

Moonlighting Ribosomal Proteins: The Control of p53 Via the Nucleolar Surveillance Pathway

Elaine Sanij¹, Nadine Hein¹ and Ross D. Hannan^{1,2,3*}

¹Research Division, Peter MacCallum Cancer Centre, St Andrews Place, East Melbourne, VIC 3002

²Department of Biochemistry and Molecular Biology, University of Melbourne, VIC 3010

³Department of Biochemistry and Molecular Biology, Monash University, VIC 3800

*Corresponding author: ross.hannan@petermac.org

Introduction

The nucleolus, which has long been considered simply the site of transcription of the ribosomal genes, is increasingly being seen as an important hub for controlling many cellular functions in addition to ribosome biogenesis. Central to these 'extra-ribosomal functions' is the nucleolar surveillance pathway, which regulates p53 function in response to nucleolar stress. Indeed, it is now apparent that an elaborate sensing mechanism exists to monitor ribosome biogenesis/assembly and nucleolar formation that is coupled to cell survival and senescence through extra-ribosomal functions of ribosomal proteins. In this brief review, we elaborate on this process and describe how ribosome biogenesis through control of the nucleolus lies at the crossroads in the control of diverse functions such as growth, genomic stability, senescence and survival.

Ribosome Biogenesis and Transcription of Ribosomal Genes

Ribosome availability is one of the most fundamental rate-limiting steps for cell growth and proliferation. The assembly of the two ribosomal subunits 40S and 60S

occurs in the nucleolus, a nuclear sub-compartment that specialises in ribosome production. Efficient production of ribosomes requires the coordinated regulation and activity of the three RNA polymerases (Pol). Pol I transcribes the 200 copies of ribosomal RNA genes (rDNA) to produce the 47S precursor of the 18S, 5.8S and 28S ribosomal RNAs (rRNAs), which together with the 5S rRNA form the RNA backbone of the ribosome. This process is considered to be the first step in ribosome biogenesis and accounts for 35-60% of all nuclear transcription in eukaryotes including yeast, plants and animals (1). On the other hand, Pol II is required to transcribe the genes encoding ribosomal proteins (RPs) and other auxiliary proteins necessary for processing, maturation and posttranslational modification of the rRNAs. In addition, Pol III synthesises the 5S rRNA, the tRNAs, as well as a number of small nucleolar RNAs (snoRNAs) involved in the processing and assembly of the ribosome.

The human genome has over 200 copies of the rRNA genes arranged in arrays of tandem repeats. These clusters of rDNA are located on the short arms of the five acrocentric chromosomes known as nucleolar organiser

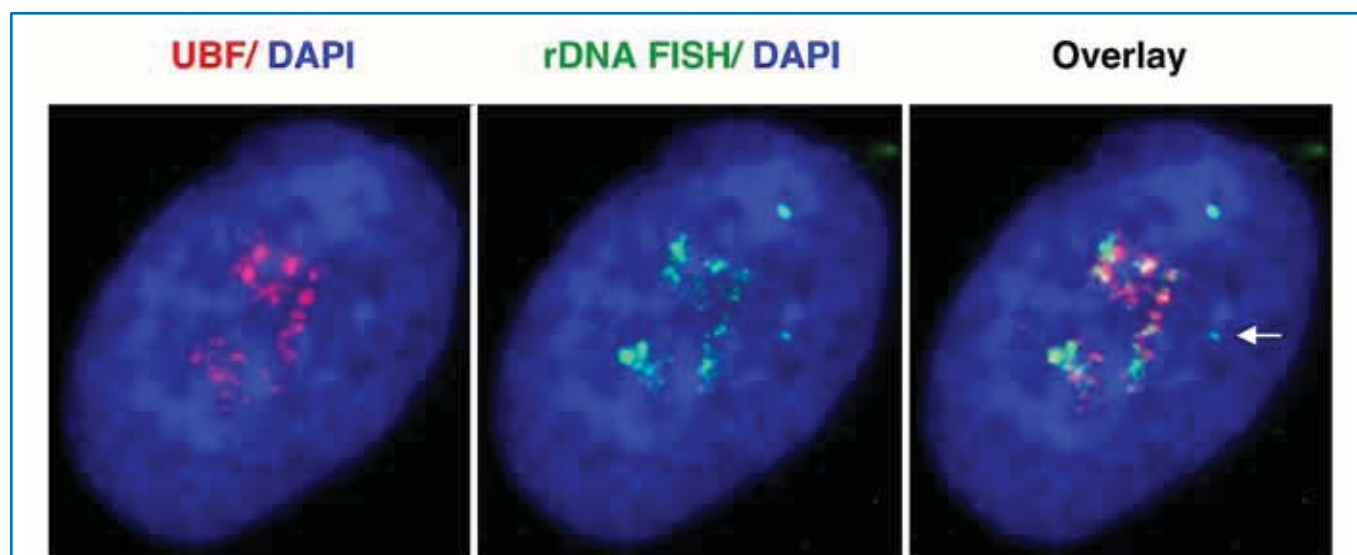


Fig. 1. The nucleolar organiser regions (NORs) are either transcriptionally active or silent.

Combined immunofluorescence of the ribosomal gene specific transcription factor, UBF, and fluorescent *in situ* hybridisation (FISH) analysis of ribosomal genes (rDNA) in interphasic primary human fibroblasts. UBF was visualised with anti-UBF rabbit sera (in-house antibody) and an Alexa-fluor-594-conjugated secondary antibody (red). rDNA was detected using a biotin-labeled probe to the intergenic spacer (kindly provided by Professor B. McStay, National University of Ireland) and visualised using Alexa-fluor-488-conjugated streptavidin (green). Active NORs are associated with UBF, while silent NORs (a representative NOR is indicated by the arrow) are devoid of UBF. Nuclei were visualised by DAPI (blue).

regions (NORs). During late telophase, the nucleoli begin to form around actively transcribed NORs. As the cells progress through the G1 phase of the cell cycle, the nucleoli acquire their mature functional morphology. In prophase, the nucleoli begin to disassemble when rDNA transcription is repressed (2). In metaphase, the NORs are condensed and the nucleoli are completely dissociated; however, mitotic NORs retain a subset of the Pol I transcription machinery including Pol I, the upstream binding factor (UBF) and the selectivity transcription factor (SL1) to allow the rapid resumption of rDNA transcription as cells re-enter the cell cycle (3). However, not all NORs are active. Intriguingly, well over half of the ribosomal genes are epigenetically silenced, are in a closed chromatin configuration and appear not to play a role in gene transcription. NORs containing silent rRNA genes are not associated with Pol I and Pol I-specific factors and appear as condensed foci of rDNA (4) (**Fig. 1**). They remain epigenetically silent throughout the cell cycle and during subsequent cell divisions (4). The reason why cells silence over half the tandem repeats of rRNA genes, although their transcription is rate limiting for growth, has until recently remained a complete mystery. However, a recent landmark paper by Ide *et al.* (5) demonstrated that high transcription rates of ribosomal genes interfere with cohesion between rDNA loci of sister chromatids. Extra copies of silent rRNA genes facilitate condensin association and sister-chromatid cohesion, thereby facilitating recombinational repair. Thus, in effect, high levels of highly transcribed genes are toxic to the cells, and therefore amplified genes, such as rDNA, have evolved.

With respect to the active portion of the rRNA gene repeats, we have recently reported that the nucleolar HMG Box containing transcription/chromatin remodeling factor UBF outcompetes histone H1 for binding to silent rDNA leading to chromatin decondensation (6, 7). Indeed, UBF associates exclusively with active rRNA genes and its binding is proposed to establish and maintain rDNA in a euchromatic open configuration indicative of active NORs (**Fig. 1**).

The Nucleolus as Site for Other Non-ribosomal Functions

In addition to ribosome biogenesis, the nucleolus has far more diverse roles mediated by factors localised to the nucleolus. A landmark mass spectrometry analysis of the nucleoli identified a total of 692 proteins that localise to the human nucleoli. Surprisingly, the fraction of proteins known to be involved in ribosome biogenesis made up only 16% of all purified nucleolar proteins, despite the fact that the existence of the nucleolus depends on ongoing rDNA transcription (8). While one third of all nucleolar proteins have no identifiable function, the remaining fraction includes factors belonging to a wide range of functional categories, including nuclear export, sequestering of oncogenes and tumour suppressors, modifying small RNAs, assembling ribonucleoprotein, controlling many forms of stress response and even aging (1, 9).

Interestingly, analysis of differences in nucleolar proteome dynamics between species shows that

the mammalian nucleoli may have acquired a new evolutionary function in its capability to sequester nucleoplasmic proteins that normally reside outside the nucleoli (9). The most famous of those 'drifters' is the oncoprotein MDM2, the p53-ubiquitin ligase. In response to oncogenic stimuli, the tumor suppressor protein ARF binds to MDM2 and sequesters it in the nucleoli, leading to the nucleoplasmic stabilisation of p53 and the induction of downstream negative regulators of cell cycle progression. However, several lines of evidence suggest that the nucleolus is not a mere sequestration site, but rather a sensitive stress centre that plays an active role in integrating various cellular stress signals through its ability to control the ARF-MDM2-p53 pathway (10, 11). This has been coined the ribosome biogenesis surveillance pathway.

Ribosome Biogenesis Surveillance Pathway and the Control of p53

The existence, size and integrity of the nucleolus appear to be tightly correlated with rDNA transcription and cell proliferation (1). Nucleoli expand when rDNA transcription is induced, and elevated levels of rRNA synthesis and subsequent increases in the number and size of nucleoli are associated with the uncontrolled proliferative state of cancer cells. In fact, morphological changes and increases in the number and the size of nucleoli are considered as a reliable prognostic marker of tumourigenic growth and a definitive measure of tumour pathology (9).

In contrast, inactivation of rDNA transcription, ribosomal protein synthesis, rRNA processing and the assembly and nucleolar export of the 40S and 60S ribosomal subunit (12) are established mechanisms for causing 'nucleolar stress' and nucleolar disruption. This stress in turn leads to cell cycle arrest, programmed cell death and rapid activation of p53 (13). The activation of the RP-p53-MDM2 pathway represents a long-unidentified cross-talk between cell growth and division. In this pathway, the nucleolus functions as a highly sensitive stress sensor mediating signals to a p53 check point, which then controls cell fate. In turn, ribosome biogenesis is inhibited by p53, primarily through impairing the formation of the preinitiation complex, which leads to inhibition of rRNA transcription (14). Under normal growth conditions, the E3 ubiquitin ligase MDM2 regulates p53's availability in two ways: first, MDM2 monoubiquitylates p53 at its C-terminus, thereby targeting it for 26S proteasomal degradation (10); second, MDM2 inhibits the transactivation activity of p53 by abrogating its interaction with the basal Pol II transcription machinery (**Fig. 2**) (12). Remarkably, MDM2 itself is a transcriptional target of p53 (12). Under conditions of 'nucleolar stress', p53 is stabilised and activated through the action of a number of nucleolar proteins, including Bop1, nucleophosmin (also termed NPM or B23), ARF and a subset of ribosomal proteins (10,12,15,16). These factors can disrupt the p53/MDM2 interaction, directly suppress MDM2 E3 ligase activity or increase the translation rate of p53 mRNA, leading to enhanced p53 stability (**Fig. 2**).

Nucleolar Proteins Regulate the 'Nucleolar Stress' Response

Depletion of the Pol I-specific transcription factors UBF and TIF-1A results in dysregulation of rDNA transcription, nucleolar disruption and, subsequently, p53-induced cell cycle arrest and apoptosis/senescence (10,13). Observations made by Pestov and colleagues that overexpression of a dominant negative mutant of Bop1, a factor implicated in pre-rRNA processing and assembly of ribosomal subunits, causes p53-dependent cell cycle arrest strongly suggested a connection between the regulation of ribosome synthesis and cell cycle (15). Another factor involved in the coordination of rRNA transcription/processing, the export of ribosomal subunit and, in particular, the subnuclear localisation of the tumour suppressor ARF is NPM (13). Stress-induced perturbation of NPM/ARF complexes inside the nucleolus enables ARF to regulate the abundance of p53 by sequestering MDM2 (17). Absence or mutation of NPM triggers destabilisation and nucleolar exclusion of ARF 1. In addition, NPM promotes the transactivation activity of p53 under stress conditions (13). Similar to ARF-dependent inhibition of MDM2 activity, a number of ribosomal proteins, including RPS5, RPS7, RPL5, RPL11, RPL23 and RPL26, are capable of interacting with MDM2, leading to p53 stabilisation (12,14,18,19). Under normal growth conditions, the RPs are assembled in the nucleolus and transported to the cytoplasm to form functional ribosomes. Following nucleolar breakdown in response to 'nucleolar stress', RPL5, RPL11 and RPL23 are released to the nucleoplasm where they can interact with the central acidic domain of MDM2, resulting in the suppression of p53 ubiquitination. However, Horn and Vousden observed a synergistic suppression of MDM2 activity through cooperation of RPL11 and RPL5, suggesting that they have distinct roles in inhibiting MDM2 function (11).

In contrast, association of RPS7 and RPL26 with MDM2 has a different regulatory function as they are both reported as substrates for MDM2 ubiquitination. RPL26 is so far unique in its ability to bind the 5' untranslated region of the p53 mRNA and enhance its translation. Its interaction with MDM2 triggers its own ubiquitination and degradation, which in turn causes down-regulation of p53 mRNA translation (19). The diverse roles of RPs in the regulation of the MDM2-p53 pathway are further supported by the finding that knockdown of RPS6 affects 40S ribosomal biogenesis, but enforces RPL11 mRNA translation. This leads to an enhanced interaction between RPL11 and MDM2, leading to the accumulation and activation of p53 (18). Since multiple RPs have separate mechanisms for activating p53, it is plausible they may have distinct roles in sensing different types of signals leading to nucleolar stress.

Conclusions

The ribosome biogenesis surveillance pathway is not just of academic interest but has important potential for the treatment of cancer. It is becoming apparent that the RP-MDM2-p53 pathway is robustly activated by inhibition of ongoing precursor rRNA synthesis through a number of chemotherapeutic agents. For instance, inhibition of rDNA transcription with the chemotherapeutic agents Actinomycin D (ActD) or 5-fluorouracil induces p53 stabilisation and cell cycle arrest (13,20). At low concentrations (<10 µg/ml), 5-fluorouracil treatment drives p53 activation by promoting the interaction of MDM2 with RPL5, RPL11 and RPL23. Similar to low doses of 5-fluorouracil, treatment with mycophenolic acid enhances the association of RPL11 and RPL5 with MDM2, leading to p53 stabilisation (12). These studies strongly suggest that the therapeutic activity of many cytotoxic agents currently used for the treatment of cancer may function, at least in

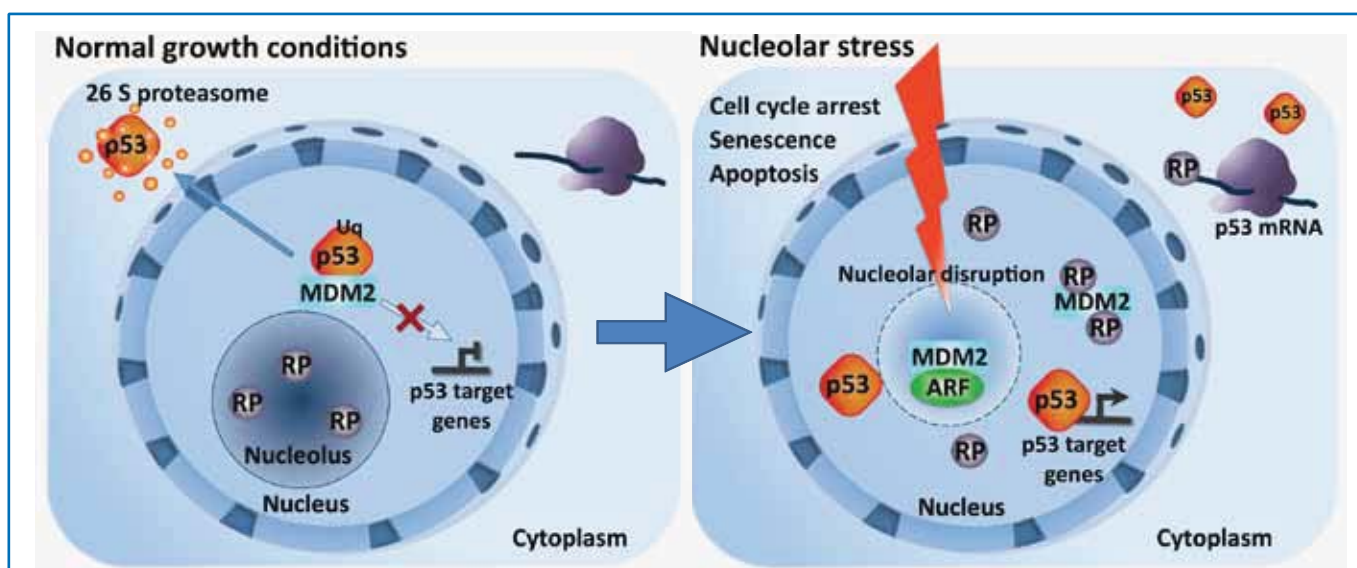


Fig. 2. Schematic presentation of the 'nucleolar stress' response.

Under normal growth conditions, the ribosomal proteins (RPs) are assembled in the nucleolus and transported to the cytoplasm to form functional ribosomes. MDM2 binds and monoubiquitylates p53 in the nucleoplasm, which promotes p53 proteasomal degradation (left panel). Under nucleolar stress conditions, RPs are released to the nucleoplasm where they can interact with MDM2 resulting in the suppression of p53 ubiquitination. RPL26 is also capable of binding p53 mRNA and enhances its translation. The activation of p53 induces cell cycle arrest, apoptosis and/or senescence.

part, through the induction of p53-dependent apoptosis/senescence following nucleolar stress. Indeed, the potential therapeutic benefit of selectively inhibiting a target such as ribosome biogenesis has prompted the development of small molecule components that selectively inhibit rRNA synthesis in cancer cells. Two such drugs, developed by Cylene Pharmaceuticals, are potent and specific inhibitors of Pol I transcription that induce apoptosis and autophagy, exhibit potent and selective antiproliferative activity *in vitro* and have antitumour activity in xenograft models (13). Together, these studies place dysregulation of ribosomal gene transcription, a much-maligned 'housekeeping' process, as a critical novel target in the treatment of malignancy.

References

1. Moss, T., and Stefanovsky, V.Y. (2002) *Cell* **109**, 545-548
2. Leung, A.K., Gerlich, D., Miller, G., Lyon, C., Lam, Y.W., Lleres, D., Daigle, N., Zomerdijk, J., Ellenberg, J., and Lamond, A.I. (2004) *J. Cell Biol.* **166**, 787-800
3. Prieto, J.L., and McStay, B. (2007) *Genes Dev.* **21**, 2041-2054
4. McStay, B., and Grummt, I. (2008) *Annu. Rev. Cell Dev. Biol.* **24**, 131-157
5. Ide, S., Miyazaki, T., Maki, H., and Kobayashi, T. (2010) *Science* **327**, 693-696
6. Sanij, E., Poortinga, G., Sharkey, K., Hung, S., Holloway, T.P., Quin, J., Robb, E., Wong, L.H., Thomas, W.G., Stefanovsky, V., Moss, T., Rothblum, L., Hannan, K.M., McArthur, G.A., Pearson, R.B., and Hannan, R.D. (2008) *J. Cell Biol.* **183**, 1259-1274
7. Sanij, E., and Hannan, R.D. (2009) *Epigenetics* **4**, 374-382
8. Leung, A.K., Andersen, J.S., Mann, M., and Lamond, A.I. (2003) *Biochem. J.* **376**, 553-569
9. Maggi, L.B., Jr., and Weber, J.D. (2005) *Cancer Invest.* **23**, 599-608
10. Rubbi, C.P., and Milner, J. (2003) *EMBO J.* **22**, 6068-6077
11. Horn, H.F., and Vousden, K.H. (2008) *Oncogene* **27**, 5774-5784
12. Zhang, Y., and Lu, H. (2009) *Cancer Cell* **16**, 369-377
13. Drygin, D., Siddiqui-Jain, A., O'Brien, S., Schwaebe, M., Lin, A., Bliesath, J., Ho, C.B., Proffitt, C., Trent, K., Whitten, J.P., Lim, J.K., Von Hoff, D., Anderes, K., and Rice, W.G. (2009) *Cancer Res.* **69**, 7653-7661
14. Deisenroth, C., and Zhang, Y. (2010) *Oncogene* **29**, 4253-4260
15. Pestov, D.G., Strezoska, Z., and Lau, L.F. (2001) *Mol. Cell. Biol.* **21**, 4246-4255
16. Sherr, C.J., and Weber, J.D. (2000) *Curr. Opin. Genet. Dev.* **10**, 94-99
17. Korgaonkar, C., Hagen, J., Tompkins, V., Frazier, A.A., Allamargot, C., Quelle, F.W., and Quelle, D.E. (2005) *Mol. Cell. Biol.* **25**, 1258-1271
18. Fumagalli, S., Di Cara, A., Neb-Gulati, A., Natt, F., Schwemberger, S., Hall, J., Babcock, G.F., Bernardi, R., Pandolfi, P.P., and Thomas, G. (2009) *Nat. Cell Biol.* **11**, 501-508
19. Ofir-Rosenfeld, Y., Boggs, K., Michael, D., Kastan, M.B., and Oren, M. (2008) *Mol. Cell* **32**, 180-189
20. Sun, X.X., Dai, M.S., and Lu, H. (2007) *J. Biol. Chem.* **282**, 8052-8059

