Furthermore, RPL11 has previously been shown to regulate the proliferative factor, c-Myc (Dai et al., 2007). We would suggest that c-Myc regulation is mediated by the 5S RNP, and we therefore propose this complex to be a critical regulator of several cellular signaling pathways.

A number of ribosomopathies, including Diamond Blackfan anemia, 5q syndrome, and Treacher Collins syndrome, all result in misregulation of p53, probably due to impaired ribosome production increasing the amount of nonribosomal 5S RNP (Figure 7; Fumagalli and Thomas, 2011). The increased levels of p53 cause apoptosis during facial development in Treacher Collins syndrome (Jones et al., 2008). In Diamond Blackfan anemia and 5q syndrome, anemia is predicted to result from an inability to meet the increased demand for ribosomes during erythropoiesis (Boulwood et al., 2012). Paradoxically, many of these diseases also result in an increased propensity for cancer (Freed et al., 2010; Narla and Ebert, 2010). However, it is possible that either the p53 response becomes desensitized or that the cells adapt to high p53 levels. Our data, therefore, highlight the 5S RNP as a future target for anticancer and antiribosomopathy drugs.

EXPERIMENTAL PROCEDURES

RNAi and the Analysis of rRNA Processing

HEK293 cell, U2OS cells, or U2OS cells expressing p14^{ARF} under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (Llanos et al., 2001) were transfected with siRNAs (see Table S1) using RNAiMAX Lipofectamine. Harvested cells were analyzed by northern or western blotting (see Tables S2 and S3). For pulse-labeling experiments, cells were treated with siRNAs or chemotherapeutic agents (ActD and 5FU) and then pulse-labeled with ³²P orthophosphate (Sloan et al., 2013). RNAs extracted from

the cells were analyzed by agarose-glyoxal and denaturing-polyacrylamide gel electrophoresis.

Immunoprecipitation and Glycerol Gradient Analysis

Whole cell extracts were separated on 10%–40% glycerol gradients, and the resultant fractions analyzed by gel electrophoresis followed by western/northern blotting or, in the case of labeled RNAs, phosphorimager analysis. Immunoprecipitation reactions were performed with whole cell extracts or pooled gradient fractions using either an anti-FLAG antibody or anti-MDM2 antibodies. Coprecipitated RNAs were extracted and analyzed by northern blotting.

Immunofluorescence

HEK293 cell lines stably expressing inducible FLAG-tagged RPL5 or RPL11 were treated with ActD or specific siRNAs and then analyzed by immunofluorescence using anti-FLAG and anti-fibrillarin antibodies. Images were captured with a Zeiss Axiovert 200M inverted microscope.

UV Crosslinking of 5S rRNA and 5S RNP Biogenesis Factors

HEK293 cells expressing FLAG/His-tagged proteins of interest were UV irradiated. Complexes were purified using anti-FLAG antibodies, eluted using PreScission Protease, and then purified using nickel-nitrilotriacetic acid (NTA) under denaturing conditions (6 M guanidium-HCl). Coprecipitated RNAs were eluted using Proteinase K and analyzed by northern blotting.

See [Extended Experimental Procedures](#) for more information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.08.049>.

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