Suprainduction of p53 by disruption of 40S and 60S ribosome biogenesis leads to the activation of a novel G2/M checkpoint

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Impairment of ribosome biogenesis leads to p53 induction and cell cycle arrest, a checkpoint involved in human disease. Induction of p53 is attributed to the binding and inhibition of human double minute 2 (Hdm2) by a subset of ribosomal proteins (RPs): RPS7, RPL5, RPL11, and RPL23. However, we found that only RPL11 or RPL5, in a mutually dependent manner, elicit this response. We show that depletion of RPS7 or RPL23, like depletion of other RPs, except for RPL11 and RPL5, induces a p53 response and that the effects of RPS7 and RPL23 on p53 induction reported earlier may be ascribed to inhibition of global translation. Moreover, we made the surprising observation that codepletion of two essential RPs, one from each subunit, but not the same subunit, leads to suprainduction of p53. This led to the discovery that the previously proposed RPL11-dependent mechanism of p53 induction, thought to be caused by abrogation of 40S biogenesis and continued 60S biogenesis, is still operating, despite abrogation of 60S biogenesis. This response leads to both a G1 block and a novel G2/M block not observed when disrupting either subunit alone. Thus, induction of p53 is mediated by distinct mechanisms, with the data pointing to an essential role for ribosomal subunits beyond translation.

[*Keywords*: 5′TOP translation; G2/M checkpoint; RPL11; p53]

Received February 16, 2012; revised version accepted March 29, 2012.

The ribosome plays a unique role in the maintenance of the species, translating mRNAs into functional proteins (Watson 1964). Moreover, it is known that the affinity of the translational apparatus for any single mRNA species is unique (Lodish 1974; Thomas 2000). Given that there is an excess in the number of mRNA transcripts to ribosomes, a decrease in ribosome number would impinge not only on the rates of translation, but also on the patterns of translation (Lodish 1974; Thomas 2000). This is because as the number of ribosomes to mRNA transcripts decreases, those mRNAs for which the translational apparatus has high affinity will continue to be translated, whereas the translation of those mRNAs for which the protein synthetic apparatus has low affinity will decrease (Thomas 2000; Volarevic and Thomas 2001; Ruggero and Pandolfi 2003). Importantly, changes in gene expression, caused by alterations in ribosome number, have been implicated in aberrant growth and human pathologies (Ferreira-Cerca and Hurt 2009; Zhang and Lu 2009). Evidence in support of this concept initially came from findings in model systems showing that ribosomal proteins (RPs) act as haploinsufficient tumor suppressors (Watson et al. 1992; Stewart and Denell 1993; Amsterdam et al. 2004). More recently, it has become evident that patients affected by Diamond-Blackfan anemia (DBA) (Draptchinskaia et al. 1999; Gazda et al. 2006) or $5q^{-}$ syndrome (Ebert et al. 2008), pathological conditions characterized by heterozygous loss-of-function mutations in RP genes, have a propensity to develop tumors later in life (Fumagalli and Thomas 2011).

Given the observations above, it is not surprising that the eukaryotic cell has developed checkpoints to monitor the fidelity and status of the translational machinery. We initially described the existence of this checkpoint following the deletion of an essential 40S RP gene, RPS6, in the liver of the adult mouse. This checkpoint blocked the ability of hepatocytes to re-enter the cell cycle and regenerate the lost liver mass following partial hepatectomy (Volarevic and Thomas 2001). Surprisingly, deletion of RPS6, although abrogating 40S ribosome biogenesis, had no effect on that of the 60S ribosome (Volarevic and

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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.189951.112.

Thomas 2001). Recently, we demonstrated that the lesion in 40S as well as 60S ribosome biogenesis results in the induction of p53 and G1 cell cycle arrest (Fumagalli et al. 2009). Notably, in the liver, the induction of the p53 response is caused by the deletion of the RPS6 gene and is not a consequence of the subsequent induction of hepatocytes to dedifferentiate and re-enter the cell cycle. This led us to conclude that it is not the number of ribosomes, but nascent ribosome biogenesis, that the cell monitors (Fumagalli et al. 2009). The importance of these findings to human pathology has been underscored in DBA and $5q^{-}$ syndrome, for which there is increasing evidence that the aberrant activation of the p53-dependent cell cycle checkpoint in erythroid precursors is responsible for the associated macrocytic anemia (Pellagatti et al. 2010; Dutt et al. 2011; Fumagalli and Thomas 2011).

The checkpoint elicited by impairment of ribosome biogenesis has been shown to be due to the ability of specific RPs to bind to human double minute 2 (Hdm2), inhibiting its E3 ligase activity, allowing p53 levels to rise in the cell and suppress cell cycle progression (Zhang and Lu 2009). Initially, based on studies of inhibition of rRNA transcription with low doses of actinomycin D, this response was attributed to nucleolar disruption and passive diffusion from the nucleolus to the nucleoplasm of RPs, particularly RPS7, RPL5, RPL11, and RPL23 (Dai et al. 2004; Jin et al. 2004; Chen et al. 2007; Zhu et al. 2009). However, we recently demonstrated that p53 up-regulation in response to impaired 40S or 60S ribosome biogenesis, despite being mediated by RPL11, does not lead to any notable alterations in nucleolar structure, leading to the conclusion that it is a regulated, rather than a passive, event (Fumagalli et al. 2009). We also demonstrated that the induction of p53 was due to the binding of RPL11 to Hdm2 and that depletion of RPL11 abolished this response (Fumagalli et al. 2009). This result raised the question of the role of RPS7, RPL5, and RPL23, which in the absence of RPL11 would have been predicted to still bind and inhibit Hdm2, maintaining high levels of p53.

As mentioned above, deletion of RPS6 abrogated 40S ribosome biogenesis, but did not alter the synthesis of 60S ribosomes (Volarevic et al. 2000). In fact, depletion of any single RP of either the 40S or 60S ribosomal subunit does not alter the production of the other subunit (Fumagalli et al. 2009). This is a surprising finding, given that ribosome biogenesis is a highly regulated process requiring the transcriptional coordination of all three RNA polymerases and expending a tremendous amount of cellular energy (Rudra and Warner 2004). Given that the extra ribosomal subunits generated under these conditions are not able to contribute to the cell's protein synthetic capacity (Volarevic et al. 2000; Fumagalli et al. 2009), the likely prediction would have been an extension of the p53 checkpoint to halt their synthesis. That the synthesis of either subunit is maintained, when the other is abrogated, suggests an alternative role for ribosomal subunits beyond translation, in agreement with the findings of others (Miyoshi et al. 2002; Steffen et al. 2008). Consistent with the continued maturation of one subunit in the absence of the other, it is known that the maturation of the two ribosomal subunits is mediated by independent processing pathways (Tschochner and Hurt 2003; Zemp and Kutay 2007). However, despite the processing pathways being insulated from one another, p53 in response to impaired 40S or 60S ribosome biogenesis is mediated by RPL11 binding to Hdm2 (Fumagalli et al. 2009). In the case of impaired 40S ribosome biogenesis, this effect requires the translational up-regulation of RPL11 to generate sufficient RPL11 protein to bind to Hdm2 in the face of continued 60S ribosome biogenesis and a sharp decrease in protein synthesis rates (Fumagalli et al. 2009). In contrast, impaired 60S ribosome biogenesis leads to an apparent suppression of RPL11 translation, since in the absence of large subunit biogenesis, there are sufficient RPL11 levels to bind Hdm2 (Fumagalli et al. 2009). Thus, despite the biogenesis of both subunits being controlled by distinct processing pathways, disruption of either leads to the suppression of Hdm2 by RPL11, suggesting a common pathway of p53 induction.

Given the importance of ribosome biogenesis to human pathology, it is critical to identify the basic molecular mechanisms by which this process is regulated and how it is integrated with mechanisms that control cell cycle progression. Here we set out to evaluate the requirement of RPS7, RPL5, RPL11, and RPL23 in inducing the p53 response to impaired ribosome biogenesis and determine whether this response is common for insults to either ribosomal subunit. We demonstrate that only RPL11 and RPL5, in a mutually dependent manner, are required for p53 induction. Moreover, we traced the apparent difference in these findings with those of earlier reports to the effect of long-term RP depletions on global protein synthesis rates (Fumagalli et al. 2009). In carrying out these studies, we made the unexpected observation that complete abrogation of ribosome biogenesis by codepletion of two essential RPs, one from each subunit, but not the same subunit, leads to suprainduction of p53. Consistent with this observation, we found that RPL11 mRNA is still selectively up-regulated at the translational level when the biosynthesis of both subunits is abrogated, despite no competition for RPL11 protein in the biogenesis of 60S ribosomes. Moreover, we demonstrate that the consequence of the suprainduction of p53 is a G1 cell cycle block and the induction of a novel G2/M cell cycle block. Thus, the mechanisms of p53 induction following disruption of the synthesis of either subunit are insulated from one another and, when simultaneously engaged, lead to full cell cycle arrest.

Results

RPL5 and RPL11 are required to induce p53

Others have recently shown that ectopic expression of either RPL11 or RPL5 is sufficient to inhibit Hdm2 and induce the p53 response, whereas our findings indicated that in the absence of RPL11, endogenous RPL5 is not sufficient to induce this response (Fumagalli et al. 2009).

Given studies in yeast showing that RPL5 and RPL11 are preassembled into a ribonucleoprotein particle before being incorporated into the nascent 90S processome, we reasoned that there may be a mutual dependence of the two proteins for inhibition of Hdm2. Such a model would explain the inability of RPL5 to inhibit Hdm2 in the absence of RPL11. To test this possibility, A549 human lung carcinoma cells (ATTC, CCL-185) were first treated with either a nonsilencing siRNA (NS siRNA) or a siRNA specific for RPS6—in the latter case to disrupt 40S ribosome biogenesis and induce the up-regulation of p53. In addition, cells were treated with siRNAs against RPL5 and RPL11, either alone or in combination with siRNAs against RPS6. As compared with cells treated with the control NS siRNA, those treated with the RPS6 siRNA showed a strong induction of p53 and its target gene, p21 (Fig. 1A), consistent with a reduction in RPS6 mRNA levels, as measured by quantitative qRT–PCR (qRT–PCR) (Fig. 1B), and the disruption of 40S ribosome biogenesis, as evidenced by the loss of native 40S ribosomes and an increase in native 60S ribosomes on polysome profiles (Fig. 1C). Likewise, depletion of either RPL5 or RPL11 caused a reduction in each of their transcript levels (Fig. 1B); however, in contrast to depletion of RPS6, their depletion was paralleled by a disruption of 60S ribosome biogenesis, as evidenced by the loss of native 60S ribosomes and an increase in native 40S ribosomes (Fig. 1C). In addition, the reduction in the amount of 60S ribosomal subunits relative to the amount of 40S ribosomal subunits leads to the increased formation of 43S preinitiation complexes, detected as half-mer polyribosomes, apparent as a pronounced shoulder on the right side of the 80S monosome and polysomal peaks (Fig. 1C; Rotenberg et al. 1988). It should also be noted that depletion of any RP leads to a decrease in total ribosomes as well as the polysome mean size (Fig. 1C). Depletion of RPL5 or RPL11, alone or together, has no effect on p53 levels (Fig. 1A; Fumagalli et al. 2009). Nevertheless, when combined with depletion of RPS6, depletion of RPL5 was as efficient in suppressing the up-regulation of p53 as depletion of RPL11 (Fig. 1A). It should be noted that depletion of RPS6 leads to the translational up-regulation of RPL5 (data not shown), as we showed for RPL11 (Fumagalli et al. 2009). Consistent with a model of mutual dependence, the rise in p53 levels was not further suppressed by codepletion of RPL5 and RPL11 (Fig. 1A). This paradigm is not limited to disruption of 40S biogenesis by RPS6 depletion, as we obtained equivalent results in cells treated with low doses of actinomycin D (Fig. 1D), which selectively inhibits rRNA polymerase I (Pol I) (Perry 1963). It should be noted that the induction of p53 by actinomycin D is always much more pronounced than that caused by depletion of an RP, such as RPS6 (see below); however, the extent to which codepletion of RPL11 rescues this response is qualitatively equivalent (Fig. 1D). These findings indicate that the effects of RPL5 and RPL11 are dependent on one another, such that neither protein alone is sufficient to inhibit Hdm2.

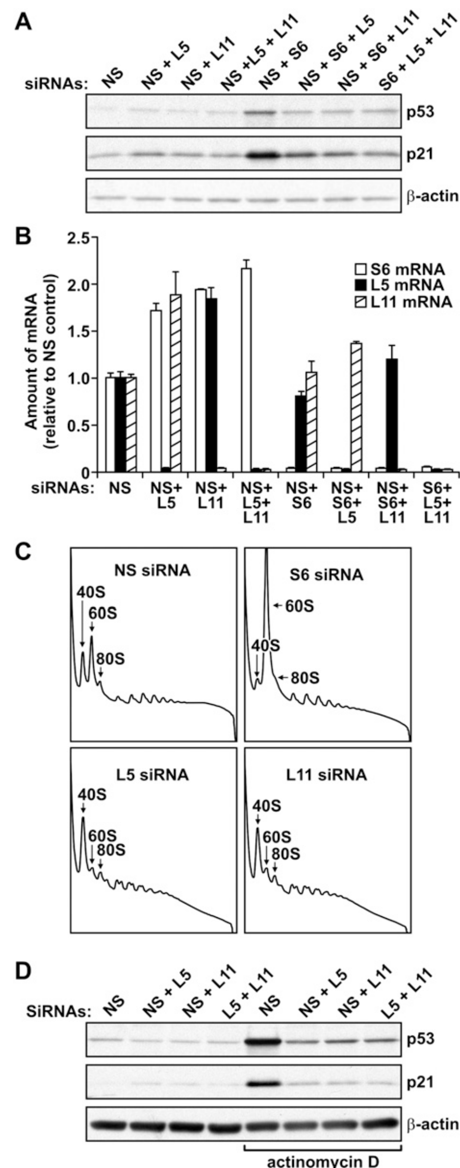

Figure 1. p53 up-regulation induced by inhibition of ribosome biogenesis requires RPL5 and RPL11. (*A*) Western blots showing the levels of p53, p21, and β-actin proteins in A549 cells transfected with the indicated siRNAs. (*B*) Levels of the RPS6, RPL5, and RPL11 mRNAs in A549 cells transfected with the indicated siRNAs, as measured by qRT–PCR. Each bar represents the average ± SEM of the ratio of the measurement of the indicated mRNA to the one of β-actin mRNA, as calculated for three independent samples. (*C*) Polysome profiles from extracts of A549 cells transfected with the indicated siRNAs. (*D*) Western blots showing the levels of p53, p21, and β-actin proteins in A549 cells transfected with the indicated siRNAs for 48 h and treated with or without 5 ng/mL actinomycin D for 6 h.

No requirement for RPS7 and RPL23 in the p53 response

RPS7 and RPL23 have been shown to bind and inhibit the E3 ligase activity of Hdm2 toward p53 (Zhang and Lu 2009). In the presence of RPS7 and RPL23, depletion of either RPL5 or RPL11 is sufficient to rescue inhibition of

Hdm2 induced by impaired ribosome biogenesis, raising the possibility that the ability of either RP to inhibit Hdm2 is also mutually dependent on RPL5 or RPL11. To test this, we depleted A549 cells of either RPS7 or RPL23 alone or in combination with RPS6 or RPL7a, both of whose depletion leads to p53 induction and disruption of 40S and 60S ribosome biogenesis, respectively (Fumagalli et al. 2009). In all cases, the extent of depletion of each RP was verified by qRT–PCR (Fig. 2A,B). Unexpectedly, unlike the depletion of RPL5 or RPL11, depletion of either RPS7 or RPL23 induced a p53 response equivalent to that observed when depleting RPs from the same ribosomal subunit, RPS6 and RPL7a, respectively (Fig. 2A,B, respectively). Moreover, the induction of p53 by depleting RPS6 or RPL7a is not suppressed by codepletion of RPS7 or RPL23 (Fig. 2A,B, respectively), which is in contrast to what others have shown when inducing p53 with actinomycin D. These findings were not unique to A549 cells, as similar results were obtained in the U-2 OS human osteosarcoma cell line (ATCC, HTB-96) (Fig. 2C). Unexpectedly, codepleting two RPs, one from each of the two ribosomal subunits, RPS7 and RPL7a or RPS6 and RPL23, leads to the suprainduction of p53 (Figs. 2A–C). This result was unexpected, as in our hands, depletion of any single RP from either subunit is sufficient to elicit a p53 response and G1 cell cycle arrest, with the exception of RPL5 and RPL11 (Figs. 1, 2; Fumagalli et al. 2009), and implies that that the mechanisms of p53 induction are independently regulated, depending on the subunit whose biosynthesis is disrupted—a question analyzed later in this study. Taken together, the results show that disruption of either 40S or 60S ribosome biogenesis by depletion of either RPS7 or RPL23, respectively, leads to the induction of p53 and that depletion of neither protein suppresses the p53 response elicited by the depletion of another RP, unlike depletion of either RPL5 or RPL11 (Fig. 1A).

Depletion of RPS7 or RPL23 has no effect on nucleolar integrity

The up-regulation of p53 by depletion of either RPS7 or RPL23 was unexpected and raised the question as to the mechanism involved. We showed previously that, distinct from the case of inhibition of Pol I transcription by actinomycin D, up-regulation of p53 by depletion of RPs such as RPS6 or RPL7a occurs in the absence of any notable alterations in nucleolar structures or in the synthesis of the other subunit (Fumagalli et al. 2009). In order to determine whether the mechanism of up-regulation of p53 by RPS7 and RPL23 depletion is similar to that of depletion of RPS6 and RPL7a or treatment with actinomycin D, we analyzed the distribution of the nucleolar protein fibrillarin by immunofluorescence. In these experiments, cells were pretreated with siRNAs directed against RPS6, RPS7, RPL7a, or RPL23. As a negative control of nucleolar disruption, we transfected cells with a NS siRNA, whereas for a positive control, cells were treated with low doses of actinomycin D. The results of these studies show that compared with cells transfected with NS siRNA, treatment with low doses of actinomycin D caused an increase in nuclear p53 staining, dispersion of fibrillarin in the nucleus, and its association with nucleolar cap structures (Fig. 3A; Hernandez-Verdun et al. 2010). Treatment of cells with siRNAs directed against RPS6, RPS7, RPL7a, or RPL23 also resulted in an increase in the levels of p53 in the nucleus, but did not affect the

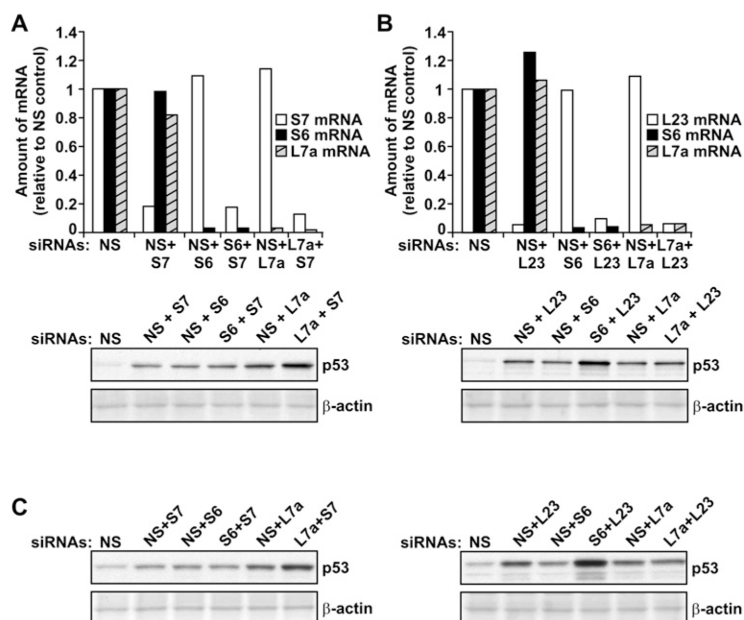

Figure 2. RPS7 and RPL23 are not required for the induction of p53 by depletion of RPs. (*A*, *top* panel) Levels of the RPS7, RPS6, and RPL7a; RPS7 and RPS6; or RPS7 and RPL7a mRNAs in A549 cells transfected with the indicated siRNAs, as measured by qRT–PCR. Each bar represents the average ± SEM of the ratio of the measurement of the indicated mRNA to the one of β-actin mRNA, as calculated for three independent samples. (*Bottom* panel) Western blots showing the levels of p53 and β-actin proteins in A549 cells transfected with the indicated siRNAs. The β-actin blot is stained with Ponceau; the prominent visible band migrates in the position of β-actin. (*B*, *top* panel) Levels of the RPL23, RPS6, and RPL7a; RPL23 and RPS6; or RPL23 and RPL7a mRNAs in A549 cells transfected with the indicated siRNAs, as measured by qRT–PCR. Each bar represents the average ± SEM of the ratio of the measurement of the indicated mRNA to the one of β-actin mRNA, as calculated for three independent samples. (*Bottom* panel) Western blots showing the levels of p53 and β-actin proteins in A549 cells transfected with the indicated siRNAs. The β-actin blot is stained with Ponceau; the prominent visible band migrates in the position of β-actin. (*C*) Western blots showing the levels of p53 and β-actin proteins in U-2 OS cells transfected with the indicated siRNAs. The β-actin blots are stained with Ponceau; the prominent visible band migrates in the position of β-actin.

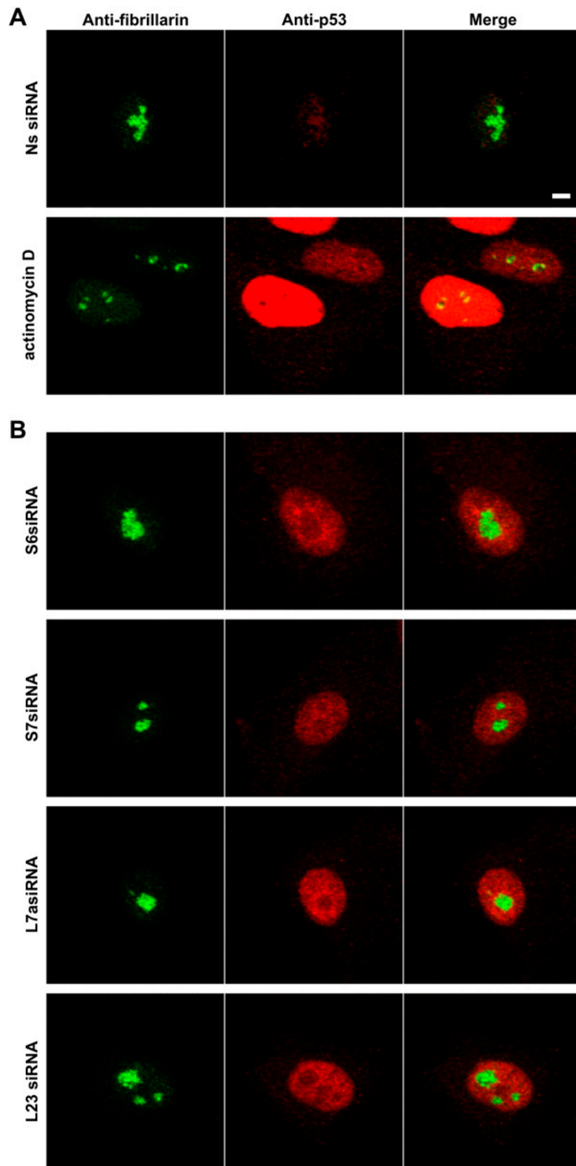

Figure 3. Depletion of RPS7 or RPL23 does not result in nucleolar disruption. (*A*) Fluorescent immunostaining with anti-p53 and anti-fibrillarin antibodies of A549 cells transfected with NS siRNA and then treated where indicated for 6 h with 5ng/mL actinomycin D. (*B*) Fluorescent immunostaining with anti-p53 and anti-fibrillarin antibodies of A549 cells transfected with the indicated siRNAs. The samples were analyzed by confocal microscopy with a Plan-Apochromat 63×/1.4 oil Dic objective (Zeiss). Bar, 5 μm.

distribution of fibrillarin, which was indistinguishable from that of NS siRNA-treated cells (Fig. 3B). Thus, even though depletion of each RP leads to abortive processing of either nascent 40S or 60S ribosomal subunits, the effects on p53 are not attributed to alterations in nucleolar integrity.

Induction of p53 by depletion of RPS7 and RPL23 is RPL5/RPL11 dependent

The findings above suggest that the induction of p53 caused by depleting RPS7 and RPL23 is due to the impairment of either 40S or 60S ribosome biogenesis, respectively, as it is for depletion of either RPS6 or RPL7a (Figs. 1, 2). Based on the latter findings, we would hypothesize that the induction of p53 triggered by depleting RPS7 or RPL23 is mediated by the binding of RPL5 and RPL11 to Hdm2. Consistent with this hypothesis, we previously showed that depletion of RPS7 leads to the translational up-regulation of RPL11 mRNA, similar to the depletion of RPS6, whereas depletion of RPL23, like that of RPL7a, led to the translational suppression of RPL11 mRNA (Fumagalli et al. 2009). To test this hypothesis, we asked whether the effect of p53 induction by depletion of RPS7 or RPL23 is reversed by codepletion of either RPL11 or RPL5. The results show that the p53 response induced by depletion of RPS7 or RPL23 is completely reversed by codepletion of either RPL11 or RPL5 (Fig. 4–C, respectively). These data support the notion that both RPL5 and RPL11 are necessary to mediate the induction of p53 by depletion of RPS7 or RPL23, but that neither alone is sufficient.

Depletion of RPs has an inhibitory effect on global protein synthesis rates

The finding that depletion of either RPS7 or RPL23 leads to the induction, rather than suppression, of p53 was hard to rationalize with earlier reports (Zhang and Lu 2009). In most cases, others have evaluated the role of RPS7 or RPL23 in suppressing p53 induction by acutely aborting nascent ribosome biogenesis with low doses of actinomycin D (Zhang and Lu 2009). In contrast, we interfered with nascent ribosome biogenesis by either deleting or depleting individual 40S or 60S RPs. We also showed that such treatments have a differential effect on the translation of 5′TOP mRNAs, including RPL11, even though they inhibited global protein synthesis rates to a similar extent (Fumagalli et al. 2009). However, acute treatment with low doses of actinomycin D would be expected to

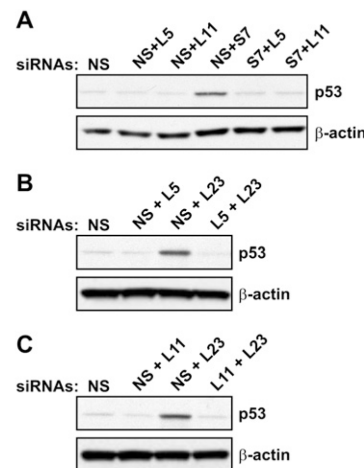

Figure 4. The up-regulation of p53 induced by depletion of RPS7 or RPL23 is dependent on RPL5 and RPL11. (*A–C*) Western blots showing the levels of p53 and β-actin proteins in A549 transfected with the indicated siRNAs.

have little or no inhibitory effect on global protein synthesis rates. Therefore, we reasoned that the suppression of the actinomycin D-induced p53 response by depleting RPS7 or RPL23 may instead be due to the more profound inhibition of global protein synthesis rates, as compared with cells treated with NS siRNA, and that the same effect would result from the depletion of any RP essential for ribosome biogenesis. To test this possibility, we compared the effects on global translation rates of A549 cells treated with siRNAs to RPS6, RPS7, RPL7a, RPL11, and RPL23, as well as an NS siRNA, followed by acute treatment with actinomycin D. The results show that whereas actinomycin D treatment of NS siRNA transfected cells had little effect on global translation, as measured by ^{3}H-leucine incorporation into nascent protein, depletion of any single RP had a pronounced effect on protein synthesis (Fig. 5A). In parallel, we found that in cells depleted of RPS6, the induction of p53 by actinomycin D was suppressed and equivalent to that in cells depleted of RPS7 (Fig. 5B). A similar inhibition of p53 induction by actinomycin D was observed for cells depleted of RPL7a or RPL23, whereas depletion of RPL11 completely abolished the p53 response (Fig. 5B) despite having an inhibitory effect on protein synthesis similar to that of depletion of other RPs (Fig. 5A). These results were not unique for A549 cells, as we obtained similar findings in U-2 OS cells (Fig. 5C). However, it should be noted that the reduction in p53 levels was less pronounced when we depleted a 40S versus a 60S RP (Fig. 5B,C). This difference most likely reflects the selective translational upregulation of RPL11 mRNA transcripts when cells are depleted of a 40S RP, RPS6, and RPS7 versus a 60S RP, RPL7a, and RPL23 (Fumagalli et al. 2009), despite a similar inhibitory effect on global protein synthesis (Fig. 5A). Such an increase in RPL11 would be expected to more strongly protect p53.

If the reduction in p53 levels is due to inhibition of global translation, this response should be recapitulated by treating cells with a general protein synthesis inhibitor, such as cycloheximide. The results show that suppression of protein synthesis by cycloheximide at increasing doses, from 0.02 to 0.1 μg/mL (Fig. 5D), is paralleled by a reduction in the p53 response to actinomycin D (Fig. 5E), similar to that observed when depleting RPs, with the exception of RPL11 (Fig. 5B,C). These data show that the effects of RPS7 and RPL23 depletion on p53 accumulation induced by actinomycin D are not unique for these RPs, as equivalent effects are obtained by depleting other RPs of the same ribosomal subunit. More importantly, they suggest that the differences between our findings reported here and those of others, with respect to the ability of RPS7 and RPL23 depletion to suppress the induction of p53 (Dai et al. 2004; Jin et al. 2004; Chen et al. 2007; Zhu et al. 2009), may be attributed to a general inhibition of global protein synthesis rates, combined with the known short half-life of p53 (Vousden and Lane 2007).

Distinct mechanisms mediate the induction of p53 in response to impaired 40S versus 60S ribosome biogenesis

As noted above, we unexpectedly observed that aborting both 40S and 60S ribosome biogenesis by depleting two essential RPs of either subunit causes a suprainduction of p53, whereas if the two proteins depleted are of the same subunit, the effect on p53 is equivalent to the depletion of any single RP (Fig. 2). These effects were not unique to the combinations used in the experiments described in Figure 2, as an equivalent result was obtained when depleting RPS6 and RPL7a (Fig. 6A), arguing that the combined effect is a consequence of the simultaneous impairment

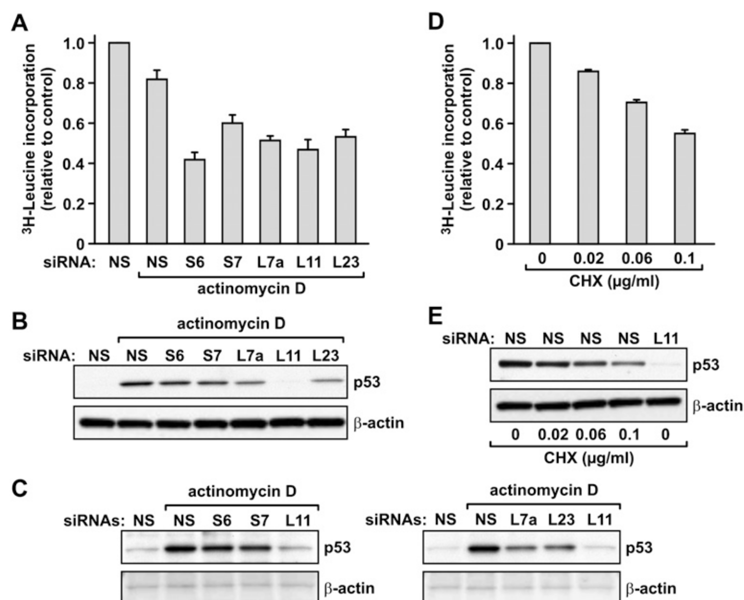

Figure 5. The effects of RPS7 and RPL23 depletion on p53 accumulation induced by actinomycin D are due to inhibition of protein synthesis. (*A*) Measurement of ^{3}H-leucine incorporation in A549 cells transfected with siRNAs and then treated where indicated with 5 ng/mL actinomycin D for 6 h. Each bar represents the average ± SEM of three independent samples. (*B*) Western blots showing the levels of p53 and β-actin proteins in A549 cells treated as in *A*. (*C*) Western blots showing the levels of p53 and β-actin proteins in U-2 OS cells transfected with the indicated siRNAs and then treated, where indicated, with 5 ng/mL actinomycin D for 12 h. In both panels, the β-actin blot is stained with Ponceau; the prominent visible band migrates in the position of β-actin. (*D*) Measurement of ^{3}H-leucine incorporation in A549 cells transfected with NS siRNA and then treated with 5 ng/mL actinomycin D for 6 h in the presence of the indicated concentrations of cycloheximide. Each bar represents the average ± SEM of three independent samples. (*E*) Western blots showing the levels of p53 and β-actin proteins in A549 cells treated as in *D*. Included is a sample of A549 cells transfected with L11 siRNA and then treated with 5ng/mL actinomycin D for 6 h.

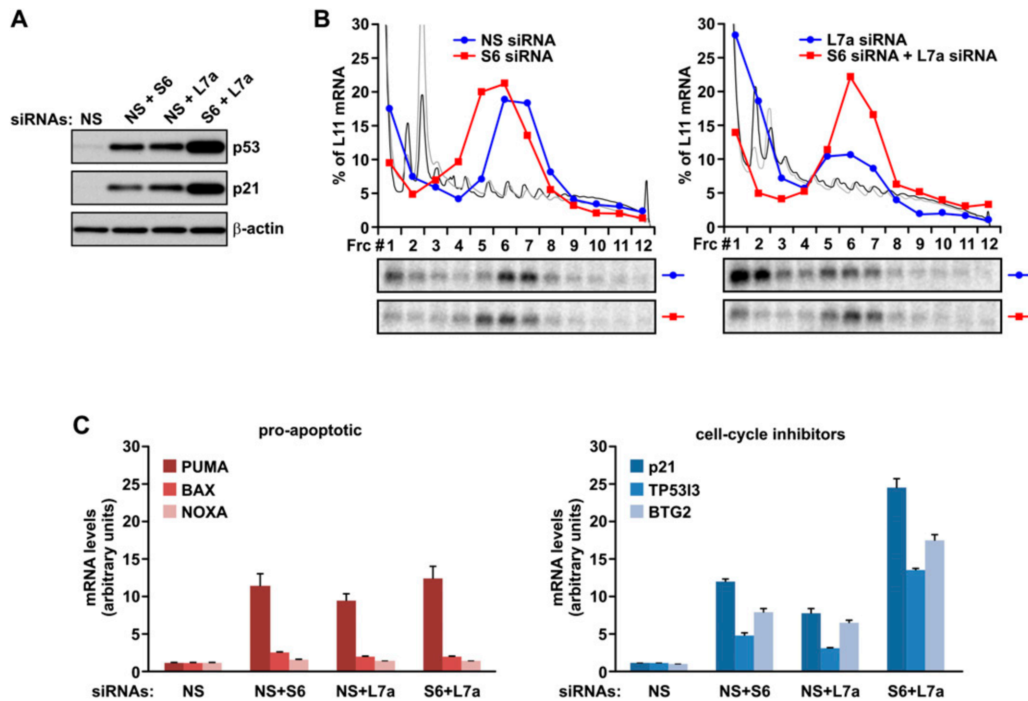

Figure 6. Concomitant inhibition of 40S and 60S ribosome biogenesis has an additive effect on the accumulation of p53. (*A*) Western blots showing the levels of p53, p21, and β-actin proteins in A549 cells transfected with the indicated siRNAs. (*B*) Quantification of the distribution of the RPL11 mRNA along polysome profiles of A549 cells transfected with NS siRNA or RPS6 siRNA for 30 h (*left* panel) and with RPL7a siRNA or RPS6 and RPL7a siRNAs (*right* panel). Superimposed are the traces of the polysome profiles (*left* panel: NS siRNA [black] and RPS6 siRNA [gray]; *right* panel: RPL7a siRNAs [black] and RPS6 and RPL7a siRNAs [gray]). (*C*) Levels of the PUMA, BAX, and NOXA mRNAs (*left* panel) and of the p21, TP53I3, and BTG2 mRNAs (*right* panel) in A549 cells transfected with the indicated siRNAs, as measured by qRT–PCR. Each bar represents the average ± SEM of the ratio of the measurement of the indicated mRNA to the one of β-actin mRNA, as calculated for the three independent samples.

of 40S and 60S ribosome biogenesis. Surprisingly, these results suggest that the mechanisms, which sense impaired 40S and 60S ribosome biogenesis, like those involved in the biogenesis of nascent ribosomes (Zemp and Kutay 2007), are insulated from one another and act independently to mediate the induction of p53. If this is the case, the prediction would be that the translational up-regulation of 5′TOP mRNAs, including RPL11 mRNA, which is required to elicit a p53 response in cells where 40S ribosome biogenesis is disrupted, is not affected by disruption of the 60S ribosome biogenesis. We would not have foreseen this, given that we had earlier rationalized that ongoing 60S ribosome biogenesis was the reason for the translational up-regulation of RPL11 mRNA, regardless of the inhibition of global protein synthesis (Fumagalli et al. 2009). To test this possibility, we depleted cells of RPS6, RPL7a, or RPS6 in combination with RPL7a. The analysis of RPL11 mRNA distribution on polysome gradients revealed that disruption of the 40S ribosome biogenesis following depletion of RPS6 leads to an almost complete recruitment of RPL11 mRNA into polysomes, as compared with NS siRNA-treated cells, despite a decrease in mean polysome size (Fig. 6B; Fumagalli et al. 2009). In contrast, depletion of RPL7a alone causes RPL11 transcripts to accumulate in the nonpolysome portion of the sucrose gradient (Fig. 6B; Fumagalli et al. 2009). In addition, depletion of RPL7a led to the appearance of half-mer polyribosomes (Fig. 6B), as observed following depletion of either RPL5 or RPL11 (Fig. 1C). Although the appearance of half-mer polyribosomes is lost by codepletion of RPS6, there is no effect on the mean polysome size (Fig. 6B), consistent with the effects on global translation rates, as measured by the incorporation of ^{3}H-leucine into nascent protein (Fig. 5A). However, the translational up-regulation of RPL11 mRNA following RPS6 depletion is not suppressed by codepletion of RPL7a (Fig. 6B). We observed equivalent results when codepleting RPS6 and RPL23 (data not shown), which also results in p53 suprainduction (Fig. 2B,C). Moreover, as we reported previously, these effects are unique to 5′TOP mRNAs (Fumagalli et al. 2009), as we do not see a similar effect on transcripts such as β-actin (data not shown). Therefore, even though 60S ribosome biogenesis is disrupted and RPL5 and RPL11 are no longer being consumed in the biogenesis of nascent 60S ribosomes, impairment of 40S ribosome biogenesis still leads to the translational up-regulation of RPL11 mRNA, consistent with the suprainduction of p53. This is a surprising observation, as it demonstrates that the checkpoint mechanisms that monitor 40S and 60S ribosomes biogenesis, despite the fact that both are dependent on RPL5/RPL11 protein levels, are regulated independently of one another.

Despite coinhibition of 40S and 60S ribosome biogenesis leading to the suprainduction of p53 (Fig. 6A), we observed no evidence of increased apoptosis up to 48 h post-siRNA treatment, as measured by either PARP or caspase 3 cleavage (data not shown). Importantly, we showed previously that A549 cells elicit a p53-dependent apoptotic response to DNA-damaging agents (Beuvink et al. 2005). To corroborate this finding, we analyzed by qRT–PCR the mRNA levels of a number of proapoptotic p53 target genes, including PUMA, NOXA, and BAX (Riley et al. 2008). Amongst these genes, PUMA mRNA was potently induced, but this effect was not enhanced by simultaneously impairing the biogenesis of both subunits and did not parallel the induction of p53 (Fig. 6C). Moreover, the effects on NOXA or BAX expression were minimal, and, like PUMA, their level of induction did not correlate with that of p53 (Fig. 6C). The modest effects on NOXA and BAX expression are consistent with our inability to detect any signs of apoptosis regardless of the extent of inhibition of ribosome biogenesis. In contrast, we found that the expression of p53 target genes involved in the inhibition of cell cycle progression, all of which were induced by impairing the synthesis of either ribosomal subunit, was further augmented by the simultaneous inhibition of both subunits (Fig. 6C). We observed similar effects in other cell lines (data not shown), consistent with the suprainduction of p53 eliciting its antiproliferative effects at the level of cell cycle progression.

A G2/M checkpoint revealed by coimpairment of 40S and 60S ribosome biogenesis

The question that arises from the findings above is whether the suprainduction of p53, caused by impairing both 40S and 60S ribosome biogenesis, has a further consequence on the cell beyond that elicited by impaired synthesis of either subunit alone. We noted that actinomycin D provokes a much stronger p53 induction than disrupting either 40S or 60S ribosome biogenesis (Figs. 1D, 3; data not shown). In parallel, like others (Choong et al. 2009), we found that actinomycin D elicits a block in both the G1 and G2/M phases of the cell cycle, with virtually no cells in S phase (Fig. 7A). In contrast, we reported that depleting cells of RPS6 and disrupting 40S ribosome biogenesis leads to a strong arrest of cells in G1, but with no apparent difference in the G2 population, and a significant amount of cells in S phase (Fumagalli et al. 2009). This raised the question as to whether the suprainduction of p53 caused by impairing both 40S and 60S ribosome biogenesis, similar to the action of actinomycin D, also leads to arrest of cells in G2/M. We found that depletion of either RPS6 or RPL7a leads to a G1 block and a decreased percentage of cells in S phase, with the level of the G1 block not further enhanced by codepleting the two RPs (Fig. 7B). However, disruption of both 40S and 60S ribosome biogenesis further decreased the percentage of cells in S phase and caused a distinct accumulation of cells in G2/M, similar to that observed for actinomycin D treatment (Fig. 7A). To determine whether the G2/M response, like the G1 response, was dependent on the induction of p53, we treated cells with NS siRNA or siRNAs against RPS6 and RPL7a in the presence of a siRNA directed against p53. The results show that both the G1 and G2/M cell cycle checkpoints are relieved by depleting cells of p53 (Fig. 7, cf. B and C). It should be noted that depletion of p21 is not sufficient to rescue either block (data not shown), consistent with the up-regulation of other cell cycle inhibitors, including BTG2 and TP53I3 (Fig. 6C). These findings demonstrate that the enforced rise in the suppression of cell cycle progression, caused by impairing both 40S and 60S ribosome biogenesis, is due to the suprainduction of p53.

Discussion

The role of p53 as a tumor suppressor in humans and other mammals is well established. Loss or mutations in the p53 gene are found in ~50% of human tumors, and alterations in p53 regulators such as Hdm2 and ARF are present in many more cancers (Vousden and Prives 2009). However, the role of p53 is not limited to that of a tumor suppressor, as it has been shown to participate in other cellular processes, including longevity, mitochondrial oxidation, and glucose metabolism (Vousden and Prives 2009). Earlier studies suggested that regulation of p53 degradation by Hdm2 plays a critical part in response to changes in the efficacy of ribosome biogenesis (Zhang and Lu 2009). The importance of understanding the mechanisms by which p53 is activated in lesions in ribosome biogenesis is underscored by a number of diseases associated with mutations in nucleolar components involved in this process, including Treacher-Collins syndrome (Jones et al. 2008), *Dyskeratosis congenita* (Ruggero et al. 2003), Shwachman-Diamond syndrome (Shimamura 2006), DBA (Draptchinskaia et al. 1999; Gazda et al. 2006), and $5q^{-}$ syndrome (Ebert et al. 2008). Studies over the last year have shown that patients suffering from either DBA (Dutt et al. 2011) or $5q^{-}$ syndrome (Pellagatti et al. 2010) have elevated levels of p53. More remarkably, it was demonstrated in mouse models of Treacher-Collins syndrome (Jones et al. 2008) and $5q^{-}$ syndrome (Pellagatti et al. 2008) that the pathogenic phenotypes can be rescued in a $p53^{+/-}$ or $p53^{-/-}$ background. These studies suggest that impairment of ribosome biogenesis, the lesion caused by these mutations, is most likely not innately responsible for the pathology, but that the cause is instead the unscheduled up-regulation of p53 (Fumagalli and Thomas 2011). Given the involvement of p53 in diseases caused by impairment of ribosome biogenesis, it is critical to elucidate the molecular mechanisms responsible for sensing the lesions in this process and identify the downstream pathways that elicit the RPL5/RPL11 checkpoint.

The studies presented here show that RPL5 and RPL11 cooperate to suppress Hdm2 and allow p53 levels to rise in the cell and that although both are required, neither one alone is sufficient to induce this response (Fig. 1). Our findings contrast with the model proposed by Horn and Vousden (2008), which is largely based on experiments involving overexpression of RPL5 and RPL11. They

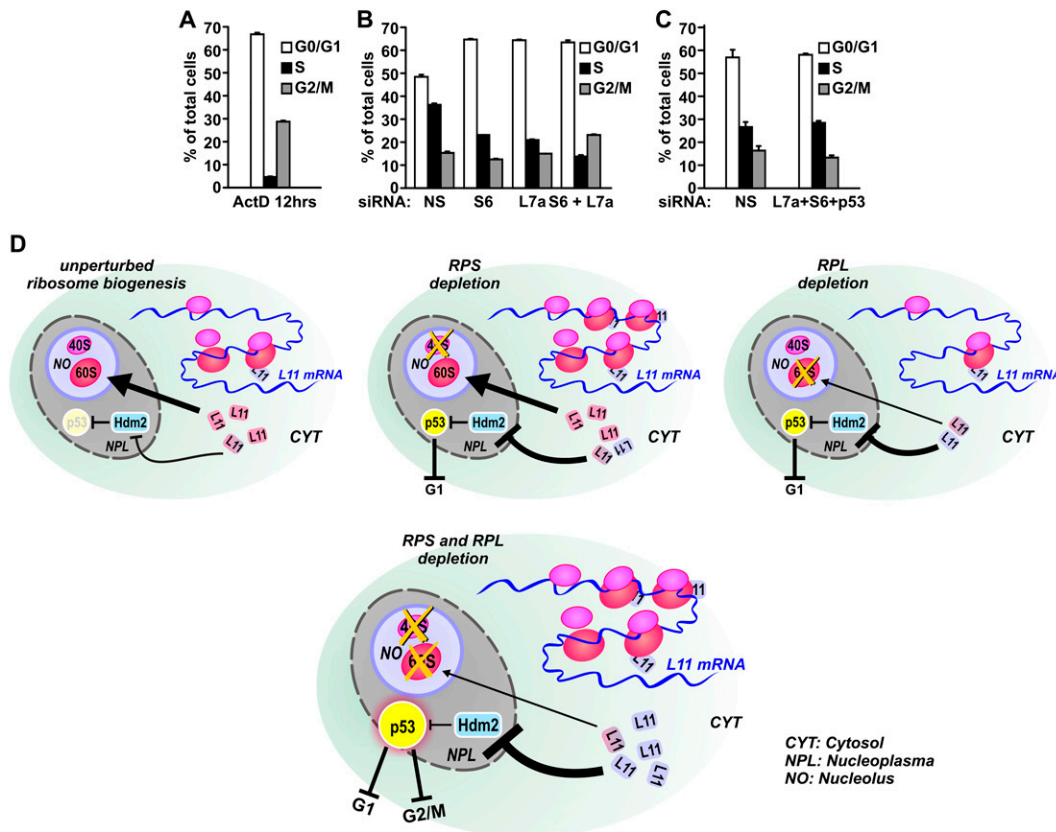

Figure 7. Concomitant inhibition of 40S and 60S ribosome biogenesis results in accumulation of cells in both the G1 and G2/M phases of the cell cycle. A549 cells treated for 12 h with 5ng/mL actinomycin D (*A*) or transfected with the indicated siRNAs for 30 h (*B*,*C*) were stained with propidium iodide. The distribution in the G1, S, and G2/M phases of the cell cycle was analyzed by flow cytometry. Each bar represents the mean of three independent samples ± SEM. Under these conditions, we depleted cells of p53 mRNA >84% and reduced p53 protein to less than basal levels, as measured by qRT–PCR and Western blot analysis, respectively (data not shown). (*D*) Model of stabilization of p53 in response to impairment of 40S, 60S, or both 40S and 60S ribosome biogenesis. The *top left* panel shows that under normal conditions, the majority of newly synthesized RPL11 is used in the synthesis of 60S ribosomes. The *top middle* panel shows that upon impairment of 40S ribosome biogenesis, there is a need to translationally up-regulate RPL11 protein expression to bind to Hdm2 to compete for the requirement of RPL11 protein in ongoing 60S ribosome biogenesis. In contrast, the *top right* panel shows that following impairment of 60S ribosome biogenesis, there is no need to up-regulate RPL11 protein expression to bind to Hdm2, as there is no competition for RPL11 protein, as 60S ribosome biogenesis has been abrogated. The *bottom middle* panel, which is drawn at slightly larger scale than the *top* ones, shows that despite abrogation of 60S ribosome biogenesis, coimpairment of 40S ribosome biogenesis still results in the translational up-regulation of RPL11, resulting in higher accumulation of free RPL11, as compared with impairing either 40S or 60S ribosome biogenesis alone. This leads to a more potent inhibition of Hdm2, suprainduction of p53, and accumulation of cells in both G1 and G2/M. The RPL11 proteins that either bind Hdm2 or are used in the synthesis of 60S ribosomes are represented in violet and pink, respectively. For simplicity, only RPL11 and not RPL5 are shown. The thickness of the black lines is directly proportional to the intensity of the stimulatory (→) or inhibitory (—|) effects.

suggested that although the effect of RPL5 and RPL11 on Hdm2 is cooperative, any single protein is sufficient to inhibit Hdm2 (Horn and Vousden 2008). Our data argue instead that RPL5 and RPL11 work in a complex to inhibit Hdm2. The concept of a RPL5/RPL11 complex is supported by studies in both bacteria (Yu and Wittmann 1973) and yeast (Zhang et al. 2007), where it has been shown that the RPL5 and RPL11 orthologs are incorporated into nascent ribosomes as part of a complex, which also includes 5S rRNA. In yeast (Deshmukh et al. 1993), amphibians (Picard and Wegnez 1979), and mammals (Steitz et al. 1988), it is known that RPL5 forms a complex with 5S rRNA, and recently, 5S rRNA has been shown to coimmunoprecipitate with RPL11 (Horn and Vousden 2008). In yeast, two assembly factors, Rpf2 and Rrs1, are essential for assembling the RPL5/RPL11/5S rRNA complex into the nascent 90S processome (Zhang et al. 2003). Loss of either protein arrests 25S rRNA maturation at the level of the $27SB_s/27SB_1$ intermediates, equivalent to mammalian 32S/36S precursors, which give rise to the mature 28S rRNA (Zhang et al. 2003). Orthologs of yeast Rpf2 and Rrs1 have been identified in the nucleolus of human cells (Scherl et al. 2002). It will be of interest to determine whether the human orthologs of Rpf2 and Rrs1 as well as 5S rRNA are part of the hypothetical RPL5/RPL11 complex that binds to and inhibits Hdm2.

Our findings suggest that neither RPS7 nor RPL23 is required for the suppression of Hdm2 and the induction of p53 caused by depletion of RPs (Fig. 2). We demonstrated previously that depleting the RPS7 level was as efficient in inducing the translational up-regulation of 5′TOP mRNAs, including RPL11 mRNA, as depleting RPS6 (Fumagalli et al. 2009). Consistent with these findings, we show that depleting RPS7 induces, rather than suppresses, the induction of p53, a response that is dependent on RPL5 and RPL11 (Fig. 4A) and occurs in the absence of alterations to nucleolar structures (Fig. 3). We observed the same response in three different cell lines, including A549, U-2 OS, and HCT116 (Fig. 4A; S Fumagalli and G Thomas, unpubl.). We observed the same effects of depleting RPL23 as we observed for depleting RPS7, (Figs. 2B,C, 3, 4; S Fumagalli and G Thomas, unpubl.). We found that RPL23 or RPS7 depletion partially suppresses p53 up-regulation by actinomycin D; however, these effects are equivalent to those of depleting a second RP of the 60S or 40S ribosome, respectively (Fig. 5B,C). We propose that inhibition of ribosome biogenesis caused by RPL23 or RPS7 depletion results in suppression of protein synthesis, similar to cycloheximide treatment, and that in parallel, this leads to an apparent inhibition on p53 induction by actinomycin D (Fig. 5A,D,E). It should be noted that Dai et al. (2004) have demonstrated previously that reducing RPL23 levels leads to the induction of p53, whereas in parallel, they found that the induction of p53 by actinomycin D was suppressed by depletion of RPL23. The investigators suggested that the first response may have been through an insult to ribosome biogenesis through an as yet unidentified pathway (Dai et al. 2004). However, the findings presented here would explain the apparent conundrum, with depletion of RPL23 inducing p53 through a canonical RPL5/RPL11-dependent pathway, and the suppression of the actinomycin D-induced p53 response due to the inhibition of global translation rates (Fig. 5), rather than a specific effect of RPL23 on p53 stability. It also should be noted that RPL23 binds to a region on Hdm2 that is distinct from that bound by RPL5 and RPL11 (Dai and Lu 2004; Jin et al. 2004). Consistent with our findings, Macias et al. (2010) have recently demonstrated that a mutant of MDM2 that binds RPL23, but not RPL5/RPL11, can still drive p53 degradation in cells treated with agents that cause inhibition of rRNA transcription, including actinomycin D. These findings show that binding of RPL23 to MDM2 is not sufficient to stabilize p53. Nevertheless, given that RPS7 and RPL23 interact with Hdm2 (Dai et al. 2004; Jin et al. 2004; Chen et al. 2007; Zhu et al. 2009), we cannot rule out that they may have a role in mediating Hdm2 function distinct from that of the induction of p53. In this context, it is important to note that RPs are highly basic proteins and the central domain of Hdm2 is very acidic, such that conclusions based on overexpression of RP genes may be misleading. Analyses of point mutations that alter the ability of RPS7 and RPL23 to bind Hdm2 would be useful in resolving these issues.

Our initial observation that disruption of 40S ribosome biogenesis in liver does not alter the rates of 60S ribosome biogenesis was unexpected (Volarevic et al. 2000). This observation has been confirmed in other mammalian systems (Fumagalli et al. 2009; O'Donohue et al. 2010) as well as in yeast (Zemp and Kutay 2007). It is well established that the 40S and 60S ribosome biogenesis are handled by distinct processing pathways (Poll et al. 2009). The unexpected observation that disruption of both pathways in concert led to suprainduction of p53 led us to suspect that the underlying molecular mechanisms, which sense damage to the processing of either ribosomal subunit, were distinct from one another. Although we cannot exclude that the effects observed are confined to unique properties of the combination of RPs that we analyzed, in the case of codepletion of RPS6 and RPL7a, the suprainduction of p53 was found to be dependent on RPL11 (data not shown). However, the requirement for both proteins is less dependent on translation in cases where 60S ribosome biogenesis is impaired as compared with impairment of 40S ribosome biogenesis (Fumagalli et al. 2009). This is evidenced in the latter case by the translational up-regulation of RPL11 mRNA and the higher sensitivity of the p53 response to a general inhibitor of global translation (Fumagalli et al. 2009). These studies clearly show that the mechanisms that respond to each insult do so through RPL5 and RPL11, but do not rely on one another (see the model in Fig. 7D). This is a surprising observation because, insofar as protein synthesis is concerned, the effects of impairing the biogenesis of either ribosomal subunit are equivalent to that of impairing that of both ribosomal subunits. Interestingly, we found that the suprainduction of p53 by concomitant inhibition of 40S and 60S ribosome biogenesis is associated with a selective supra-up-regulation of p53 targets involved in cell cycle arrest but not of those involved in apoptosis. This is consistent with the effects of suprainduction of p53 having an effect on cell cycle (Figs. 7A,B) but not survival (data not shown). It is known that target specificity of p53 is regulated at the level of post-translational modification of p53, such as acetylation and phosphorylation, as well as by its interaction with cofactors (Vousden and Prives 2009). It will be interesting to determine which mechanisms (Vousden and Prives 2009) are responsible for the selective effects of p53 on gene expression in systems of inhibition of ribosome biogenesis. Interestingly, the suprainduction of p53 correlates with a selective accumulation of cells in G2/M, arguing that a threshold of p53 response exists beyond which not only a G1, but also a G2/M, checkpoint is activated. In support of this hypothesis, we observed that incomplete depletion of p53 rescues the G2/M but not the G1 checkpoint in cells codepleted of RPS6 and RPL7a (data not shown).

The fact that p53 induction and cell cycle arrest elicited by impairing the synthesis of either subunit does not extend to inhibiting the synthesis of the other subunit is consistent with both subunits playing an essential role beyond that of protein synthesis. In yeast, it was demonstrated that a defect in the secretory pathway, which is required for cell wall growth, leads to the coordinate transcriptional suppression of ribosome biogenesis (Zhao

et al. 2003). This response is selectively suppressed by impaired 60S but not 40S ribosome biogenesis or global protein synthesis (Zhao et al. 2003). Moreover, suppression of this checkpoint is only observed when interfering with an early and not a late step of 60S ribosome biogenesis, arguing that it is pre-60S ribosomes that are critical, but at an early processing step. Interestingly, the loss of Rrs1, required for the assembly of the RPL5/RPL11 complex into the 60S subunit, also suppresses the secretory checkpoint (Miyoshi et al. 2002). Recent studies in yeast demonstrate that inhibition of 60S ribosome biogenesis, but not 40S ribosome biogenesis or protein synthesis, increases replicative life span, the number of mitotic cycles completed by a mother cell before senescence (Steffen et al. 2008). Thus, it may be that both ribosomal subunits play essential roles that are distinct from one another and that are not related to protein synthesis, which would explain how impairment of either does not suppress the synthesis of the other.

Materials and methods

Cell culture, siRNA transfection, and drug treatments

A549 and U-2 OS cells were cultured in Dulbecco Modified Essential Medium containing 4500 mg/mL L-glucose and 110 mg/L sodium pyruvate supplemented with 10% fetal calf serum and 2 mM L-glutamine. Transfection of siRNAs (Qiagen) was performed by using the calcium phosphate method as described (Fumagalli et al. 2009). For transfection in 6-cm dishes, 20 pmol of each siRNA was used. For each treatment, the total amount of siRNA was adjusted to the same value by the addition of NS siRNA (Qiagen). The target sequences of the siRNAs for human RPS6, RPS7, RPL7a, RPL11, RPL23, and p53 have been published (Fumagalli et al. 2009). The target sequence of the RPL5 siRNA is 5′-ACGCTTGGTGATACAAGATAA-3′. Cells were treated with drugs and processed for analyses 48 h after transfection unless indicated otherwise. Actinomycin D is from Sigma (catalog no. A9415), and cycloheximide is from Calbiochem (catalog no. 239763)

Antibodies

The rabbit anti-p53 antibody and mouse monoclonal anti-p21 antibody used for Western blots have been previously described (Fumagalli et al. 2009). The same rabbit anti-p53 antibody was used for the immunofluorescences at a 1:500 dilution. The anti-β-actin antibody is from Cell Signaling (catalog no. 4967) and was used at a 1:1000 dilution. The mouse monoclonal anti-fibrillarin antibody is from Thermo Scientific (catalog no. MAI-22000) and was used at a 1:500 dilution. Secondary antibodies for both Western blot and immunofluorescence have been used as described (Fumagalli et al. 2009).

Protein extraction and Western blot analysis

Preparation of protein extracts and Western blot analysis have been performed as described (Fumagalli et al. 2009).

Immunofluorescence

Fixation of cells, staining with antibodies, and analysis by confocal microscopy were performed as described (Fumagalli et al. 2009).

Polysome profiling

Preparation of the cellular extracts for polysome profiling and analysis of distribution of L11 mRNA have been performed as described (Fumagalli et al. 2009).

Labeling of cells with ^{3}H-leucine

Cells were incubated with 10 μCi/mL ^{3}H-leucine (Perkin Elmer, catalog no. NET116600) for 30 min. Cells were washed with phosphate-buffered saline (PBS) and then incubated with cold 10% trichloroacetic acid (TCA) for 10 min. TCA-insoluble proteins were then washed twice with 5% TCA, solubilized with 0.1 M NaOH, and analyzed using a liquid scintillation counter (Tri-Carb 2100TR, Perkin Elmer).

Analysis of mRNA expression by qRT–PCR

RNA purification was performed by using the RNAeasy kit from Qiagen (catalog no. 74104). Reverse transcription was performed as previously described (Fumagalli et al. 2009). The resulting cDNAs were used in qRT–PCR reactions containing Fast SYBR Green master mix from Applied Biosystems (catalog no. 4385612). The reactions were analyzed in an Applied Biosystems 7500 Fast Real-Time PCR system. The standard curves were generated for each assay using the PCR-amplified fragments of the targets. Reactions were performed on the following cycle: 20 sec at 95°C, followed by 40 cycles of 3 sec at 95°C, and 30 sec at 60°C. Melt-curve analysis was performed after each run to verify the production of a single product. The primers used were L5 forward primer, 5′-GGTGTGAAGGTTGGCCTGAC-3′; L5 reverse primer, 5′-GGCACCTGGCTGACCATCAA-3′; β-actin forward primer, 5′-AATGTGGCCGAGGACTTTGATTGC-3′; β-actin reverse primer, 5′-AGGATGGCAAGGGACTTCCTGTAA-3′; S7 forward primer, 5′-AGTTCAGTGGGAAGCATGTCGTCT-3′; S7 reverse primer, 5′-AAGACCAAGTCCTCAAGGATGGCA-3′; L23 forward primer, 5′-TCCAGCAGTGGTCATTCGACAA-3′; L23 reverse primer, 5′-TGCTACTGGTCCTGTAATGGCA-3′; PUMA forward primer, 5′-TTGCGATTGGGTGAGACCCAGTAA-3′; PUMA reverse primer, 5′-TTACTTCCTGCCCTGCTCTGGTTT-3′; BAX forward primer, 5′-TCTACTTTGCCAGCAAACTGGTGC-3′; BAX reverse primer, 5′-TGTCCAGCCCATGATGGTTCTGAT-3′; NOXA forward primer, 5′-TCTCAGGAGGTGCACGTTTCATCA-3′; NOXA reverse primer, 5′-ATTCCATCTTCCGTTTCCAAGGGC-3′; p21 forward primer, 5′-AAATCGTCCAGCGACCTTCCTCAT-3′; p21 reverse primer 5′-TCTGACTCCTTGTTCCGCTGCTAA-3′; TP53I3 forward primer, 5′-AAGCGAGGAAGTCTGATCACCAGT-3′; TP53I3 reverse primer, 5′-AGGCAGAATTTGCTCCGTGAAAGC-3′; BTG2 forward primer, 5′-TTCCCAGACCTGCTTCCAGTCTTT-3′; and BTG2 reverse primer, 5′-ACAAGATGCAAGAACACAGCCTGC-3′. The primers for RPS6, RPL7a, and RPL11 have been previously described (Fumagalli et al. 2009).

Flow cytometry

Staining of fixed cells with propidium iodide and flow cytometry analysis were performed as described (Fumagalli et al. 2009).

Acknowledgments

We thank S. Schwemberger, P. Hexley, and G. F. Babcock for carrying out the fluorescence-activated cell-sorting analyses. We are also indebted to G. Doerman for preparing figures. We thank J. Warner and R. Hannan for their critical reading of the manuscript. These studies were supported by grants from

La Ligue Contre le Cancer to S.F., and grants from the Spanish Ministry of Science and Innovation (SAF2011-24967), the Instituto de Salud Carlos III (ISIS) (IIS10/00015/P), and NIH/NIDDK (1RC1-DK087680).

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