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Nucleolar stress as an aging hallmark: the role of NOL12 and FoxM1.

Marta Andreia Ribeiro de Pinho

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Marta Andreia Ribeiro de Pinho

SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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Marta Andreia Ribeiro de Pinho

NUCLEOLAR STRESS AS AN AGING HALLMARK: THE ROLE OF NOL12 AND FOXM1

Tese de Candidatura ao grau de Doutor em
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Instituição de Acolhimento – Instituto de Investigação
e Inovação em Saúde

Orientador – Doutor Paulo S. Pereira
Categoria – Investigador Principal
Afiliação – Instituto de Investigação
e Inovação em Saúde

Co-orientador – Doutora Elsa Logarinho
Categoria – Investigador Principal
Afiliação – Instituto de Investigação
e Inovação em Saúde

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Pinho, M., Logarinho, E., Pereira, P.S., 2020. Forkhead box M1 transcriptional activity regulates nucleolar homeostasis (*in preparation*).

Dado que os artigos foram feitos em colaboração com outros autores, a candidata clarifica que, em todos eles, participou ativamente no desenho, trabalho experimental, obtenção, análise e discussão dos dados, preparação e publicação dos artigos.

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This thesis contains results from the following original publications:

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Neste barco, eu sei lá,

Quem me leva, quem me traz

Mas eu sei que quando a tempestade chegar

Ao meu mar

Eu sozinho não ficarei

Pois sozinho não embarquei (…)

Esta letra é parte de uma música de cariz cristão escrita e composta pelas minhas duas manas e reflecte bem a minha filosofia de vida! E é também, aliás, o reflexo do que foram estes 4 anos de Doutoramento e destes 6 anos desde que a aventura com o NOL12 começou! Olhando em retrospectiva, foram anos de muita aprendizagem, quer profissional quer pessoal, marcados por marés mais calmas e mais agitadas, por tempos de bonança e por tempestades, …! Graças a Deus, foram muitas as pessoas que contribuíram e enriqueceram esta viagem: aquelas que já iam comigo no barco, aquelas que foram embarcando e aquelas que partiram para uma outra viagem… É a elas que dedico esta minha Tese. Guardo-vos a todos no meu coração!

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ABSTRACT

The nucleolus is a subnuclear compartment that has been increasingly investigated. Since its original identification as the cell's ribosome factory, many other functions were attributed to the nucleolus. Some of these functions were implicated in the establishment and development of different human diseases, from cancer to neurodegeneration. Importantly, the disruption of nucleolar homeostasis, either by defective morphology or impaired nucleolar function, has been implicated in cellular senescence and aging. Here, we further explored how nucleolar stress is linked to aging.

First, we found that the nucleolar protein NOL12 is required for maintenance of nucleolar homeostasis. Particularly, we demonstrated that its downregulation causes increased expression of key nucleolar proteins such as FBL and NCL, impacting nucleolar ultrastructure, organization and function, as well as global protein synthesis. Under this nucleolar stress, we showed that the ribosomal protein RPL11 is needed for P53 activation, which we found to mediate decreased cell proliferation, cell cycle arrest at G2 phase and consequent senescence accrual. Finally, we found NOL12 repression in fibroblasts from elderly donors, with nucleoli recapitulating the phenotypes observed in NOL12-depleted cells. This revealed NOL12 repression as a biomarker in human chronological aging.

Secondly, we further explored the link between nucleolar stress and aging. We investigated the role of FoxM1 in nucleolar homeostasis, a transcription factor whose activity was reported as critical in aging. Similarly to NOL12 repression, FoxM1 repression in human primary fibroblasts elicited significant morphological alterations in the nucleolus. Interestingly, we also found FoxM1 as being required for proper nucleolar function, although in a distinct fashion when compared with NOL12. Additionally, FoxM1 repression induced the upregulation of several nucleolar proteins, including FBL that was required for NCL nucleolar recruitment. Nucleolar stress induced by FoxM1 repression also activates P53, mediated by RPL11. Importantly, we found FBL function to be an additional requirement for P53 activation. Similarly to NOL12-depleted human primary fibroblasts, we found that FoxM1 downregulation decreases the proliferative capacity of cells and promotes the accumulation of senescent cells. However, P53 activation was not a major contributor for decreased cell proliferation. Interestingly, although FoxM1 seems to regulate ribosome biogenesis distinctly from NOL12, FoxM1 repression also results in a decreased global protein synthesis, which likely supports the observed decreased cell proliferation. As putative direct transcriptional nucleolar targets of FoxM1 linking nucleolar homeostasis and cell proliferation, we found PIM1 and PARP1. Altogether, our findings strongly support nucleolar stress as an aging hallmark.

RESUMO

O nucléolo é um compartimento subnuclear que cada vez mais tem sido investigado. Desde a sua identificação original como a fábrica de ribossomas da célula, muitas outras funções foram-lhe sendo atribuídas. Algumas destas funções foram implicadas no estabelecimento e desenvolvimento de diferentes doenças humanas, desde o cancro à neurodegeneração. De modo particularmente importante, a disrupção da homeostase nucleolar, quer pela alteração da morfologia, quer pela desregulação da função nucleolar, foi implicada na senescência celular e no envelhecimento. Aqui, nós explorámos a forma como o stress nucleolar se relaciona com o envelhecimento.

Em primeiro lugar, descobrimos que a proteína nucleolar NOL12 é necessária para a manutenção da homeostase nucleolar. Em particular, demonstrámos que a sua diminuição provoca o aumento da expressão de proteínas nucleolares chave como a FBL e a NCL, afectando a ultraestrutura, organização e função nucleolares, assim como a síntese proteica global. Mostrámos que, sob este stress nucleolar, a proteína ribossomal RPL11 é necessária para a activação de P53, que por sua vez medeia a diminuição da proliferação celular, a paragem das células na fase G2 do ciclo celular e a consequente acumulação de células senescentes. Finalmente, descobrimos que NOL12 está reduzido em fibroblastos de dadores envelhecidos, cujos nucléolos reproduzem os fenótipos observados nas células em que depletámos NOL12. Isto revelou que a diminuição de NOL12 é um biomarcador do envelhecimento cronológico humano.

Em segundo lugar, explorámos a ligação entre stress nucleolar e envelhecimento. Investigámos o papel de FoxM1 na homeostase nucleolar, um factor de transcrição cuja actividade foi reportada como central no envelhecimento. De forma similar à depleção de NOL12, a depleção de FoxM1 em fibroblastos primários humanos induziu alterações significativas na morfologia do nucléolo. De forma relevante, descobrimos também que FoxM1 é necessária para a correcta função nucleolar, embora de forma diferente quando comparada com NOL12. Adicionalmente, a depleção de FoxM1 induziu o aumento de várias proteínas nucleolares, incluindo FBL, que mostrou ser requisito para o recrutamento nucleolar de NCL. O stress nucleolar induzido pela depleção de FoxM1 também activa P53 mediada por RPL11. Descobrimos ainda que a função de FBL é um requisito adicional para a activação de P53. De forma similar aos fibroblastos primários humanos depletados em NOL12, mostrámos que a diminuição de FoxM1 reduz a capacidade proliferativa das células e promove a acumulação de células senescentes. Contudo, a activação de P53 não foi o maior potenciador da diminuição da proliferação celular. Embora FoxM1 pareça regular a biogénese ribossomal de forma distinta de NOL12, a depleção de FoxM1 também

resulta na diminuição da síntese proteica global que, provavelmente, suporta a redução da proliferação celular observada, o que foi uma descoberta muito interessante. Como alvos transcricionais nucleolares de FoxM1, que relacionam a homeostase nucleolar e a proliferação celular, encontrámos PIM1 e PARP1. De forma sólida, todas estas descobertas suportam o stress nucleolar enquanto marca do envelhecimento.

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ABBREVIATIONS

µl	**microlitre**
µM / mM / M	micromolar / milimolar / molar
ºC	degree Celsius
DNA	Deoxyribonucleic acid
FBL	Fibrillarin
mRNA	Messenger RNA
NCL	Nucleolin
NPM	Nucleophosmin
NS	Nucleostemin
Pre-rRNA	Precursor ribossomal RNA
qPCR	Real-time Polymerase chain reaction
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
RNA Pol I / II / III	RNA Polymerase I / II / III
RNAi	RNA interference
RNP	Ribonucleoprotein
rRNA	Ribosomal RNA
snoRNA	Small nucleolar RNA

CHAPTER 1

General Introduction

In this first chapter, I invite you to travel in the amazing nucleolar world! The central goal is to know the structure and the multiple functions of the nucleolus and then to understand the numerous potential roles of this subnuclear membraneless organelle in the context of different human diseases.

1. THE NUCLEOLUS: FROM STRUCTURE TO FUNCTION

1.1 The nucleolus structure

A typical eukaryotic cell is composed of a single nucleus surrounded by cytoplasm (Gorlich and Kutay 1999). A selectively permeable membrane with pore complexes, the nuclear envelope, separates the nucleus from the cytoplasm (Figure 1.1A). Importantly, the nucleus is considered a central hub for gene expression and regulation. Despite not containing any membrane-enclosed sub-compartment, the nucleus is composed of several subnuclear aggregates, containing RNA and proteins (Feric, Vaidya et al. 2016). These aggregates include the Cajal bodies, the promyelocytic leukemia (PML) bodies and the nucleolus, among others (Spector 2001). Alterations on their composition or organization result in severe diseases, as Huntington's (Savas, Makusky et al. 2008) or spinal muscular dystrophy (Gall 2000). Although the nucleolus has been the first subnuclear structure identified and formally described by 1830's (Pederson 2011), its dynamics, behaviour and biological relevance began to be highlighted only in recent years. It is now clear that the nucleolus and other subnuclear bodies behave like liquid droplets, which explains how they remain separated from each other and how they fuse, a centenary scientific observation (Brangwynne, Mitchison et al. 2011) (Figure 1.1B). Particularly, a recent study used a combination of nuclear and nucleolar fluorescently labels and computational mathematics to further confirm the liquid-like nature of the nucleolus and more importantly to deeply explore the dynamics of the nucleolar fusion (Caragine, Haley et al. 2018).

The human nucleolus is assembled around the nucleolar organizer regions (NORs), which are genomic regions in acrocentric chromosomes (13, 14, 15, 21 and 22) (McStay 2016) composed of clusters of ribosomal DNA (rDNA) repeats (Kalmarova, Smirnov et al. 2007) (Figure 1.2). Consequently, the number of nucleoli cannot exceed ten per each diploid cell (Farley, Surovtseva et al. 2015). Interestingly, the average number of nucleoli per nucleus varies among mammalian cells, as well as between different human cell types (Farley, Surovtseva et al. 2015). Also, there is significant variation on this number even inside the same cell population, which suggests the number of nucleoli per nucleus to be cell- and tissue-dependent (Farley, Surovtseva et al. 2015). Despite these observations, the mechanisms that determine the number of nucleoli remain elusive (Farley, Surovtseva et al. 2015).

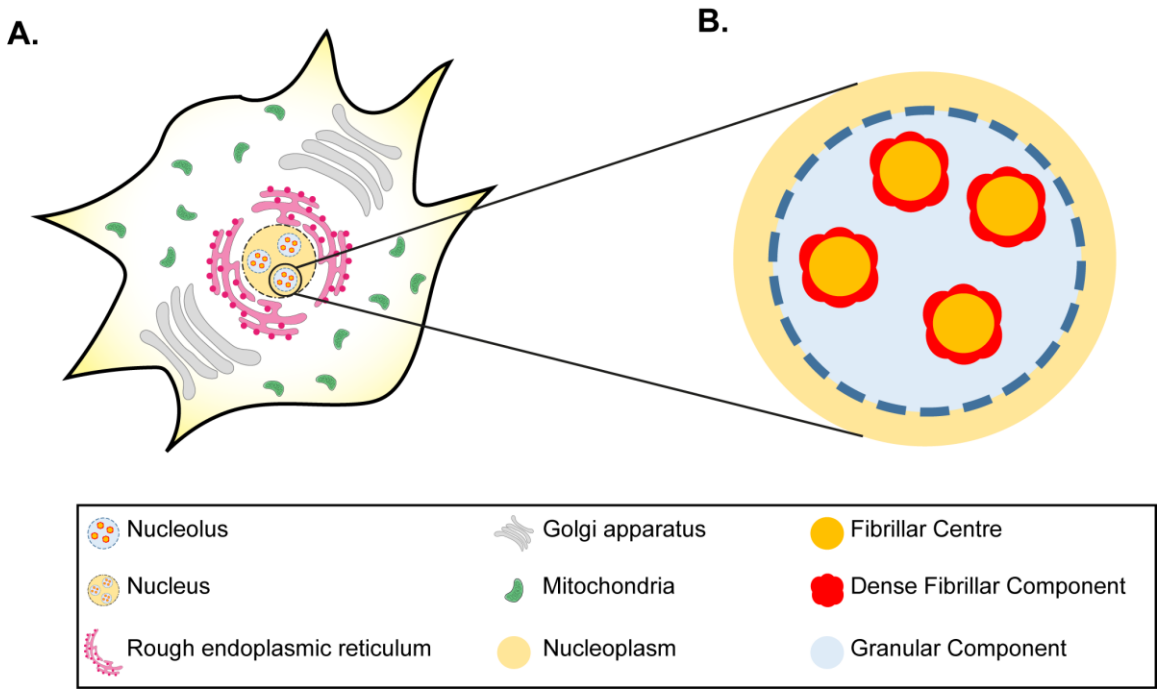

Figure 1.1. The nucleolus is a subnuclear compartment. (A) Schematic and simplified illustration of a typical mammalian cell showing cytoplasm containing mitochondria, rough endoplasmic reticulum, Golgi apparatus and nucleus containing nucleoli embedded in nucleoplasm (light yellow). **(B)** Schematic illustration of a single nucleolus comprising fibrillar centre (FC, yellow))/dense fibrillar component (DFC, red) units embedded in the granular component (GC, light blue).

By transmission electron microscopy, three morphologically distinct sub-compartments inside the nucleolus were identified: the fibrillar centre (FC), surrounded by the dense fibrillar component (DFC), all embedded within the peripheral granular component (GC) (Figure 1.1B). Interestingly, while human and plant cells display this tripartite organization, yeast cells exhibit a bipartite configuration (Shaw and Doonan 2005), and the *Drosophila melanogaster* nucleolus has a homogeneous appearance (Knibiehler, Mirre et al. 1982, Orihara-Ono, Suzuki et al. 2005). Nevertheless, in human cells, the tripartite organization of the nucleolus follows the dynamics of ribosome biogenesis, the originally described and major function of the nucleolus.

1.2 Ribosome biogenesis

During the second half of the 20th century, the nucleolus was extensively studied and described as the “ribosome factory” within the cell (Brown and Gurdon 1964). The rDNA is organized into transcriptional units separated by intergenic sequences (IGS) and repeated several hundred times, although their sequences display some differences (Raska, Shaw et al. 2006, Tseng, Chou et al. 2008). In somatic cells, only about half of these copies are transcribed (Grummt 2007) (Figure 1.2). How and why the cell decides

that a copy is inactivated is still unknown, but it likely involves different epigenetic mechanisms, from DNA methylation to noncoding RNAi or late replication timing (Schlesinger, Selig et al. 2009). Schlesinger et al (Schlesinger, Selig et al. 2009) suggest that during development one allele is randomly chosen to undergo late replication, meaning that one allele will be replicated in S phase earlier than the other one (Singh, Ebrahimi et al. 2003), generating the "replication timing pattern" (Ensminger and Chess 2004). Once established, this pattern is maintained in somatic cells and it may function as an epigenetic template, determining which allele will be silenced (Schlesinger, Selig et al. 2009).

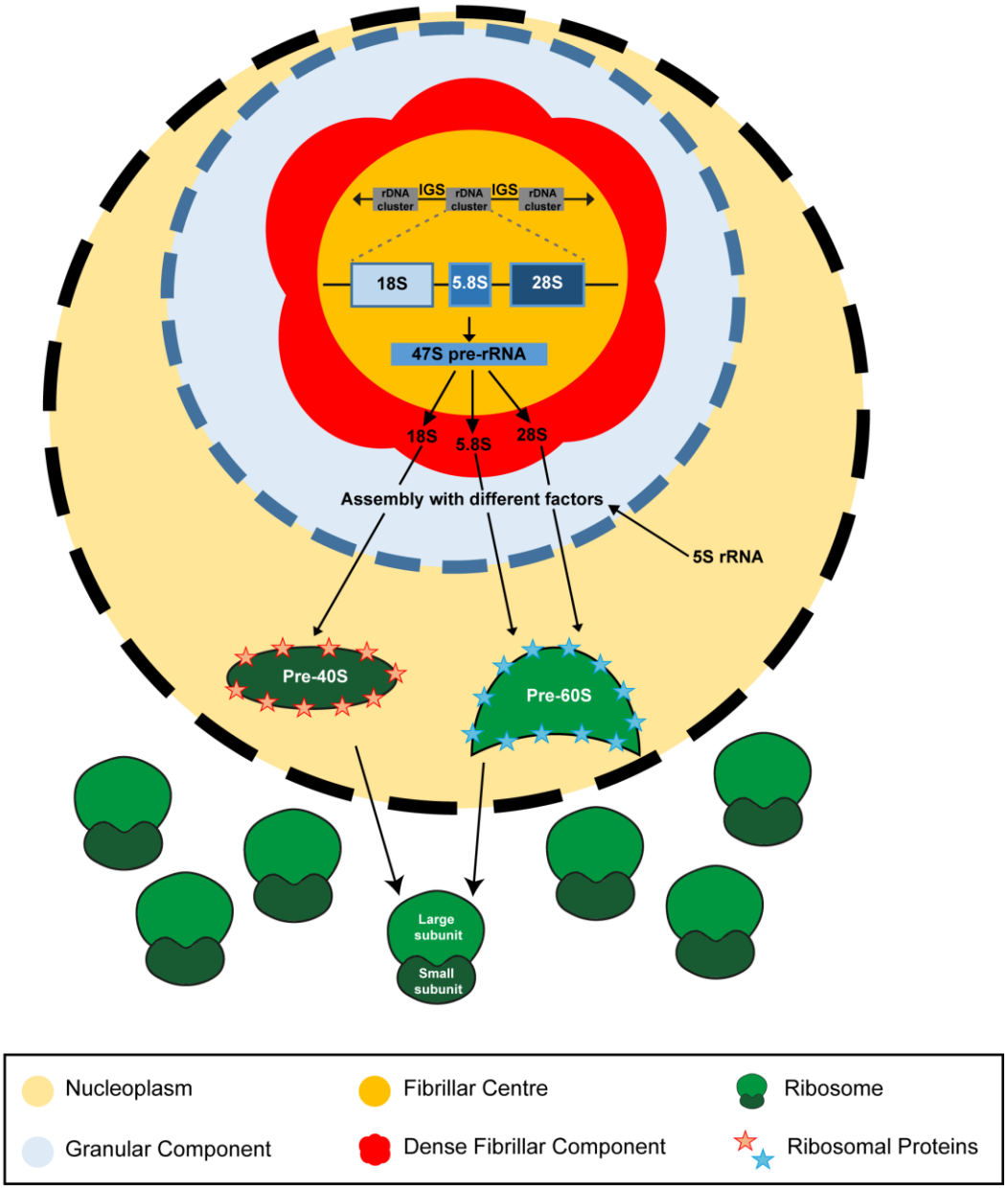

Figure 1.2. Ribosome biogenesis is a sequential process which follows the tripartite organization of the nucleolus. The ribosomal DNA (rDNA) is organized into multiple clusters (rDNA cluster, gray) separated by the intergenic sequence (IGS). Each rDNA cluster is a unit containing sequences that code for 18S, 5.8S and 28S rRNAs. Ribosome biogenesis begins with the transcription of each cluster into a primary transcript – the 47S

pre-rRNA – at the FC (yellow)/DFC (red) border. The rRNA processing and assembly with different factors such as 5S rRNA, ribosomal proteins, pre-ribosomal factors and small nucleolar ribonucleoprotein particles occur at the DFC and end at the GC (light blue). Those complexes are then transported to nucleoplasm (light yellow), where 18S rRNA assembles with small subunit ribosomal proteins (red stars) to generate the small pre-40S subunit (dark green), whereas 5.8S, 28S and 5S rRNAs assemble with large subunit ribosomal proteins (blue stars) to form the large pre-60S subunit (light green). Both subunits are then exported to the cytoplasm, where they maturate into functional ribosomes.

In eukaryotes, ribosome biogenesis begins with the transcription of an entire rDNA repeat into a single polycistronic transcript, the 47S pre-rRNA, by RNA Polymerase I (RNA Pol I) (Boisvert, van Koningsbruggen et al. 2007) (Figure 1.2). The initiation of transcription also requires the recruitment and binding of other transcription factors at the rDNA promoter, which comprises a core element and an upstream control element (UCE), ensuring precise transcription initiation (Russell and Zomerdijk 2005). Among those transcription factors, the human selectivity factor 1 (SL1) and the upstream binding factor (UBF) are the most relevant in assuring the recruitment of RNA Pol I to the rDNA promoter (Russell and Zomerdijk 2005) and the correct architecture of the entire complex that drives the synthesis of 47S pre-rRNA (Bazett-Jones, Leblanc et al. 1994, O'Sullivan, Sullivan et al. 2002), respectively.

The 47S pre-rRNA transcript comprises 5’ and 3’ external transcribed spacers (ETS), two internal transcribed spacers (ITS1 and ITS2) and the sequences for 18S, 5.8S and 28S (Lafontaine 2015) (Figure 1.3). Through a set of multiple endo- and exonucleolytic cleavages at the level of both ETS and ITS, methylation in sugar residues (2’-O-ribose methylations) and conversion of uridine residues to pseudouridines (pseudouridylations) (Thomson, Ferreira-Cerca et al. 2013), the ETS and ITS spacers are gradually removed, originating intermediate rRNAs which are chemically modified, ultimately giving rise to the 18S, 5.8S and 28S rRNAs (Figure 1.3). There are several nucleases involved in 47S pre-rRNA processing: the endonuclease NOB1 is required for the last step of 18S maturation (Pertschy, Schneider et al. 2009); XRN2 is a 5’-3’ exonuclease required for removal of 5’-ETS fragments and ITS1 and ITS2 sequences (Sloan, Mattijssen et al. 2013); NOL12 is a 5’-3’ exonuclease required for ITS1 processing (Sloan, Mattijssen et al. 2013); among many others. Interestingly, these cleavages do not entirely follow a hierarchical sequence of events (Aubert, O'Donohue et al. 2018): there is a major pathway for rRNA processing that is paralleled by alternative processing pathways, evidencing the high complexity of this process in human cells (Henras, Plisson-Chastang et al. 2015) (Figure 1.3). Concurrently, two different families of small nucleolar ribonucleoproteins (snoRNPs), the H/ACA and C/D boxes, catalyse pseudouridylation and 2’-O-methylation post-transcriptional modifications,

respectively (Sloan, Warda et al. 2017). Both are composed of an enzyme, the pseudouridyl synthase dyskerin and the methyltransferase Fibrillarin (FBL), respectively, assisted by other proteins, NHP2, NOP10, GAR1 and NOP56, NOP58, 15.5K/NHPX, and a small nucleolar RNA sequence (snoRNA), which binds pre-rRNA, guiding and allowing enzymes to catalyse the reaction (Aubert, O'Donohue et al. 2018). FBL is a highly conserved protein (Newton, Petfalski et al. 2003), which is the unique known methyltransferase in eukaryotes, in the context of rRNA processing (Marcel, Ghayad et al. 2013).

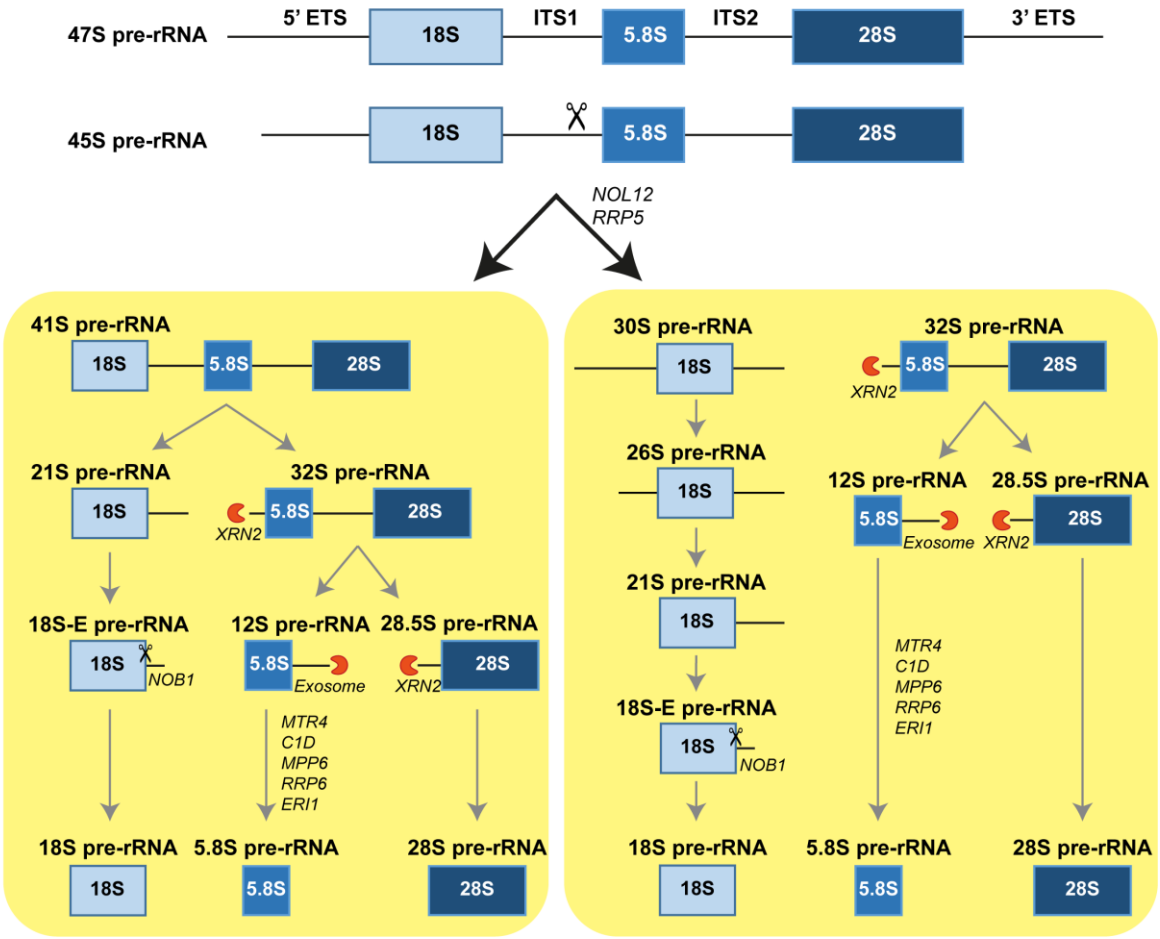

Figure 1.3. rRNA processing is a complex cascade of cleavage events. Schematic representation of rRNA processing, showing the multiple cleavages that occur in the nascent and intermediate rRNA transcripts at the level of ETS and ITS sequences. The two alternative processing pathways are shown in different yellow boxes. Several exo- (red pacmans) and endonucleases (scissors) ensure the proper production of 18S, 5.8S and 28S rRNAs by removing both ETS and ITS.

Besides the three different rRNA species, proper ribosome biogenesis requires another rRNA: the 5S rRNA. 5S rDNA is organized as clusters of tandem repeats (Torres-Machorro, Hernandez et al. 2010) and is located in a different chromosomal locus, on chromosome 1 (Sorensen and Frederiksen 1991, Ciganda and Williams 2011). However,

the 5S rDNA transcription is driven independently by RNA Polymerase III and its processing, despite involving several exonucleases (van Hoof, Lennertz et al. 2000) and binding of different partners (Zhang, Harnpicharnchai et al. 2007, Ciganda and Williams 2011), rarely includes base modification (Ciganda and Williams 2011). Whereas transcription and processing of 47S pre-rRNA occurs at the FC/DFC boundary, and DFC and GC (Thiry and Lafontaine 2005, Hernandez-Verdun 2006, Hernandez-Verdun 2006), respectively, 5S rDNA transcription occurs in the nucleoplasm and the 5S rRNA is then imported into the nucleolus (Ciganda and Williams 2011) (Figure 1.2).

While rRNA is processed and modified, the assembly of ribosomal proteins, pre-ribosomal factors and snoRNPs takes place at the GC (Henras, Plisson-Chastang et al. 2015). Particularly, 5.8S, 28S and 5S rRNA, and 18S assemble with approximately 80 ribosomal proteins, which were transcribed by RNA Pol II and imported from cytoplasm into the nucleolus, originating the pre-60S and pre-40S ribosome subunits (Venema and Tollervey 1999) (Figure 1.2). Depending on whether they will be part of the large or the small ribosomal subunit (LSU or SSU), ribosomal proteins are divided into two different groups, RPLs or RPSs, respectively. After assembly with ribosomal proteins, both ribosomal subunits are then exported to cytoplasm, through nuclear pore complexes (Lafontaine and Tollervey 2001), where they undergo key steps of maturation (Thomson, Ferreira-Cerca et al. 2013). After that, both large and small ribosomal subunits (60S LSU and 40S SSU, respectively) are finally ready to translate messenger RNAs (mRNAs) in the cytoplasm (Thomson, Ferreira-Cerca et al. 2013) (Figure 1.2).

In order to ensure that ribosomes are functional, there is a quality control system of rRNA, in which rRNA is polyadenylated when it is unfolded, incorrectly processed or excluded from functional ribosomes (Slomovic, Laufer et al. 2006). This post-transcriptional modification will then lead to the rRNA degradation by exonucleolytic digestion (Slomovic, Fremder et al. 2010). This is interesting since previously it had been shown that: i) in general, polyadenylation is a stable post-transcriptional modification that confers mRNA stability and allows initiation of the mRNA translation (Colgan and Manley 1997); ii) in yeast, 5S rRNA polyadenylation is a mark for degradation (Kuai, Fang et al. 2004); iii) in prokaryotes and organelles, polyadenylation of transcripts is transient and leads to their degradation (Kushner 2004, Slomovic, Laufer et al. 2005, Slomovic, Portnoy et al. 2006). This transient polyadenylation works as a surveillance system, not only in nucleus but also in cytoplasm (Slomovic, Fremder et al. 2010).

Once produced, functional ribosomes translate mRNAs into proteins through a process comprising three different steps: initiation, elongation and termination (Cooper 2000). The two different ribosomal subunits play a distinct role during translation: the SSU ensures the proper base-pairing between the codon (registered in mRNA) and anticodon

(carried by tRNA), whereas the LSU monitors the addition of aminoacids into the newly synthetized polypeptide chain (Cooper 2000). Interestingly, mRNA translation has been shown to be modulated by different ribosome composition, which could result from modifications in rRNA chemical patterning, variations in the RP's expression and post-translational modifications and deregulated activity of ribosome-associated factors (Xue and Barna 2012, Sulima, Hofman et al. 2017, Bastide and David 2018). The emerged concept of ribosome heterogeneity in the cell means that ribosome composition differs between different tissues, and even within an individual cell it is modified along time or in response to different conditions (Gupta and Warner 2014, Mills and Green 2017, Shi, Fujii et al. 2017). Indeed, these different ribosome subpopulations have been shown to be necessary to, or to promote, the translation of specific mRNAs (Mills and Green 2017). Several interesting examples could be found in literature: different human tissues display a highly variable expression of different ribosomal protein genes and from a set of 89 RPs, 13 were found to be tissue-specific (Bortoluzzi, d'Alessi et al. 2001); particularly, RPS4Y2 was found to be specifically expressed in testis and prostrate, suggesting that there are ribosomes specific of these tissues (Lopes, Miguel et al. 2010); in mice, RPL38 expression was found to be required for translation of the development-associated HOX mRNAs, leading to the proper establishment of the body plan (Kondrashov, Pusic et al. 2011). Another example came from the isolated congenital asplenia, a developmental defect characterized by the specific absence of a spleen in humans (Mahlaoui, Minard-Colin et al. 2011). Importantly, mutations in the RPSA, a ribosomal protein involved in rRNA processing (O'Donohue, Choesmel et al. 2010) and a component of the SSU (Ben-Shem, Garreau de Loubresse et al. 2011), were found in these patients hence suggesting that it may interfere with the translation of specific mRNAs contributing for the pathophysiology of this disease (Bolze, Mahlaoui et al. 2013). Furthermore, the downregulation of the ribosome-associated protein GYS1 showed to affect the translation of specific mRNA subsets in HeLa cells (Fuchs, Diges et al. 2011). Finally, the abolishment of the rRNA pseudouridylation by DKC1 mutation or repression decreases the translational efficiency (Jack, Bellodi et al. 2011).

1.3 Nucleolar proteins

Besides being the hub for ribosome biogenesis, the nucleolus has been increasingly reported to play central roles in other important cell events such as cell differentiation, cell cycle, senescence and apoptosis (Olson, Dundr et al. 2000, Boisvert, van Koningsbruggen et al. 2007, Rosete, Padros et al. 2007, Watanabe-Susaki, Takada et al. 2014). Supporting this, there are two main findings: i) the nucleolar proteome in human cells revealed the presence of an extended set of proteins with different functions, from ribosomal proteins to

kinases/phosphatases and chromatin-related factors (Andersen, Lam et al. 2005); ii) multiple nucleolar proteins are able to shuttle between the nucleolus and other cell compartments, such as the nucleoplasm or cytoplasm (Borer, Lehner et al. 1989). This dynamic nature of the nucleolar proteome strongly suggests that the nucleolus is able to respond to different cellular growth conditions (Andersen, Lam et al. 2005). Currently, the function of several nucleolar proteins is not completely understood, as they interact with each other and are often multifunctional proteins, being involved and crosstalking with different cell processes (Pederson and Tsai 2009).

Altogether, this firstly means that the mere localization of a protein in the nucleolus does not necessarily imply a role in ribosome biogenesis. An example is **nucleostemin** (**NS**), a nucleolar protein encoded by the GNL3 gene, whose depletion was initially shown to perturb ribosome biogenesis, particularly pre-rRNA processing (Romanova, Grand et al. 2009). However, it was recently proposed that this could be a secondary effect of cell cycle arrest rather than a direct effect of NS in ribosome biogenesis (Tsai 2014). Interestingly, NS is involved in DNA repair (Scott and Oeffinger 2016) by recruiting RAD51 to DNA damage foci, therefore ensuring genomic stability in stem cells (Meng, Lin et al. 2013, Yamashita, Nitta et al. 2013).

Secondly, data strongly support that nucleolar proteins involved in ribosome biogenesis actually play other roles. One example is **nucleolin** (**NCL**), an abundant phosphoprotein encoded by the NCL gene (Srivastava, McBride et al. 1990) and mainly localized at the GC, thus used as a marker for this nucleolar compartment (Tajrishi, Tuteja et al. 2011). This localization reflects its function in initiation of rRNA processing (Turner, Knox et al. 2009), although a role in rDNA transcription and ribosome assembly was also reported (Salvetti, Coute et al. 2016). Additionally, NCL is also present in nucleoplasm and cytoplasm, and it was shown to regulate mRNA translation through binding to untranslated regions (Chen and Xu 2016). Interestingly, two studies revealed an additional role of NCL during mitosis: NCL downregulation elicits centrosome amplification and multipolar mitotic spindles (Ugrinova, Monier et al. 2007), and NCL is required for proper chromosome congression (Ma, Matsunaga et al. 2007). Moreover, NCL was implicated in DNA repair, by disrupting nucleosome (Goldstein, Derheimer et al. 2013), interacting with γH2AX and MDC1 signalling pathway (Kobayashi, Fujimoto et al. 2012).

Another abundant nucleolar phosphoprotein at the GC is **nucleophosmin** (**NPM**, also known as B23), which is encoded by the NPM1 gene (Umekawa, Chang et al. 1993). During ribosome biogenesis, NPM is essential for nuclear export of the ribosomal protein RPL5 (Yu, Maggi et al. 2006) and of both the pre-40S and pre-60S ribosomal subunits (Maggi, Kuchenruether et al. 2008). NPM also plays additional roles during rRNA processing (Savkur and Olson 1998) and transcription (Murano, Okuwaki et al. 2008).

Importantly, its nucleolar localization was shown to be dependent on RNA binding, and its phosphorylation during mitosis disables RNA binding and disrupts nucleolar localization (Okuwaki, Tsujimoto et al. 2002). Similarly to other nucleolar proteins, NPM plays several non-ribosomal roles. One example is the NPM association with polyadenylated mRNA, which strongly suggests a role in controlling gene expression (Palaniswamy, Moraes et al. 2006, Mukudai, Kubota et al. 2008). Other roles include DNA repair and chromosome stability (Colombo, Alcalay et al. 2011) and chromatin remodelling (Okuwaki, Matsumoto et al. 2001).

Another important nucleolar protein is **XRN2**, encoded by XRN2 gene (Zhang, Yu et al. 1999), which is a 5'-3' exonuclease. During pre-rRNA processing, endonucleases activity exposes 5' ends which are then processed by XRN2 (Wang and Pestov 2011) to generate both 5.8S and 18S (Sloan, Mattijssen et al. 2013). Additionally, data support a role for XRN2 in active degradation of failed RNA Pol I transcripts, spacer fragments and abnormal pre-rRNA intermediates (Wang and Pestov 2011). Furthermore, XRN2 was also shown to be important for processing of snoRNAs (Kufel and Grzechnik 2019), whose highly conserved sequences determine the group of proteins that bind them, establishing different snoRNPs as the mentioned above C/D and H/ACA boxes (Kufel and Grzechnik 2019). Beyond these functions in ribosome biogenesis, XRN2 is involved in termination of RNA Pol II-mediated transcription during protein synthesis (Wang and Pestov 2011). Although several models are proposed for RNA Pol II termination (Proudfoot 2016), XRN2 was shown to be required for the 'torpedo model' (West, Gromak et al. 2004). Specifically, it was shown that XRN2 recognizes an RNA 5' end exposed by the co-transcriptional cleavage sequence, which is downstream of a poly(A) site, leading to the degradation of the nascent transcript (West, Gromak et al. 2004). The effective termination of RNA Pol II-mediated transcription happens when XRN2 meets RNA Pol II, eliciting the removal of the transcription machinery from the DNA template (Proudfoot 2016).

The NOL12 protein family plays key roles in and beyond ribosome biogenesis. The relevance of this protein gradually increased with reports in different models. In *Mus musculus*, NOP25 was shown to be constitutively nucleolar and a 28S binding protein (Suzuki, Kanno et al. 2006). In *Saccharomyces cerevisiae*, RRP17p displayed a 5'-3' exonucleolytic activity, required for proper 60S pre-rRNA processing and export (Oeffinger, Zenklusen et al. 2009). In *Drosophila melanogaster*, *viriato*, the single NOL12 homologue, was described as a dMyc target (Marinho, Casares et al. 2011), and a positive regulator of the Dpp signalling, accounting for both cell growth and photoreceptor differentiation during eye development (Marinho, Martins et al. 2013). In human HeLa and HCT116 cell lines, NOL12 was reported as being essential for both cleavages in ITS1, contributing for proper generation of 5.8S, 18S and 28S pre-rRNAs (Sloan, Mattijssen et al. 2013, Scott, Trahan

et al. 2017). Moreover, human NOL12 was able to rescue both cell viability in yeast, which is lost in the rrp17Δ strain (Oeffinger, Zenklusen et al. 2009), and the size of eye imaginal discs in fruit fly, which is decreased in viriatoRNAi larvae (Marinho, Casares et al. 2011), suggesting NOL12 as a protein with a conserved functional role. Recently, in HCT116 cells, NOL12 was also found to co-localize with nucleoplasmic and cytoplasmic proteins, consistent with a role in DNA damage repair, ensuring the preservation of genomic integrity (Scott, Trahan et al. 2017).

1.4 Nucleolar localization

When nucleolar proteome studies revealed a large number of proteins localized in this subnuclear compartment, a question was raised up: how are these factors sent to the nucleolus?

The existence of signals targeting those proteins to the nucleolus was initially explored. A study analysed 46 sequences reported as nucleolar localization signals (NoLSs) and proposed that using a prediction software for this kind of sequences could work as first approach (Scott, Boisvert et al. 2010). Five years later, a set of biochemical properties that ensure nucleolar localization was reported, e.g. the existence of 6 arginines in the peptide sequence (Martin, Ter-Avetisyan et al. 2015), which was in accordance with the previously observed high proportion of basic aminoacids in NoLSs (Scott, Boisvert et al. 2010). The acidic nature of the nucleolus when compared with the nucleoplasm provides the suitable environment for proteins with basic aminoacids, such as arginine, to localize there (Martin, Ter-Avetisyan et al. 2015). Importantly, arginines mainly bind RNA inside the nucleolus, a mechanism that seems to be highly conserved along evolution, from insect to human cells (Martin, Ter-Avetisyan et al. 2015).

Another process by which proteins localize into the nucleolus is through transporters. For instance, NPM was shown to bind the cell cycle protein P120 to drive its nucleolar localization (Valdez, Perlaky et al. 1994). However, a NoLS is not sufficient to localize P120 in the nucleolus, it also requires a nuclear localization sequence (NLS) (Valdez, Perlaky et al. 1994). Interestingly, a recent study reported that high levels of NPM and arginine-rich proteins is sufficient for their phase separation into liquid-like droplets, proposing phase separation as an additional mechanism through which proteins localize into the nucleolus (Mitrea, Cika et al. 2016). Another relevant recent finding is that most of RNA-binding proteins contain intrinsically disordered regions that enable those proteins to bind RNA, particularly allowing RNA retention into the nucleolus (Jarvelin, Noerenberg et al. 2016). These disordered regions are now under research focus since they were shown

to be important for RNA metabolism and biology (Xie, Vucetic et al. 2007), and their disruption was associated with disease (reviewed in (Jarvelin, Noerenberg et al. 2016)).

2. THE NUCLEOLAR DYNAMICS DURING CELL CYCLE

The nucleolus was identified as playing a role during the cell cycle. As a cell enters mitosis, specifically in early prophase, the nucleolus is disrupted since the rRNA processing machinery leaves the nucleolus (Hernandez-Verdun, Roussel et al. 1993) (Figure 1.4A) to localize at the perichromosomal layer of all the chromosomes at the end of prophase (Dimario 2004, Angelier, Tramier et al. 2005) or disperse into the cytoplasm (Figure 1.4B). Besides, the activation of the CDK1-cyclin B complex was shown to phosphorylate some key components of both rRNA processing (Peter, Nakagawa et al. 1990), such as FBL, NCL and NOP52 (Hernandez-Verdun, Roussel et al. 1993), and RNA Pol I transcription machineries, such as SL1 and TTF-1, leading to their silencing (Heix, Vente et al. 1998, Sirri, Roussel et al. 1999). However, at early prophase, this transcription machinery maintains the association with rDNA in the NORs (Roussel, Andre et al. 1996), being fully silenced only at the end of the prophase, when both the nuclear envelope and the nucleolus are no longer visible (Guttinger, Laurell et al. 2009, Gavet and Pines 2010). Thus, inhibition of rRNA processing occurs prior to rDNA transcription silencing (Dousset, Wang et al. 2000), presumably due to different local concentrations of CDK1-cyclin B complex (Hernandez-Verdun 2011). During metaphase, rRNA processing components are still in association with the perichromosomal layer (Gautier, Robert-Nicoud et al. 1992) (Figure 1.4C) and remain during anaphase, when chromosomes migrate to spindle poles (Savino, Gebrane-Younes et al. 2001) (Figure 1.4D). Interestingly, the components of rRNA processing machinery that do not associate with chromosomes start to aggregate into numerous large cytoplasmic foci – the nucleolus-derived foci (NDF) – during anaphase (Dundr, Misteli et al. 2000) (Figure 1.4D). Contrarily, the RNA Pol I machinery was found to be highly dynamic during mitosis, as it travels between rDNA clusters and cytoplasm (Chen, Dundr et al. 2005). In telophase, characterized by the reassembly of the nuclear envelope and the determination of the cleavage furrow (Pollard, Earnshaw et al. 2017), the nucleolar reassembly begins, taking about two hours to be fully completed in HeLa cells (Muro, Gebrane-Younis et al. 2010) (Figure 1.4E), whereas nucleolar disassembly only needs about thirty minutes (Gavet and Pines 2010). This reflects the strict coordination that is needed to reactivate both rDNA transcription and rRNA processing machineries (Hernandez-Verdun, Roussel et al. 2002). Regarding rDNA transcription, it is resumed

during telophase, when CDK1-cyclin B activity is inhibited by PP1 and PP2A phosphatases (Heix, Vente et al. 1998, Sirri, Roussel et al. 1999). This reactivation occurs in each of the NOR-containing chromosomes (Hernandez-Verdun, Roussel et al. 2002), although only in those where rDNA transcription machinery is associated, the termed “competent” NORs (Roussel, Andre et al. 1996) (Figure 1.4F).

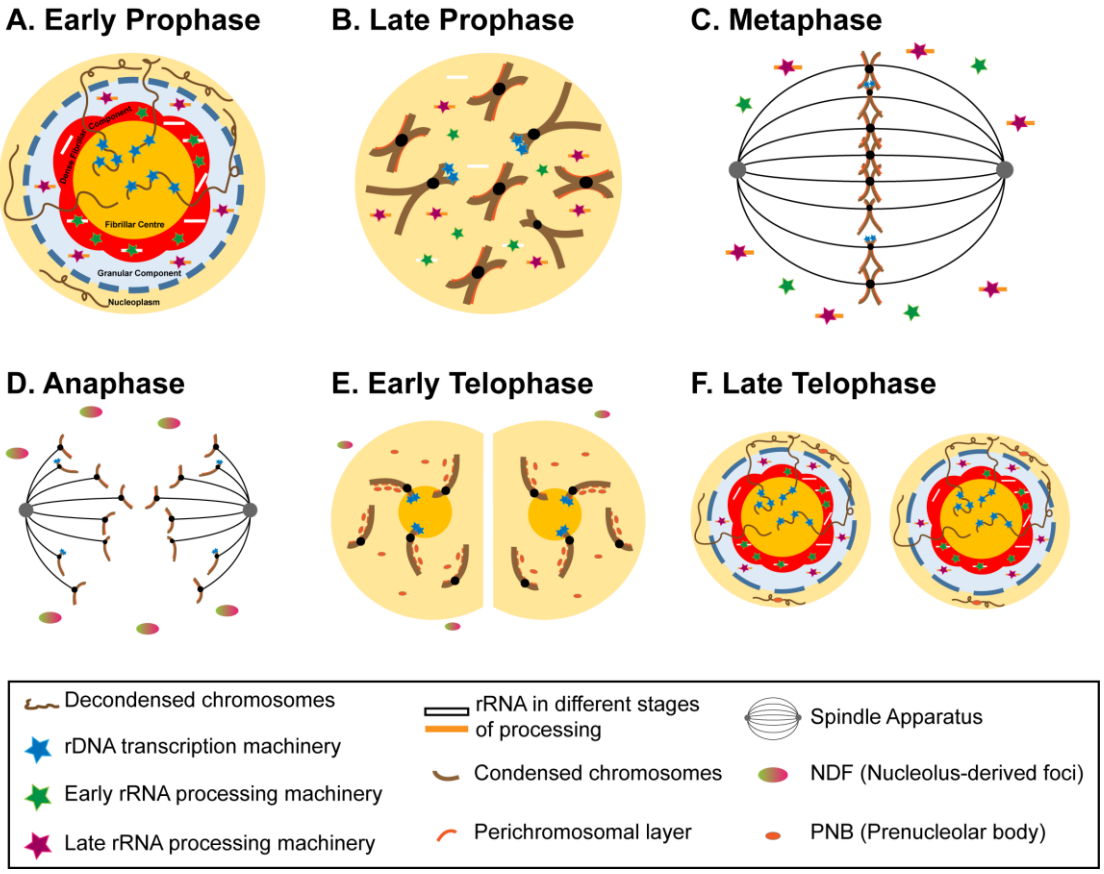

Figure 1.4. The nucleolus disassembles and reassembles during the cell cycle. (A) During early prophase, rDNA transcription machinery (blue stars) is associated with NORs at the FC (yellow). **(B)** In late prophase, as the nucleolus is disrupted, some components of rRNA transcription machinery associate with condensed chromosomes at the perichromosomal layer (orange), while others are dispersed in the nucleus. **(C)** As the cell enters in metaphase, chromosomes align at the metaphase plate and rRNA processing components remain in the perichromosomal layer. **(D)** During anaphase, rRNA processing components that are not in the perichromosomal layer of the condensed chromosomes are packed in nucleolus-derived foci (NDF, green-to-red gradient ovals). **(E)** In early telophase, FC starts to be reassembled around NOR-containing chromosomes. rDNA transcription is reactivated in those chromosomes where the transcription machinery is associated. As the chromosomes start to decondense, perichromosomal layer breaks down and rRNA processing components are packed into prenucleolar bodies (PNBs, orange ovals). **(F)** In late telophase, chromosomes are fully decondensed and nuclear envelope is rebuilt. Along with this, PNBs release their content into nucleus, allowing the later re-formation of DFC (red) and GC (light blue).

Even though each NOR gives rise to an individualized nucleolar focus (Hernandez-Verdun, Roussel et al. 2002), the number of foci decreases during G1, due to nucleolar fusion (Hernandez-Verdun 2011), likely driven by their liquid-like properties (Brangwynne, Mitchison et al. 2011). Despite this, reactivation of rDNA transcription is not enough to achieve a fully assembled functional nucleolus, being also dependent on the reactivation of rRNA processing machinery (Hernandez-Verdun 2011). This begins during telophase, when chromosomes start to decondense, leading to the release of nucleolar components from the perichromosomal regions into prenucleolar bodies (PNB) (Savino, Gebrane-Younes et al. 2001) (Figure 1.4E). Interestingly, there are different types of PNBs with distinct composition, which are orderly recruited to the nucleolus during G1 (Hernandez-Verdun, Roussel et al. 2002), later originating the DFC compartment (Dundr, Misteli et al. 2000). Concerning NDFs, they are similar to PNBs and it is generally accepted that they just exist in cells with high expression of rRNA processing components, which are not able to associate with perichromosomal region (Dundr, Meier et al. 1997). Contrarily to PNBs, NDFs were found to disappear once they contact with the nuclear envelope, releasing their components into the nucleus (Dundr, Misteli et al. 2000). Regarding their composition, both NDFs and PNBs are similar, containing components of rRNA processing machinery as well as partially processed pre-rRNAs (Hernandez-Verdun, Roussel et al. 2002). These pre-rRNAs are inherited by daughter cells to support the assembling of a functional nucleolus (Hernandez-Verdun, Roussel et al. 2002). Altogether, these data strongly suggest formation of PNBs as a cellular strategy to finely regulate the nucleolar assembly upon mitosis (Hernandez-Verdun 2011).

3. RESPONSE MECHANISMS TO NUCLEOLAR STRESS

As stated above, the nucleolus has been increasingly recognized as a highly dynamic subnuclear compartment, where a huge amount of proteins come in and/or go out. This dynamic protein traffic reflects and explains why the nucleolus is not just the ribosome factory but instead plays important roles on cell homeostasis, from maintenance of genome integrity to cell cycle regulation (James, Wang et al. 2014). The additional involvement of the nucleolus in apoptosis and senescence highlighted it as a hub for the integration of changes in external environment, as well as for the coordination of a suitable response, ensuring nucleolar and cell homeostasis.

3.1 Nucleolar stress

The perturbation of ribosome biogenesis caused by deregulation of ribosomal and/or nucleolar proteins (Holmberg Olausson, Nister et al. 2012) triggers a nucleolar response that ultimately impacts cell growth (Boulon, Westman et al. 2010, James, Wang et al. 2014). This response often activates P53, the primary mediator of cellular stress (Olson 2004). Given that the nucleolus is able to regulate MDM2, the main negative regulator of P53, studies have focused on understanding the mechanisms by which the nucleolus regulates P53. On the other hand, other studies aim to unveil the P53-independent mechanisms because they are also able to induce similar cell responses, such as cell cycle arrest (James, Wang et al. 2014). Comprehension of these mechanisms could unveil new targets for cancer therapy since more than half of human cancers are characterized by a non-functional P53 (James, Wang et al. 2014). Along with biochemical alterations, changes in the architecture of the nucleolus lead to a cell state commonly defined as “nucleolar stress”.

3.1.1 P53-dependent responses

P53 was initially described as an oncogene (Eliyahu, Raz et al. 1984) but posterior studies came out to attribute a tumour suppressor function (Baker, Fearon et al. 1989, Nigro, Baker et al. 1989, Malkin, Li et al. 1990, Donehower, Harvey et al. 1992). Since then, P53 has been extensively studied and many pathways involving it were unravelled. Still, many questions remain such as why some cells die and others arrest in response to P53, how tumour microenvironment favours or prejudices a P53 mutated cell, among others (Vogelstein, Sur et al. 2010).

P53 is a transcription factor containing five different domains (Figure 1.5): the transactivation domain (TAD), the proline-rich domain (PRD), the DNA-binding domain (DBD), the tetramerization domain (TD) and the basic domain (BD) (Raj and Attardi 2017). The TAD guarantees the proper activity and function of P53 and it is described that MDM2, an E3 ubiquitin ligase, binds to this region, targeting P53 to proteasomal degradation (Shimizu, Burch et al. 2002, Chi, Lee et al. 2005). Additionally, it ensures the proper correction of damaged DNA (Kannappan, Mattapally et al. 2018) and is crucial for tumour suppression activity (Raj and Attardi 2017). The other N-terminal domain, PRD, is essential for inducing apoptosis (Chipuk, Kuwana et al. 2004) and for protein stability (Toledo, Krummel et al. 2006). The core domain of P53 is occupied by the DBD which is the responsible for the ability of P53 to act as a transcription factor (Natan, Baloglu et al. 2011). Interestingly, this domain was found to contain a mutational hotspot where most of the P53 mutations observed in cancer cells occur (Pavletich, Chambers et al. 1993). Next to DBD, P53 contains the TD which ensures the proper conformation to allow the binding to several

proteins (Chene 2001). Importantly, TD determinates P53-based cell fate, such as cell growth or apoptosis, by regulating the oligomeric status of P53 (Fischer, Prodeus et al. 2016). Finally, BD is a regulatory domain whose localization at C-terminus allows it to either fold, when a target protein binds it, or function as a flexible linker (Harms and Chen 2006). BD is able to positively and negatively regulate P53 activity, depending on the post-translational modification occurred (Harms and Chen 2006). For instance, while phosphorylation of serine 15 is essential for P53 transcriptional activity (Loughery, Cox et al. 2014), C-terminal neddylation of P53 was shown to decrease transcriptional activity (Abida, Nikolaev et al. 2007).

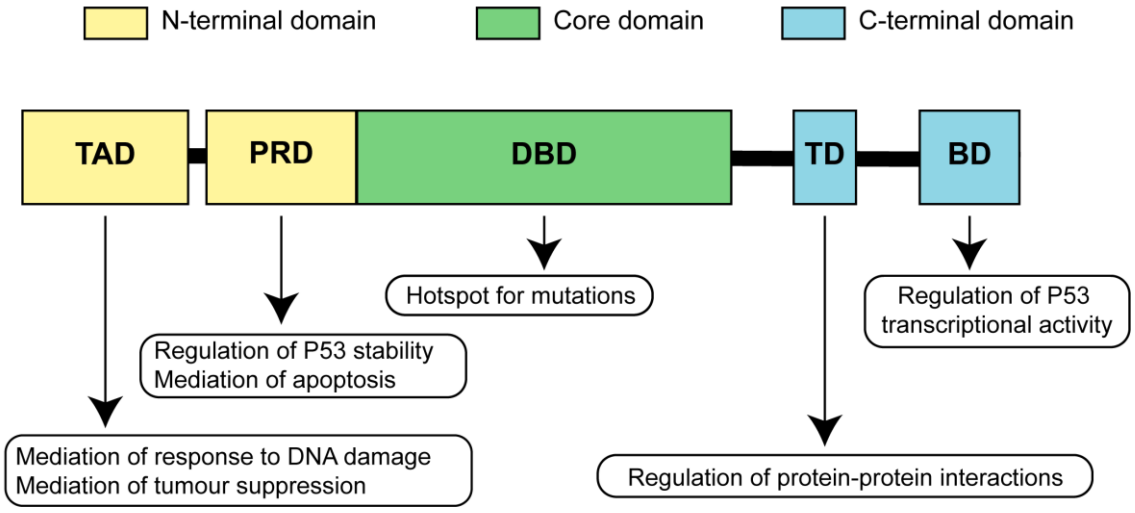

Figure 1.5. P53 protein domains and their main functions. The N-terminus (yellow) contains the transactivation (TAD) and proline-rich (PRD) domains, while the DNA binding domain (DBD) mainly represents the core domain (green). The C-terminus (blue) comprises the tetramerization (TD) and basic (BD) domains. The main functions of each domain are indicated.

MDM2 is a very interesting protein, with increasing relevance due to its ability to target P53 for degradation. A common feature to most E3 ligases is their capacity to ubiquitinate themselves. Unexpectedly, MDM2 self-ubiquitination does not result in its degradation *in vivo* (Itahana, Mao et al. 2007). But this ability potentiates substrate ubiquitination by strongly recruiting the E2-conjugating enzymes (Ranaweera and Yang 2013). Interestingly, MDM2 displays a signal for nucleolar localization, which is not important for MDM2 ubiquitination or P53 monoubiquitination (which signals P53 to be exported from nucleus to cytoplasm (Brooks, Li et al. 2004)), but is absolutely required for P53 polyubiquitination, which targets P53 for proteasomal degradation (Xirodimas, Saville et al. 2001, Moll and Petrenko 2003, Brooks and Gu 2011).

Until 2004, it was believed that P53 was able to both promote and repress transcription of its target genes. However, by meta-analysis of genome-wide data, it was found that P53 behaves primarily as a transcription activator. Its activity as a repressor requires the activation of the P53-P21-DREAM/RB pathway (Fischer, Steiner et al. 2014). Some studies also suggest that P53 is able to induce apoptosis through a transcription-independent pathway (Chipuk, Kuwana et al. 2004).

Concerning its role in cellular stress, earlier studies reported P53 as the main protector of the genome and of the cell itself (Holmberg Olausson, Nister et al. 2012), although P53 could be activated in a DNA damage-independent manner (Bursac, Brdovcak et al. 2012). Consistently with this, several reports showed P53 activation upon very different cellular stresses, including nucleolar stress, which in turn suggests that, in a certain way, the nucleolus is required for P53 degradation via MDM2. There is a current model stating that nucleolar disruption redistributes nucleolar proteins, from the nucleolus to the nucleoplasm (James, Wang et al. 2014). Eventually, these proteins will bind MDM2, preventing its binding to P53 and consequent P53 degradation. For instance, once released from the nucleolus to the nucleoplasm, ARF is able to bind to the central region of MDM2, inhibiting P53 degradation (James, Wang et al. 2014, Box, Paquet et al. 2016) (Figure 1.6A). Also, NS overexpression induces P53-dependent G1 cell cycle arrest by directly binding the central acidic domain of MDM2, preventing its negative regulatory function over P53 (Dai, Sun et al. 2008). On the other hand, ribosomal proteins are delocalized from cytoplasm to nucleoplasm, no more being able to assemble ribosomes (James, Wang et al. 2014) (Figure 1.6B). Notably, a broad range of these proteins are reported to bind MDM2, namely RPS3, RPS7, RPL23 and RPL37, among others (Dai, Zeng et al. 2004, Chen, Zhang et al. 2007, Yadavilli, Mayo et al. 2009, Daftuar, Zhu et al. 2013). Therefore, altogether these results show that nucleolar disruption leads to the dramatic changing of the nucleoplasmic pool of ribosomal proteins, leading to P53 activation (Deisenroth and Zhang 2010, Deisenroth and Zhang 2011).

During nucleolar stress, most of MDM2-binding ribosomal proteins are targeted for proteasomal degradation (Warner 1977). Among them, RPL11 and RPL5 stand out because, actually, they tend to accumulate at the ribosome-free fraction, due to the continuous import into the nucleolus of *de novo* synthesized RPL11 and RPL5 and their non-degradation (Bursac, Brdovcak et al. 2012, James, Wang et al. 2014). An important complex – the 5S RNP– has been identified, which links both ribosome biogenesis and cell proliferation pathways (Sloan, Bohnsack et al. 2013) (Figure 1.6C). This complex is composed of the ribosomal proteins RPL11 and RPL5, and 5S rRNA, and in the HEK293 cell line it is localized mainly at the nucleoplasm, in response to ribosome biogenesis inhibition (Sloan, Bohnsack et al. 2013). The existence of this complex has been also

reported in U2OS cells, and shown to shift from its assembly with 60S pre-ribosomes to MDM2 binding upon impairment of ribosome biogenesis (Donati, Peddigari et al. 2013) (Figure 1.6C). Knockdown of any of the three components of this complex decreases the binding of the other two to MDM2, therefore suggesting that efficiency of the binding depends on all the components (Donati, Peddigari et al. 2013). P53 activation mediated by the assembly of the 5S RNP complex with MDM2 has been extensively reported in response to different triggers of nucleolar stress and in different cell types. For instance, in A549 cells, both RPL11 and RPL5 co-localize with MDM2 in the nucleolus in response to rDNA transcription inhibition by low doses of actinomycin D (Bursac, Brdovcak et al. 2012). Also, both replicative and oncogenic stresses were shown to drive MCF-7 human breast cancer cells through cell senescence by activating P53 in a 5S RNP-dependent manner (Nishimura, Kumazawa et al. 2015). Additionally, the G1 cell cycle arrest induced by downregulation of NS depends on the interaction of RPL11 and RPL5 with MDM2 to activate P53 (Dai, Sun et al. 2008).

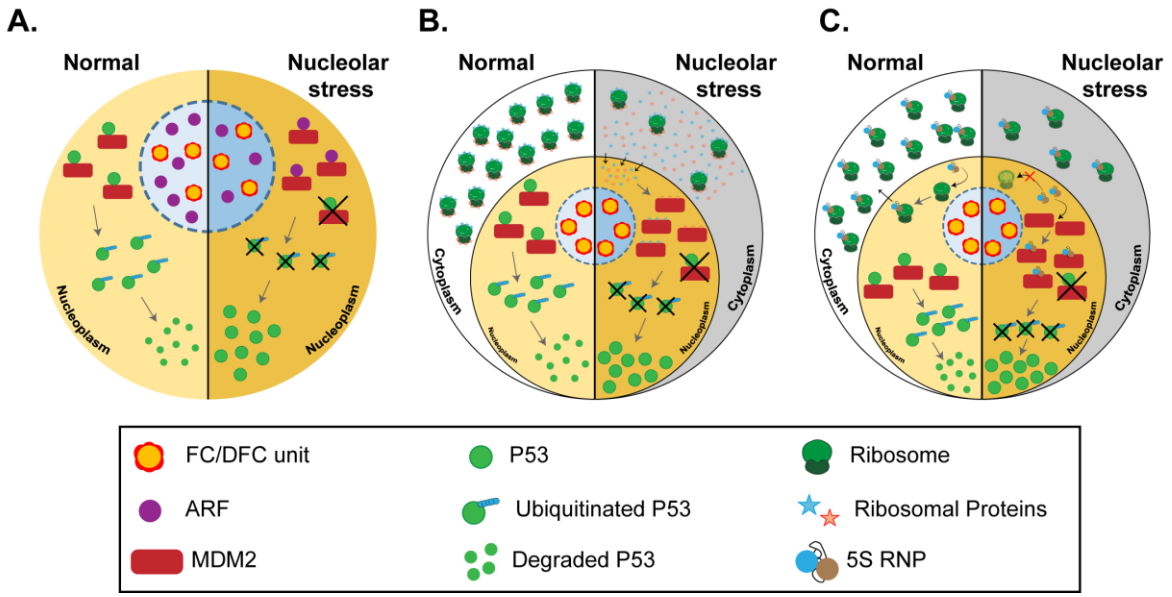

Figure 1.6. **Different mechanisms by which nucleolar stress activates P53. (A)** Under normal conditions, nucleolar proteins are in the nucleolus, which keep MDM2 (red rectangle) free to bind P53 (green circles), leading to its polyubiquitination and consequent degradation. In response to nucleolar stress, some nucleolar proteins, such as ARF (purple circles), are delocalized to the nucleoplasm and bind MDM2, precluding its binding to P53 and allowing P53 stabilization. **(B)** Under normal conditions, ribosomal proteins are assembled into ribosomes, to produce a functional pool of ribosomes in the cytoplasm. In nucleoplasm, MDM2 binds P53 and leads to its degradation. When ribosome biogenesis is impaired, a lower number of ribosomes are produced and some ribosomal proteins (blue and red stars) delocalize from cytoplasm to nucleoplasm, where a large number of them are able to bind MDM2, preventing P53 degradation, ultimately leading to P53 activation. **(C)** Under normal conditions, RPL11, RPL5 and 5S rRNA form a complex – the 5S RNP – which binds the ribosomal 60S large subunit (light green). In response to nucleolar stress, such as impairment of ribosome biogenesis, the 5S RNP shifts to MDM2 binding, preventing P53 degradation and allowing its activation.

3.1.2 P53-independent responses

In mammals, there are P53-independent pathways leading to cell cycle arrest and autophagy during nucleolar stress (Pfister 2019). For instance, double knockdown of POLR1A (which codes for the catalytic subunit of RNA Polymerase I) and P53 in U2OS and HCT-116 cancer cell lines inhibits S-phase, the same phenotype observed in POLR1A-depleted cells, suggesting that cell cycle arrest induced by POLR1A depletion occurs in a P53-independent manner (Donati, Brighenti et al. 2011). Another study using P53-proficient and P53-deficient cells showed that, upon impairment of ribosome biogenesis, cells underwent cell cycle arrest due to deregulation of PIM1 kinase, a sensor for ribosomal stress, which increases P27 protein levels (Iadevaia, Caldarola et al. 2010). Interestingly, NPM overexpression was shown to drive cells into senescence due to its ability to bind P53 and increase its transcriptional activity and protein stability (Colombo, Marine et al. 2002). However, in different tumours, NPM arises as a fused protein (Morris, Kirstein et al. 1994, Redner, Rush et al. 1996, Yoneda-Kato, Look et al. 1996) becoming unable to bind P53, which could eventually explain the P53 inactivity in such tumours (Colombo, Marine et al. 2002). Importantly, nucleolar stress was shown to activate the NF-κB signalling pathway in a P53-independent manner (Chen and Stark 2019). In fact, different stimuli that activate the NF-κB signalling also disrupt nucleoli (Chen and Stark 2019). Particularly, treatment with aspirin promotes nucleolar disruption, induces degradation of the RNA Pol I component, TIF-IA, activates the NF-κB signalling and ultimately promotes apoptosis (Chen, Lobb et al. 2018).

Meanwhile, these P53-independent mechanisms were also investigated in other organisms. For instance, yeasts do not express P53 or MDM2 as mammalian cells (Koerte, Chong et al. 1995, Di Ventura, Funaya et al. 2008), which could explain their different response to nucleolar stress induced by ribosome biogenesis inhibition (James, Wang et al. 2014). On the other hand, these studies using yeast as a model may provide important information about the existence of an ancestral pathway and how it works. Most of these reports are based on the observation of different phenotypes in terms of cell cycle and morphology induced by impairment of ribosome biogenesis, which emerge through a relatively reduced number of pathways (James, Wang et al. 2014). *Caenorhabditis elegans* or *Drosophila melanogaster* are other interesting organism models that do not express MDM2 and ARF, although they express P53 (James, Wang et al. 2014).

3.1.3 Disruption of the nucleolar architecture

As mentioned above, nucleolar stress is frequently associated with alterations in the organization of the nucleolus. For instance, loss-of-function of Drosophila NOL12/VIRIATO

was shown to upregulate FBL and to result in a substantial reduction in the packaging of nucleolar components and increased granular organisation (Marinho, Casares et al. 2011). In *Mus musculus*, the VIRIATO homologue, NOP25, had been previously implicated in the maintenance of nucleolar architecture, since its knockdown led to nucleolar fragmentation (Suzuki, Fujiwara et al. 2007).

Another interesting protein is FBL, whose knockdown in HeLa cells was shown to induce an abnormal nuclear morphology, to slow down cell proliferation and to cause G2/M arrest without changing the P53 protein levels (Amin, Matsunaga et al. 2007). While its downregulation reduces cell proliferation and decreases nucleolar number in prostate cancer cells (Koh, Gurel et al. 2011), its upregulation induced by the proto-oncogene Myc overexpression, increases nucleolar size and number in prostate intraepithelial neoplasia (Koh, Gurel et al. 2011).

In addition, NCL downregulation also impacts nucleolar ultrastructure in HeLa cells, increasing P53 levels and ultimately arrest cells at G2/M (Ugrinova, Monier et al. 2007).

Altogether, these data indicate the nucleolus as a very malleable and plastic membraneless organelle, with the ability to easily fuse or separate. This is actually what has been reported recently, pinpointing this and other membraneless organelles as possessing liquid-like properties and resulting from liquid-liquid phase separation (Brangwynne, Eckmann et al. 2009, Brangwynne, Mitchison et al. 2011). These are the features that indeed support and allow cellular components to be fast and reversibly concentrated, making the cell able to quickly respond to a stimulus (Hyman, Weber et al. 2014). Despite evidence showing that specific sequence motifs are responsible for highly structural disordered domains in proteins able to be phase separated, the requirements for this process are still poorly understood (Vernon and Forman-Kay 2019).

4. PATHOPHYSIOLOGICAL RELEVANCE OF THE NUCLEOLUS

The observation that the nucleolus is more than just a ribosome factory or an indicator of cell malignancy opened new avenues in the study of human diseases. Its ability to control many different cell processes has highlighted it as being either crucial for cell homeostasis or a potential deregulated target in several human diseases (Nunez Villacis, Wong et al. 2018). Therefore, several recent studies have allowed a deeper knowledge of pathophysiology of human diseases and sometimes have revealed the nucleolus as a very promising therapeutic target.

4.1 The nucleolus and cancer

Early, scientists realized that there was a connection between proliferation and the nucleolus: highly proliferating mammalian cells display nucleolar hypertrophy and increased ribosome biogenesis (Derenzini and Ploton 1991). This increase would result in greater protein synthesis, ensuring that daughter cells have all cell components necessary to their normal functioning (Thomas 2000). In addition to this crosstalk between ribosome biogenesis and cell proliferation, there is evidence suggesting that both share some common pathways (Montanaro, Trere et al. 2008). For instance, in cancer cells, proto-oncogenes and tumour suppressors regulate positively and negatively the uncontrolled cell proliferation, respectively, and at the same time control the rate of ribosome biogenesis (Sulic, Panic et al. 2005). Already in the 19th century, nucleoli from cancer cells were reported as bigger and morphologically abnormal (Pianese and Teuscher 1896). More recently, this nucleolar enlargement was shown to be due to MYC overexpression in prostate cancer cells (Koh, Gurel et al. 2011) (Figure 1.7A). Even nowadays, an increased number and size of nucleoli is a bad prognosis for tumour development (Derenzini, Trere et al. 2000, Ruggero 2012). However, this notion that nucleolar hypertrophy is always associated with higher rates of cell proliferation is still controversial (Montanaro, Trere et al. 2008). For instance, nucleolar hypertrophy was observed upon treatment with an anti-proliferative agent, MLN4924 (Bailly, Perrin et al. 2016). Besides, the impairment of ribosome biogenesis was found to enlarge nucleoli and induce senescence (Nishimura, Kumazawa et al. 2015). In addition, enlarged nucleoli were found in intra-epithelial prostate cancer cells which showed lower proliferative capacity when compared with other intra-epithelial cancer cells exhibiting smaller nucleoli (Orsolic, Jurada et al. 2016)

MYC is a very important transcription factor which targets and regulates several genes involved in cell growth (Schmidt 1999, van Riggelen, Yetil et al. 2010) and its overexpression is a common feature of several cancers (Dai and Lu 2008, Meyer and Penn 2008). Interestingly, in *Drosophila melanogaster* it was shown that cells containing high levels of MYC (thus displaying higher translational capacity) are more efficient in the uptake of survival and growth factors, leading to the death, or at least the proliferative slowdown, of the surrounding cells (Moreno and Basler 2004). MYC was also extensively reported as directly controlling the rDNA transcription (Poortinga, Hannan et al. 2004, Arabi, Wu et al. 2005, Grandori, Gomez-Roman et al. 2005), and a further role in regulation of the rRNA processing efficiency was found in human B-cell line P493-6 (Schlosser, Holzel et al. 2003). MYC also targets genes involved in ribosome biogenesis, particularly ribosome proteins from large and small subunits (Ruggero 2009). In mice, it was shown that the ability of MYC to regulate ribosomal proteins L24 and L38 is essential for cell growth induced by MYC

(Ruggero 2009). Also, RPL11 is a transcriptional target of MYC but one study demonstrated that there is a negative feedback loop, where RPL11 is able to inhibit MYC (Dai, Sears et al. 2007). Moreover, it was reported that RPL15 is also overexpressed in gastric cancer cell lines and that both cell growth and tumorigenicity are inhibited upon RPL15 knockdown (Wang, Zhao et al. 2006). Other examples include RPLP0, RPLP1 and RPLP2, whose mRNA upregulation is positively correlated with lymph metastasis in gynecologic tumours (Artero-Castro, Castellvi et al. 2011), and RPS11 and RPS20, whose upregulation is a poor prognostic marker in glioblastoma (Yong, Shabihkhani et al. 2015). Nevertheless, one paradigm exists in tumour cells since some of them display downregulation of several ribosomal proteins, which is contrary to what would be expected for cells with increased protein synthesis (Kasai, Nadano et al. 2003, Choi and Chen 2005) (Figure 1.7B). This suggests that these ribosomal proteins in particular account for cell proliferation and/or transformation rather than for translation machinery (Wang, Huang et al. 2010), which is in line with the observation that some ribosomal proteins play extra-ribosomal functions (Zimmermann 2003). One example is RPL41, which is described as the smallest and most basic eukaryotic protein (Yu and Warner 2001) and is downregulated in 9 out of 12 primary breast cancers (Wang, Huang et al. 2010). This study showed that this downregulation correlates with a very premature centrosome splitting, leading to abnormal mitosis and eventually triggering cell transformation (Wang, Huang et al. 2010). Other examples of this association between downregulation of ribosomal proteins and increased tumour formation include RPS6 in Drosophila (Watson, Konrad et al. 1992), RPL9 and RPL26 in mice (Beck-Engeser, Monach et al. 2001) and several ribosomal proteins in zebrafish (Amsterdam, Sadler et al. 2004).

Another interesting field in the study of cancer tries to understand how different interactions and subcellular localization impact cell transformation and proliferation (Figure 1.7C). For instance, depending on the binding partners, NPM could act as an oncogenic repressor or activator. Particularly, its association with nucleolar ARF inhibits ribosome biogenesis and cell proliferation whereas its interaction with nucleolar MYC promotes oncogenesis (Weeks, Metge et al. 2019). Lastly, the upregulation of the nucleolar protein PICT1 in wild-type P53-expressing cancers showed to sequester RPL11 into the nucleolus, preventing the activation of P53, then promoting cell proliferation (Lu 2011).

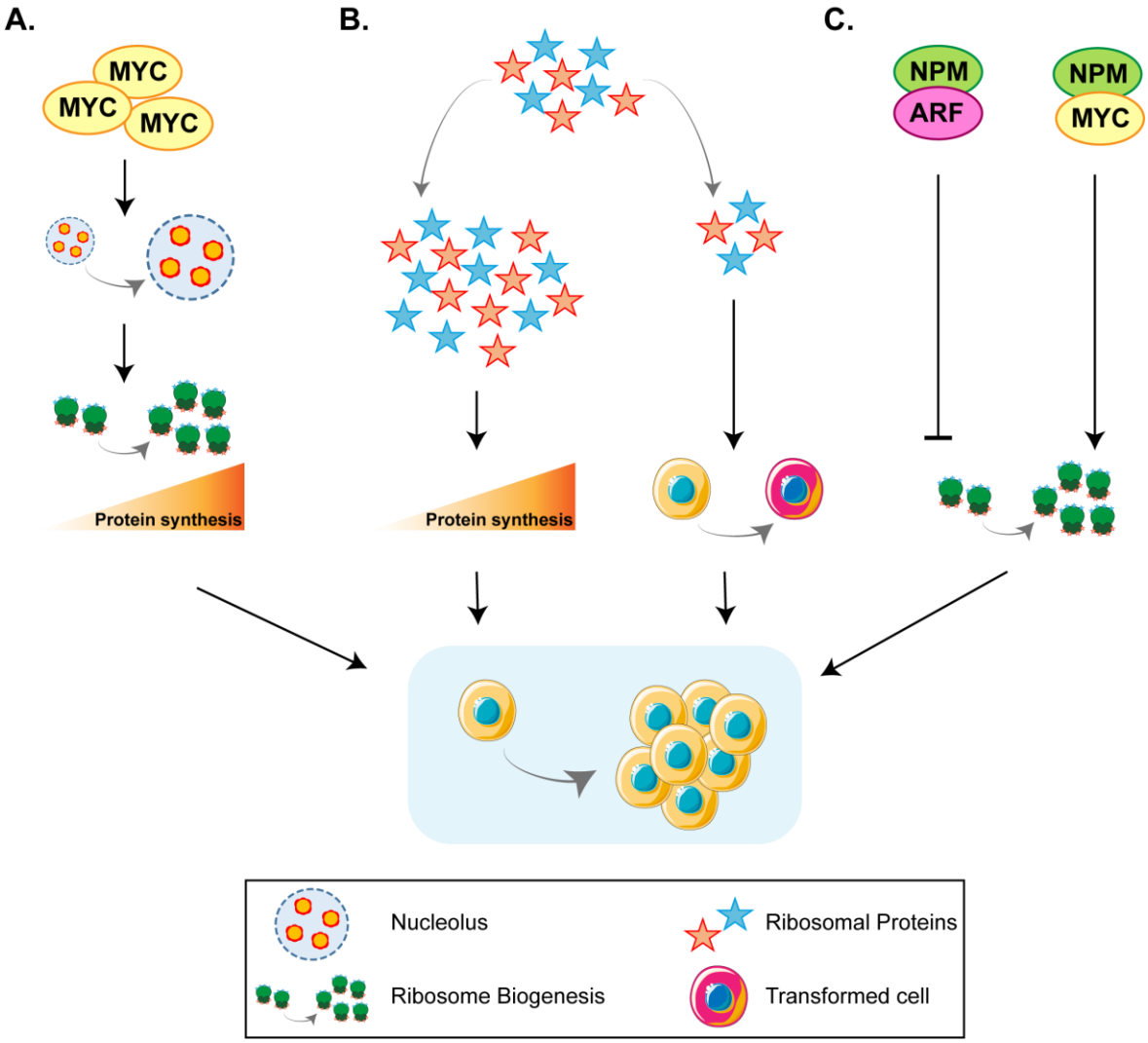

Figure 1.7. Nucleolar structure and function are involved in cancer development. (A) The MYC protein is overexpressed in several tumours. Enlarged nucleoli in MYC-overexpressing cells lead to increased ribosome production and protein synthesis, which support the increased proliferation of these cells. **(B)** Several tumours display an upregulation of ribosomal proteins (red and blue stars) which leads to increased protein synthesis, supporting the higher proliferative capacity of cells. On the other hand, some tumours exhibit a downregulation of some ribosomal proteins, which triggers cell transformation, a key step for cancer progression. **(C)** Some proteins, such as NPM, exhibit opposite effects depending on the binding partner. When NPM is associated with ARF, ribosome biogenesis and cell proliferation are prevented, whereas its association with MYC increases ribosome biogenesis and proliferation of cancer cells.

Apart from these most direct effects on ribosome production, cell proliferation or apoptosis, a mechanism recently proposed by Marcel et al. postulates P53 as a key protein for controlling the quality of ribosomes. Particularly, they propose that in cancer cells with no functional P53, FBL expression levels increase which leads to an alteration of the rRNA methylation pattern (Marcel, Ghayad et al. 2013). On the one hand, this will lead to the production of ribosomes which will translate mRNA with a lower fidelity; on the other hand,

this altered methylation pattern will lead to a preferential and biased translation initiation of mRNA coding for pro-oncogenic, anti-apoptotic and survival proteins through IRES (Internal Ribosome Entry Sites) rather than the CAP (Marcel, Ghayad et al. 2013), which is considered the canonical way for translation initiation (Komar and Hatzoglou 2011). Indeed, the disequilibrium towards any of the two modes of translation initiation has been increasingly implicated in cancer initiation and progression. During mitosis, translation initiation through CAP decreases and the IRES-dependent translation ensures the expression of specific proteins essential for mitotic progression and proper cytokinesis (Wilker, van Vugt et al. 2007, Barna, Pusic et al. 2008). Relevantly, MYC overexpressing cells do not do this shift leading to genomic instability, cytokinesis failure and ultimately cell transformation (Barna, Pusic et al. 2008). In this context, it is interesting to take into account the existence of an IRES element at the MYC 5'UTR (Stoneley, Paulin et al. 1998), which might be a chance for cell to downregulate MYC levels since IRES-dependent translation is reduced in MYC-overexpressing cells (Ruggero 2009). Another study found that decreased levels of ARL2 (ADP ribosylation factor like 2) are associated with an increased aggressiveness of breast tumour both *in vitro* and *in vivo* (Beghin, Belin et al. 2009). By using a cell line knockdown for ARL2, authors found IRES-dependent translation initiation decreased, including P53 mRNA, along with a reduced fidelity of ribosomes (Belin, Beghin et al. 2009), contributing for tumour aggressiveness.

Altogether, these results show that the nucleolus plays an important role in cancer and, in accordance, it is now recognized as a new and potential target for cancer therapy (Quin, Devlin et al. 2014, Lindstrom, Jurada et al. 2018).

4.2 The nucleolus and aging

Aging can be defined as phenotypic alterations that occur progressively in all of the individuals (Guarente 1997), leading to a gradual functional decline with loss of viability (Partridge and Mangel 1999). Therefore, "aging" means that there is an intrinsic cell viability limit that does not allow immortality. Nowadays, the scientific community is trying to understand the mechanisms behind this aging process to provide new reliable therapeutic targets, improving human health during aging by minimizing its effects (Lopez-Otin, Blasco et al. 2013). However, aging involves many different cell events turning difficult to find out "the" trigger. By now, hallmarks of aging have been described which include genomic instability, epigenetic alterations, telomere erosion, proteotoxic stress, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (Lopez-Otin, Blasco et al. 2013). Outstandingly, nucleolar function has emerged as a key regulator of some of these hallmarks of aging. Particularly,

it plays a role in the maintenance of genomic stability (Ogawa and Baserga 2017). For instance, NCL was shown to interact with the DNA damage response proteins γH2AX, RPA32 and PCNA (Kawamura, Qi et al. 2019), and NOL12 was described as an RNA-binding protein which colocalizes with the DNA repair proteins DHX9 and TOPBP1, contributing for the resolution of DNA stress and preservation of the genomic integrity (Scott, Trahan et al. 2017). Additionally, the observation that the nucleolus accumulates some of the components of the telomerase complex, which confers protection to the telomere DNA, suggests that the nucleolus is somewhat involved in the synthesis of this complex, although that was not clearly demonstrated. Nevertheless, there are studies reporting telomerase components as playing roles beyond telomere length maintenance. For instance, the nucleolar protein NOLC1 regulates the nucleolar accumulation of the TRF2 protein, a component of mammalian telomerase complex, which impacts the rDNA transcription and cell cycle progression (Yuan, Xu et al. 2018). Therefore, the regulation of cell senescence might be related to the nucleolar localization of telomerase complex components (Rosete, Padros et al. 2007). Contrarily to the other hallmarks of aging, the relationship between nucleolus and cellular senescence has been largely explored and it is described in the section below.

4.2.1 Nucleolar regulation of senescence and lifespan

A senescent cell is defined as a cell which is no more able to divide again (Campisi and d'Adda di Fagagna 2007). This cell state was described by Hayflick in 1965, when he realized that cells are not able to proliferate indefinitely in culture (Hayflick 1965). Forty years later, Campisi et al described the “senescent phenotype”, a set of features commonly found in senescent cells: i) growth arrest, as these cells stop cycling even if favourable growth conditions are present; ii) resistance to apoptosis; iii) altered gene expression, including upregulation of cell cycle kinase inhibitors (Campisi and d'Adda di Fagagna 2007). Interestingly, the “senescent gene expression program” includes the overexpression of proteins able to change tissue microenvironment, which are not directly related with growth arrest (Yoon, Kim et al. 2004). So, it is believed that senescent cells play a role in alteration of structure and function of the aging tissues, supported by the fact that the number of senescent cells increases with age (Campisi 2005).

Notably, studies have demonstrated the power of the nucleolus to regulate cellular senescence. In yeast, premature senescence is induced by mutations in nucleolar proteins and DNA helicases (Rosete, Padros et al. 2007), and chronological aging is associated with nucleolar stress, characterized mainly by nucleolar fragmentation, altered nucleolar size and dysregulated expression of nucleolar proteins (Lewinska, Miedziak et al. 2014). Despite

the presence of changes in nucleolar structure and function found in both humans and yeast, these changes are different. Nucleolar fragmentation does not usually characterize old human cells (Comai 1999). A human senescent cell is morphologically enlarged, displays a flat irregular shape and accumulates stress granules and vacuoles (Goldstein 1990, Campisi 1997). In addition, it often presents an increased activity of the acidic senescence-associated β-galactosidase (SA-β-gal), which is a useful senescence marker (Dimri, Lee et al. 1995).

The link between nucleolus, senescence and aging was originally reported in 1978, in a study showing a decrease in the number of nucleoli concomitant with an increase of nucleolar area and nucleolar dry mass in replicative senescent WI-38 human lung fibroblasts (Bemiller and Lee 1978). But this connection was just resumed in the last two decades, being under focus since then. Recently, nucleolar function has been correlated with lifespan, with small nucleoli being associated with increased lifespan in different organism models such as fruit fly, nematode worm and mouse (Tiku, Jain et al. 2017). Moreover, diminished 60S ribosomal subunit in yeast (Steffen, MacKay et al. 2008) and reduced ribosomal proteins or translation-initiation factors in nematode worm, extended the lifespan (Hansen, Taubert et al. 2007). Importantly, i) as decreased FBL expression was observed in different longevity models and ii) mild repression of FBL by small interference RNA decreased nucleoli size and extended the longevity of adult nematode worm (Tiku, Jain et al. 2017), data strongly suggest longevity as being regulated by FBL expression and nucleolar function (Tiku and Antebi 2018) (Figure 1.8A). Significantly, cells from Hutchinson-Gilford progeria syndrome patients showed increased rDNA transcription, rRNA synthesis, ribosomal proteins production and protein synthesis concomitant with reduced number but enlarged nucleoli, while FBL expression was not consistently upregulated (Buchwalter and Hetzer 2017) (Figure 1.8B).

Furthermore, different signalling pathways are emerging as mediators of this connection between nucleolar function and aging. For instance, downregulation of the insulin/insulin-like growth factor (IGF) signalling pathway was shown to enhance longevity in different models (Kenyon, Chang et al. 1993, Clancy, Gems et al. 2001, Bartke 2008, Kappeler, De Magalhaes Filho et al. 2008, Selman, Lingard et al. 2008), and rDNA transcription was shown to be a major target of this pathway (Wu, Tu et al. 2005). Accordingly, smaller nucleoli were observed in nematode worms exhibiting declined IGF receptor signalling and increased lifespan (Tiku, Jain et al. 2017) (Figure 1.8A). Also, a key component of this pathway, the serine/threonine kinase AKT, was shown to enhance rRNA synthesis, whereas its downregulation diminishes rRNA transcription (Chan, Hannan et al. 2011). Another central linker of nucleolar function and aging is the target of rapamycin (TOR) signalling pathway, which responds directly to nutrient availability by inversely

regulating cell growth and autophagy, i.e., under nutrient deficiency conditions it signals to abolish cell growth while inducing autophagy (Kapahi, Chen et al. 2010). Interestingly, nucleolar factors have risen as key players of autophagy, mainly regulated by the TOR signalling pathway (Pfister 2019), whereas autophagy was shown to promote the production of senescence-associated interleukins (Young, Narita et al. 2009, Kuilman, Michaloglou et al. 2010). Similarly to IGF signalling pathway, TOR downregulation also decreases nucleolar size (Sheaffer, Updike et al. 2008), reduces RNA Pol I-mediated transcription (Grummt, Smith et al. 1976) and increases lifespan of adult nematode worms presumably by decreasing RNA Pol III-dependent transcription (Filer, Thompson et al. 2017) (Figure 1.8A). Another emergent signalling pathway is the NF-κB, which is chronically activated in aging (Osorio, Soria-Valles et al. 2016). Particularly, this signalling pathway elicits senescent cells to secrete several different factors such as cytokines, chemokines, proteases and growth factors (Acosta, Banito et al. 2013), which together constitute the senescence-associated secretory phenotype (SASP) (Borodkina, Deryabin et al. 2018). The SASP is a very powerful mechanism for senescence spreading by supporting cell cycle arrest and showing both autocrine and paracrine effects over neighbouring cells (Borodkina, Deryabin et al. 2018). On the other hand, TIF-IA degradation was shown to induce both increased nucleolar size and NF-κB activation (Chen, Lobb et al. 2018). Despite these important findings, it remains unclear the correlation of NF-κB activation, enlargement of nucleoli and increased senescence (Figure 1.8A). Nevertheless, studies have unveiled a role for NF-κB activation for lifespan of different organisms: in fruit fly brain, high levels of NF-κB signals cause neurodegeneration and shorten the lifespan (Kounatidis, Chtarbanova et al. 2017) and pharmacological suppression of NF-κB extends the fruit fly longevity (Moskalev and Shaposhnikov 2011); in mice, either the downregulation of P65, a subunit of NF-κB, by allelic removal, or pharmacological inhibition of NF-κB resulted in extended lifespan, by decreasing DNA damage and cellular senescence as well as by delaying the onset of age-related pathologies in progeroid model (Tilstra, Robinson et al. 2012). Outstandingly, studies from yeast and nematode worm showed that SIRT1 and FOXO proteins are able to prevent the activation of NF-kB signalling, extending the lifespan of organisms, by counteracting the inflammaging process (Salminen, Ojala et al. 2008), an age-related activation of innate immunity mainly controlled by NF-κB signalling (Salminen, Huuskonen et al. 2008). Altogether, NF-κB configures a very promising and powerful therapeutic target for both extending lifespan (Osorio, Soria-Valles et al. 2016) and even for rejuvenation purposes (Tilstra, Clauson et al. 2011).

Despite the advances on establishing and understanding the connection between nucleolar function and human aging, the literature lacks studies using primary naturally aged human cells at low passages, in order to avoid replicative senescence.

Notwithstanding all limitations that an *in vitro* culture involves (de Magalhaes 1997-2013), that approach will allow to address specific questions about human chronological aging.

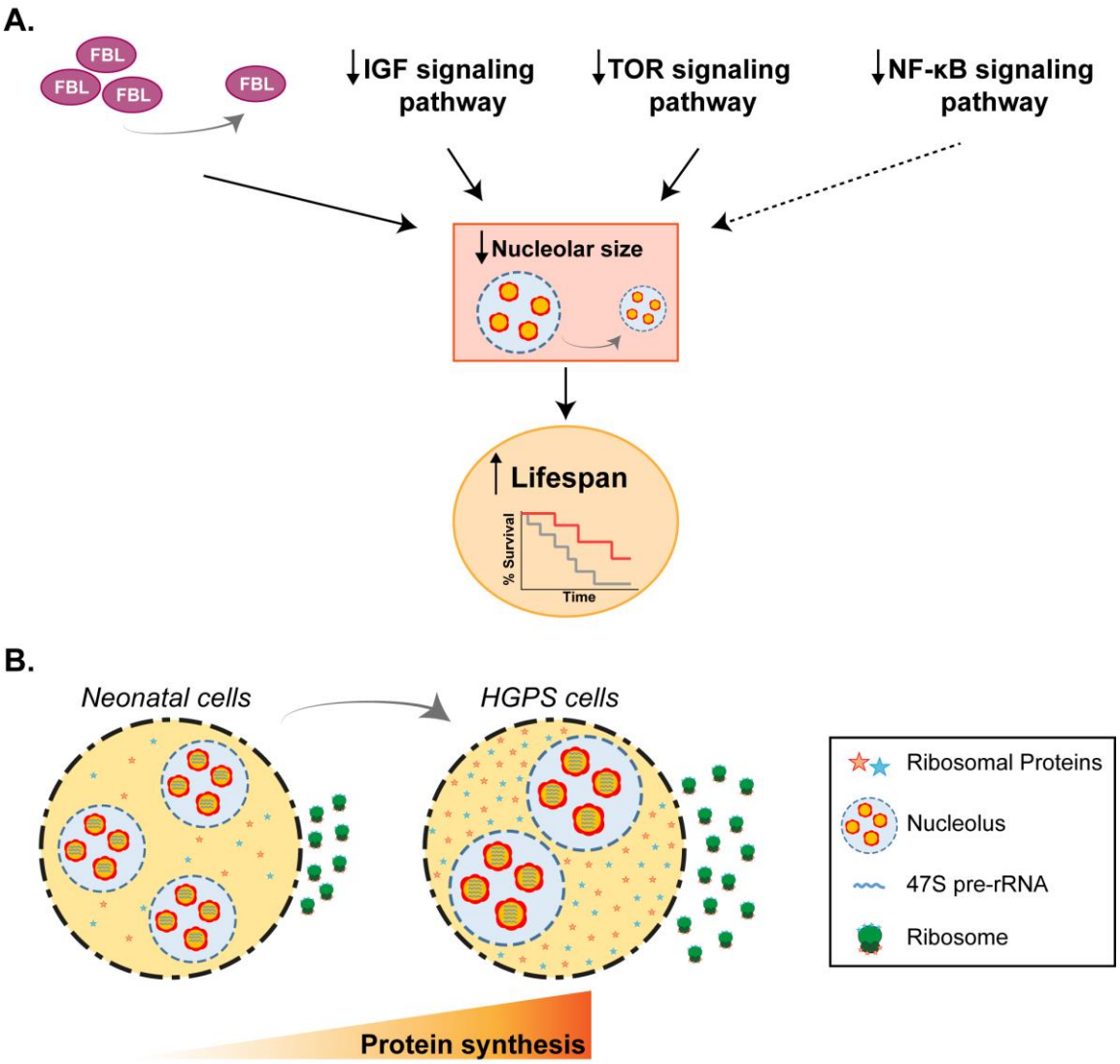

Figure 1.8. Smaller nucleoli are associated with increased lifespan. (A) Downregulation of the FBL nucleolar protein or IGF and TOR signaling leads to the reduction of nucleolar size and ultimately promotes the lifespan extension in different organisms. Downregulation of NF-κB signaling pathway increases lifespan but it is still unclear a direct effect in decreased nucleolar size and increased lifespan (dashed arrow) (see text for details). **(B)** Cells from HGPS (Hutchinson–Gilford progeria syndrome) patients exhibit enlarged nucleoli concomitant with increased 47S pre-rRNA levels, expression of ribosomal proteins (red and blue stars), ribosome production and protein synthesis.

4.3 Nucleolar deregulation in human diseases

Since nucleoli have revealed to be multifunctional, evidence emerged showing their involvement in different human pathologies. Any spontaneous mutation in ribosomal proteins or factors involved in ribosome biogenesis is included on a group of diseases

termed ribosomopathies. For instance, in Diamond-Blackfan anemia (DBA) patients, mutations were only observed in ribosomal proteins, such as RPS19, RPS24, RPS17, RPL5, RPL11 and RPL35A (Narla and Ebert 2010). Although patients could exhibit different clinical phenotypes depending on the ribosomal protein affected, they all show defective production of mature ribosomes (Narla and Ebert 2010), ultimately leading to erythroid failure and anemia, congenital anomalies and susceptibility to develop cancer (Lipton and Ellis 2009) (Figure 1.9A). Another example is the Shwachman-Diamond syndrome, caused by a mutation in the SBDS gene, which was reported to be involved in ribosome biogenesis (Ganapathi, Austin et al. 2007, Wong, Traynor et al. 2011). These patients exhibit bone marrow dysfunction, exocrine pancreatic insufficiency and increased risk to develop leukemia (Shwachman, Diamond et al. 1964, Ganapathi, Austin et al. 2007) (Figure 1.9A). Another ribosomopathy is dyskeratosis congenita, which is characterized by bone marrow failure, skin hyperpigmentation, nail dystrophy and oral leukoplakia (Walne and Dokal 2008). Although it is caused by mutations in different components of the telomerase complex, the more severe phenotype is observed in patients carrying a mutation in DKC1 gene, which encodes for dyskerin protein (Heiss, Knight et al. 1998). Interestingly, beyond its role in preventing telomere shortening (Narla and Ebert 2010), dyskerin was shown to support the pseudouridylation of rRNAs (Liu and Ellis 2006). By now, the contribution of each dyskerin function for the pathophysiology of this disease remains unknown (Narla and Ebert 2010).

Another important set of human diseases whose pathophysiology could be, at least in part, explained by nucleolar deregulation are the neurodegenerative diseases (Yang, Yang et al. 2018). Particularly, the impairment of nucleolar function has been implicated in the process through which degeneration or loss of neurons occurs (Hetman and Pietrzak 2012). For instance, the dopaminergic neurons, which are lost in Parkinson's disease, display decreased nucleolar volume (Mann and Yates 1982) (Figure 1.9B), inversely correlating with the duration of the disease (Gertz, Siegers et al. 1994). Furthermore, NCL was suggested to play a key role in Parkinson's disease, particularly by interacting with mutated RNAs, leading to a decrease of rDNA transcription (Weeks, Metge et al. 2019). Also, the overexpression of NCL in a cellular model of Parkinson's disease revealed a neuroprotective effect, by targeting both oxidative and proteotoxic stress (Caudle, Kitsou et al. 2009). Similarly, in Alzheimer's disease, nucleolar volume of neurons from the CA1-hippocampus area is also decreased (Iacono, O'Brien et al. 2008) (Figure 1.9B). Interestingly, the SIRT1 protein, mentioned above as a positive lifespan contributor, is diminished in both Parkinson and Alzheimer disease patients, which supports the decrease of rDNA transcription that in turn contributes for neurodegeneration (Kreiner, Sonmez et al. 2019). In agreement, several models of both Parkinson's and Alzheimer's disease are

based on loss of function of TIF-IA in specific neuron subpopulations (Yang, Yang et al. 2018). In dopaminergic neurons, downregulation of TIF-IA represses TOR activity, impacting on protein translation (Parlato and Kreiner 2013). Moreover, it was shown that under nucleolar stress these neurons retain the signals for P53-dependent apoptosis (Rieker, Engblom et al. 2011). Based on the liquid-like properties of the nucleolus, a recent work used an optogenetic protein system and showed that, by modulating material properties of the nucleolus, it is possible to affect rRNA processing (Zhu, Richardson et al. 2019). Given that both neurodegenerative diseases and ribosomopathies alter the interaction of molecules inside nucleolus (Kwon, Xiang et al. 2014, Elbaum-Garfinkle and Brangwynne 2015), the modulation of the nucleolar properties appears as a very powerful approach for therapeutic interventions (Zhu, Richardson et al. 2019). Also cardiac diseases have been shown to be associated with nucleolar deregulation (Hariharan and Sussman 2014). Previously, both number and size of nucleoli were indicated as early predictors of myocardial hypertrophy onset (Neuburger, Herget et al. 1998). In a recent study using a model of heart failure induced by pressure overload, authors found that cardiac fibroblasts become senescent, displaying increased nucleolar size and activation of P53 (Kumazawa, Nishimura et al. 2017) (Figure 1.9C). These senescent fibroblasts will then secrete SASP factors, which will induce hypertrophy of cardiomyocytes paracrinally. Therefore, senescent fibroblasts ultimately lead to both cardiac fibrosis and hypertrophy, aggravating heart failure (Kumazawa, Nishimura et al. 2017) (Figure 1.9C). Lastly, a model was proposed in which the nucleolus plays a role in autoimmune diseases (Brooks 2017). This model is based on the observation that nucleolar size increases in response to stress and predicts that this leads to the engulfment of the inactive X-chromosome. Consequently, this chromosome will be exposed to a large number of nucleolar polyamines, ultimately leading to the overexpression of X-linked genes, generating abnormal DNA, RNA and RNP conformations. Once stabilized, they will give rise to autoantigenic complexes (Brooks 2017).

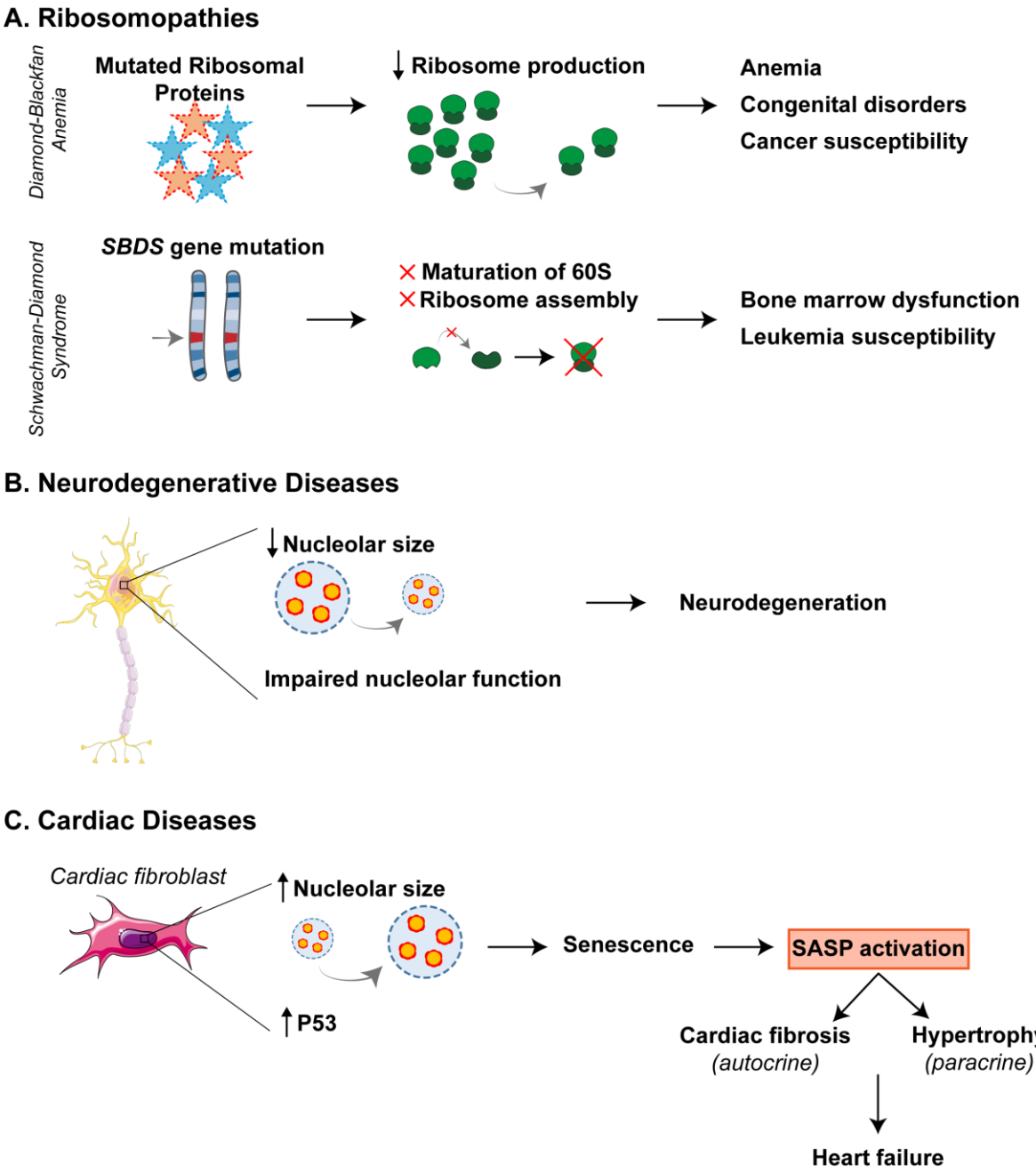

Figure 1.9. **Nucleolar structure and function are involved in several human diseases. (A)** In the Diamond-Blackfan anemia *(top line)*, mutations in different ribosomal proteins (red and blue stars) decrease ribosome biogenesis leading to a common phenotype. In the Schwachman-Diamond syndrome, a recessive mutation on *SBDS* gene blunts the maturation of the ribosomal 60S large subunit (light green), preventing its assembly with the ribosomal 40S small subunit (dark green), resulting in several dysfunctions in patients. **(B)** Neurons displaying a reduced nucleolar size or impaired nucleolar function, as those from Parkinson's and Alzheimer's diseases, have been associated with neurodegeneration. **(C)** Cardiac fibroblasts from a heart failure model exhibit an increased nucleolar size along with P53 accumulation, becoming senescent. Once senescent, they secrete SASP factors that will aggravate heart failure by promoting fibrosis (in an autocrine manner) and eliciting hypertrophy of cardiomyocytes (in a paracrine manner).

5. AIMS

The main goal of this thesis was to explore the role of nucleolar stress in senescence and aging. Specifically, we aimed to: i) characterize the role of NOL12 in nucleolar structure and function by measuring morphological nucleolar parameters and ribosome biogenesis in NOL12-depleted human dermal fibroblasts from neonatal skin; ii) ascertain the correlation between NOL12 downregulation-driven nucleolar stress and human advancing age by using human primary fibroblasts from healthy donors with different ages; iii) dissect the role of the age-associated nuclear protein, FoxM1, in nucleolar homeostasis; iv) determine the role of P53-dependent and P53-independent mechanisms in response to nucleolar stress, particularly in cell proliferation and senescence; v) identify FoxM1 nucleolar transcriptional targets that link nucleolar homeostasis, cell proliferation and aging by interrogating RNA sequencing datasets from FoxM1-depleted neonatal fibroblasts and from elderly donors' fibroblasts.

6. REFERENCES

Abida, W. M., A. Nikolaev, W. Zhao, W. Zhang and W. Gu (2007). "FBXO11 promotes the Neddylation of p53 and inhibits its transcriptional activity." J Biol Chem 282(3): 1797-1804.

Acosta, J. C., A. Banito, T. Wuestefeld, A. Georgilis, P. Janich, J. P. Morton, D. Athineos, T. W. Kang, F. Lasitschka, M. Andrulis, G. Pascual, K. J. Morris, S. Khan, H. Jin, G. Dharmalingam, A. P. Snijders, T. Carroll, D. Capper, C. Pritchard, G. J. Inman, T. Longerich, O. J. Sansom, S. A. Benitah, L. Zender and J. Gil (2013). "A complex secretory program orchestrated by the inflammasome controls paracrine senescence." Nat Cell Biol 15(8): 978-990.

Amin, M. A., S. Matsunaga, N. Ma, H. Takata, M. Yokoyama, S. Uchiyama and K. Fukui (2007). "Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells." Biochem Biophys Res Commun 360(2): 320-326.

Amsterdam, A., K. C. Sadler, K. Lai, S. Farrington, R. T. Bronson, J. A. Lees and N. Hopkins (2004). "Many ribosomal protein genes are cancer genes in zebrafish." PLoS Biol 2(5): E139.

Andersen, J. S., Y. W. Lam, A. K. Leung, S. E. Ong, C. E. Lyon, A. I. Lamond and M. Mann (2005). "Nucleolar proteome dynamics." Nature 433(7021): 77-83.

Angelier, N., M. Tramier, E. Louvet, M. Coppey-Moisan, T. M. Savino, J. R. De Mey and D. Hernandez-Verdun (2005). "Tracking the interactions of rRNA processing proteins during nucleolar assembly in living cells." Mol Biol Cell 16(6): 2862-2871.

Arabi, A., S. Wu, K. Ridderstrale, H. Bierhoff, C. Shiue, K. Fatyol, S. Fahlen, P. Hydbring, O. Soderberg, I. Grummt, L. G. Larsson and A. P. Wright (2005). "c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription." Nat Cell Biol 7(3): 303-310.

Artero-Castro, A., J. Castellvi, A. Garcia, J. Hernandez, S. Ramon y Cajal and M. E. Lleonart (2011). "Expression of the ribosomal proteins Rplp0, Rplp1, and Rplp2 in gynecologic tumors." Hum Pathol 42(2): 194-203.

Aubert, M., M. F. O'Donohue, S. Lebaron and P. E. Gleizes (2018). "Pre-Ribosomal RNA Processing in Human Cells: From Mechanisms to Congenital Diseases." Biomolecules 8(4).

Bailly, A., A. Perrin, L. J. Bou Malhab, E. Pion, M. Larance, M. Nagala, P. Smith, M. F. O'Donohue, P. E. Gleizes, J. Zomerdijk, A. I. Lamond and D. P. Xirodimas (2016). "The NEDD8 inhibitor MLN4924 increases the size of the nucleolus and activates p53 through the ribosomal-Mdm2 pathway." Oncogene 35(4): 415-426.

Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. vanTuinen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White and B. Vogelstein (1989). "Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas." Science 244(4901): 217-221.

Barna, M., A. Pusic, O. Zollo, M. Costa, N. Kondrashov, E. Rego, P. H. Rao and D. Ruggero (2008). "Suppression of Myc oncogenic activity by ribosomal protein haploinsufficiency." Nature 456(7224): 971-975.

Bartke, A. (2008). "Insulin and aging." Cell Cycle 7(21): 3338-3343.

Bastide, A. and A. David (2018). "The ribosome, (slow) beating heart of cancer (stem) cell." Oncogenesis 7(4): 34.

Bazett-Jones, D. P., B. Leblanc, M. Herfort and T. Moss (1994). "Short-range DNA looping by the Xenopus HMG-box transcription factor, xUBF." Science 264(5162): 1134-1137.

Beck-Engeser, G. B., P. A. Monach, D. Mumberg, F. Yang, S. Wanderling, K. Schreiber, R. Espinosa, 3rd, M. M. Le Beau, S. C. Meredith and H. Schreiber (2001). "Point mutation in essential genes with loss or mutation of the second allele: relevance to the retention of tumor-specific antigens." J Exp Med 194(3): 285-300.

Beghin, A., S. Belin, R. Hage-Sleiman, S. Brunet Manquat, S. Goddard, E. Tabone, L. P. Jordheim, I. Treilleux, M. F. Poupon, J. J. Diaz and C. Dumontet (2009). "ADP ribosylation factor like 2 (Arl2) regulates breast tumor aggressivity in immunodeficient mice." PLoS One 4(10): e7478.

Belin, S., A. Beghin, E. Solano-Gonzalez, L. Bezin, S. Brunet-Manquat, J. Textoris, A. C. Prats, H. C. Mertani, C. Dumontet and J. J. Diaz (2009). "Dysregulation of ribosome

biogenesis and translational capacity is associated with tumor progression of human breast cancer cells." PLoS One 4(9): e7147.

Bemiller, P. M. and L. H. Lee (1978). "Nucleolar changes in senescing WI-38 cells." Mech Ageing Dev 8(6): 417-427.

Ben-Shem, A., N. Garreau de Loubresse, S. Melnikov, L. Jenner, G. Yusupova and M. Yusupov (2011). "The structure of the eukaryotic ribosome at 3.0 A resolution." Science 334(6062): 1524-1529.

Boisvert, F. M., S. van Koningsbruggen, J. Navascues and A. I. Lamond (2007). "The multifunctional nucleolus." Nat Rev Mol Cell Biol 8(7): 574-585.

Bolze, A., N. Mahlaoui, M. Byun, B. Turner, N. Trede, S. R. Ellis, A. Abhyankar, Y. Itan, E. Patin, S. Brebner, P. Sackstein, A. Puel, C. Picard, L. Abel, L. Quintana-Murci, S. N. Faust, A. P. Williams, R. Baretto, M. Duddridge, U. Kini, A. J. Pollard, C. Gaud, P. Frange, D. Orbach, J. F. Emile, J. L. Stephan, R. Sorensen, A. Plebani, L. Hammarstrom, M. E. Conley, L. Selleri and J. L. Casanova (2013). "Ribosomal protein SA haploinsufficiency in humans with isolated congenital asplenia." Science 340(6135): 976-978.

Borer, R. A., C. F. Lehner, H. M. Eppenberger and E. A. Nigg (1989). "Major nucleolar proteins shuttle between nucleus and cytoplasm." Cell 56(3): 379-390.

Borodkina, A. V., P. I. Deryabin, A. A. Giukova and N. N. Nikolsky (2018). ""Social Life" of Senescent Cells: What Is SASP and Why Study It?" Acta Naturae 10(1): 4-14.

Bortoluzzi, S., F. d'Alessi, C. Romualdi and G. A. Danieli (2001). "Differential expression of genes coding for ribosomal proteins in different human tissues." Bioinformatics 17(12): 1152-1157.

Boulon, S., B. J. Westman, S. Hutten, F. M. Boisvert and A. I. Lamond (2010). "The nucleolus under stress." Mol Cell 40(2): 216-227.

Box, J. K., N. Paquet, M. N. Adams, D. Boucher, E. Bolderson, K. J. O'Byrne and D. J. Richard (2016). "Nucleophosmin: from structure and function to disease development." BMC Mol Biol 17(1): 19.

Brangwynne, C. P., C. R. Eckmann, D. S. Courson, A. Rybarska, C. Hoege, J. Gharakhani, F. Julicher and A. A. Hyman (2009). "Germline P granules are liquid droplets that localize by controlled dissolution/condensation." Science 324(5935): 1729-1732.

Brangwynne, C. P., T. J. Mitchison and A. A. Hyman (2011). "Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes." Proc Natl Acad Sci U S A 108(11): 4334-4339.

Brooks, C. L. and W. Gu (2011). "p53 regulation by ubiquitin." FEBS Lett 585(18): 2803-2809.

Brooks, C. L., M. Li and W. Gu (2004). "Monoubiquitination: the signal for p53 nuclear export?" Cell Cycle 3(4): 436-438.

Brooks, W. H. (2017). "Viral Impact in Autoimmune Diseases: Expanding the "X Chromosome-Nucleolus Nexus" Hypothesis." Front Immunol 8: 1657.

Brown, D. D. and J. B. Gurdon (1964). "Absence of Ribosomal Rna Synthesis in the Anucleolate Mutant of Xenopus Laevis." Proc Natl Acad Sci U S A 51: 139-146.

Buchwalter, A. and M. W. Hetzer (2017). "Nucleolar expansion and elevated protein translation in premature aging." Nat Commun 8(1): 328.

Bursac, S., M. C. Brdovcak, M. Pfannkuchen, I. Orsolic, L. Golomb, Y. Zhu, C. Katz, L. Daftuar, K. Grabusic, I. Vukelic, V. Filic, M. Oren, C. Prives and S. Volarevic (2012). "Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress." Proc Natl Acad Sci U S A 109(50): 20467-20472.

Campisi, J. (1997). "The biology of replicative senescence." Eur J Cancer 33(5): 703-709.

Campisi, J. (2005). "Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors." Cell 120(4): 513-522.

Campisi, J. and F. d'Adda di Fagagna (2007). "Cellular senescence: when bad things happen to good cells." Nat Rev Mol Cell Biol 8(9): 729-740.

Caragine, C. M., S. C. Haley and A. Zidovska (2018). "Surface Fluctuations and Coalescence of Nucleolar Droplets in the Human Cell Nucleus." Phys Rev Lett 121(14): 148101.

Caudle, W. M., E. Kitsou, J. Li, J. Bradner and J. Zhang (2009). "A role for a novel protein, nucleolin, in Parkinson's disease." Neurosci Lett 459(1): 11-15.

Chan, J. C., K. M. Hannan, K. Riddell, P. Y. Ng, A. Peck, R. S. Lee, S. Hung, M. V. Astle, M. Bywater, M. Wall, G. Poortinga, K. Jastrzebski, K. E. Sheppard, B. A. Hemmings, M. N. Hall, R. W. Johnstone, G. A. McArthur, R. D. Hannan and R. B. Pearson (2011). "AKT promotes rRNA synthesis and cooperates with c-MYC to stimulate ribosome biogenesis in cancer." Sci Signal 4(188): ra56.

Chen, D., M. Dundr, C. Wang, A. Leung, A. Lamond, T. Misteli and S. Huang (2005). "Condensed mitotic chromatin is accessible to transcription factors and chromatin structural proteins." J Cell Biol 168(1): 41-54.

Chen, D., Z. Zhang, M. Li, W. Wang, Y. Li, E. R. Rayburn, D. L. Hill, H. Wang and R. Zhang (2007). "Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function." Oncogene 26(35): 5029-5037.

Chen, J., I. T. Lobb, P. Morin, S. M. Novo, J. Simpson, K. Kennerknecht, A. von Kriegsheim, E. E. Batchelor, F. Oakley and L. A. Stark (2018). "Identification of a novel TIF-IA-NF-kappaB nucleolar stress response pathway." Nucleic Acids Res 46(12): 6188-6205.

Chen, J. and L. A. Stark (2019). "Insights into the Relationship between Nucleolar Stress and the NF-kappaB Pathway." Trends Genet 35(10): 768-780.

Chen, Z. and X. Xu (2016). "Roles of nucleolin. Focus on cancer and anti-cancer therapy." Saudi Med J 37(12): 1312-1318.

Chene, P. (2001). "The role of tetramerization in p53 function." Oncogene 20(21): 2611-2617.

Chi, S. W., S. H. Lee, D. H. Kim, M. J. Ahn, J. S. Kim, J. Y. Woo, T. Torizawa, M. Kainosho and K. H. Han (2005). "Structural details on mdm2-p53 interaction." J Biol Chem 280(46): 38795-38802.

Chipuk, J. E., T. Kuwana, L. Bouchier-Hayes, N. M. Droin, D. D. Newmeyer, M. Schuler and D. R. Green (2004). "Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis." Science 303(5660): 1010-1014.

Choi, P. and C. Chen (2005). "Genetic expression profiles and biologic pathway alterations in head and neck squamous cell carcinoma." Cancer 104(6): 1113-1128.

Ciganda, M. and N. Williams (2011). "Eukaryotic 5S rRNA biogenesis." Wiley Interdiscip Rev RNA 2(4): 523-533.

Clancy, D. J., D. Gems, L. G. Harshman, S. Oldham, H. Stocker, E. Hafen, S. J. Leevers and L. Partridge (2001). "Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein." Science 292(5514): 104-106.

Colgan, D. F. and J. L. Manley (1997). "Mechanism and regulation of mRNA polyadenylation." Genes Dev 11(21): 2755-2766.

Colombo, E., M. Alcalay and P. G. Pelicci (2011). "Nucleophosmin and its complex network: a possible therapeutic target in hematological diseases." Oncogene 30(23): 2595-2609.

Colombo, E., J. C. Marine, D. Danovi, B. Falini and P. G. Pelicci (2002). "Nucleophosmin regulates the stability and transcriptional activity of p53." Nat Cell Biol 4(7): 529-533.

Comai, L. (1999). "The nucleolus: a paradigm for cell proliferation and aging." Braz J Med Biol Res 32(12): 1473-1478.

Cooper, G. M. (2000). The Cell: A Molecular Approach. S. M. S. Associates.

Daftuar, L., Y. Zhu, X. Jacq and C. Prives (2013). "Ribosomal proteins RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network." PLoS One 8(7): e68667.

Dai, M. S. and H. Lu (2008). "Crosstalk between c-Myc and ribosome in ribosomal biogenesis and cancer." J Cell Biochem 105(3): 670-677.

Dai, M. S., R. Sears and H. Lu (2007). "Feedback regulation of c-Myc by ribosomal protein L11." Cell Cycle 6(22): 2735-2741.

Dai, M. S., X. X. Sun and H. Lu (2008). "Aberrant expression of nucleostemin activates p53 and induces cell cycle arrest via inhibition of MDM2." Mol Cell Biol 28(13): 4365-4376.

Dai, M. S., S. X. Zeng, Y. Jin, X. X. Sun, L. David and H. Lu (2004). "Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition." Mol Cell Biol 24(17): 7654-7668.

de Magalhaes, J. P. (1997-2013). "senescence.info: http://www.senescence.info."

Deisenroth, C. and Y. Zhang (2010). "Ribosome biogenesis surveillance: probing the ribosomal protein-Mdm2-p53 pathway." Oncogene 29(30): 4253-4260.

Deisenroth, C. and Y. Zhang (2011). "The Ribosomal Protein-Mdm2-p53 Pathway and Energy Metabolism: Bridging the Gap between Feast and Famine." Genes Cancer 2(4): 392-403.

Derenzini, M. and D. Ploton (1991). "Interphase nucleolar organizer regions in cancer cells." Int Rev Exp Pathol 32: 149-192.

Derenzini, M., D. Trere, A. Pession, M. Govoni, V. Sirri and P. Chieco (2000). "Nucleolar size indicates the rapidity of cell proliferation in cancer tissues." J Pathol 191(2): 181-186.

Di Ventura, B., C. Funaya, C. Antony, M. Knop and L. Serrano (2008). "Reconstitution of Mdm2-dependent post-translational modifications of p53 in yeast." PLoS One 3(1): e1507.

Dimario, P. J. (2004). "Cell and molecular biology of nucleolar assembly and disassembly." Int Rev Cytol 239: 99-178.

Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith and et al. (1995). "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." Proc Natl Acad Sci U S A 92(20): 9363-9367.

Donati, G., E. Brighenti, M. Vici, G. Mazzini, D. Trere, L. Montanaro and M. Derenzini (2011). "Selective inhibition of rRNA transcription downregulates E2F-1: a new p53-independent mechanism linking cell growth to cell proliferation." J Cell Sci 124(Pt 17): 3017-3028.

Donati, G., S. Peddigari, C. A. Mercer and G. Thomas (2013). "5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint." Cell Rep 4(1): 87-98.

Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel and A. Bradley (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature 356(6366): 215-221.

Dousset, T., C. Wang, C. Verheggen, D. Chen, D. Hernandez-Verdun and S. Huang (2000). "Initiation of nucleolar assembly is independent of RNA polymerase I transcription." Mol Biol Cell 11(8): 2705-2717.

Dundr, M., U. T. Meier, N. Lewis, D. Rekosh, M. L. Hammarskjold and M. O. Olson (1997). "A class of nonribosomal nucleolar components is located in chromosome periphery and in nucleolus-derived foci during anaphase and telophase." Chromosoma 105(7-8): 407-417.

Dundr, M., T. Misteli and M. O. Olson (2000). "The dynamics of postmitotic reassembly of the nucleolus." J Cell Biol 150(3): 433-446.

Elbaum-Garfinkle, S. and C. P. Brangwynne (2015). "Liquids, Fibers, and Gels: The Many Phases of Neurodegeneration." Dev Cell 35(5): 531-532.

Eliyahu, D., A. Raz, P. Gruss, D. Givol and M. Oren (1984). "Participation of p53 cellular tumour antigen in transformation of normal embryonic cells." Nature 312(5995): 646-649.

Ensminger, A. W. and A. Chess (2004). "Coordinated replication timing of monoallelically expressed genes along human autosomes." Hum Mol Genet 13(6): 651-658.

Farley, K. I., Y. Surovtseva, J. Merkel and S. J. Baserga (2015). "Determinants of mammalian nucleolar architecture." Chromosoma 124(3): 323-331.

Feric, M., N. Vaidya, T. S. Harmon, D. M. Mitrea, L. Zhu, T. M. Richardson, R. W. Kriwacki, R. V. Pappu and C. P. Brangwynne (2016). "Coexisting Liquid Phases Underlie Nucleolar Subcompartments." Cell 165(7): 1686-1697.

Filer, D., M. A. Thompson, V. Takhaveev, A. J. Dobson, I. Kotronaki, J. W. M. Green, M. Heinemann, J. M. A. Tullet and N. Alic (2017). "RNA polymerase III limits longevity downstream of TORC1." Nature 552(7684): 263-267.

Fischer, M., L. Steiner and K. Engeland (2014). "The transcription factor p53: not a repressor, solely an activator." Cell Cycle 13(19): 3037-3058.

Fischer, N. W., A. Prodeus, D. Malkin and J. Gariepy (2016). "p53 oligomerization status modulates cell fate decisions between growth, arrest and apoptosis." Cell Cycle 15(23): 3210-3219.

Fuchs, G., C. Diges, L. A. Kohlstaedt, K. A. Wehner and P. Sarnow (2011). "Proteomic analysis of ribosomes: translational control of mRNA populations by glycogen synthase GYS1." J Mol Biol 410(1): 118-130.

Gall, J. G. (2000). "Cajal bodies: the first 100 years." Annu Rev Cell Dev Biol 16: 273-300.

Ganapathi, K. A., K. M. Austin, C. S. Lee, A. Dias, M. M. Malsch, R. Reed and A. Shimamura (2007). "The human Shwachman-Diamond syndrome protein, SBDS, associates with ribosomal RNA." Blood 110(5): 1458-1465.

Gautier, T., M. Robert-Nicoud, M. N. Guilly and D. Hernandez-Verdun (1992). "Relocation of nucleolar proteins around chromosomes at mitosis. A study by confocal laser scanning microscopy." J Cell Sci 102 (Pt 4): 729-737.

Gavet, O. and J. Pines (2010). "Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis." Dev Cell 18(4): 533-543.

Gertz, H. J., A. Siegers and J. Kuchinke (1994). "Stability of cell size and nucleolar size in Lewy body containing neurons of substantia nigra in Parkinson's disease." Brain Res 637(1-2): 339-341.

Goldstein, M., F. A. Derheimer, J. Tait-Mulder and M. B. Kastan (2013). "Nucleolin mediates nucleosome disruption critical for DNA double-strand break repair." Proc Natl Acad Sci U S A 110(42): 16874-16879.

Goldstein, S. (1990). "Replicative senescence: the human fibroblast comes of age." Science 249(4973): 1129-1133.

Gorlich, D. and U. Kutay (1999). "Transport between the cell nucleus and the cytoplasm." Annu Rev Cell Dev Biol 15: 607-660.

Grandori, C., N. Gomez-Roman, Z. A. Felton-Edkins, C. Ngouenet, D. A. Galloway, R. N. Eisenman and R. J. White (2005). "c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I." Nat Cell Biol 7(3): 311-318.

Grummt, I. (2007). "Different epigenetic layers engage in complex crosstalk to define the epigenetic state of mammalian rRNA genes." Hum Mol Genet 16 Spec No 1: R21-27.

Grummt, I., V. A. Smith and F. Grummt (1976). "Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase." Cell 7(3): 439-445.

Guarente, L. (1997). "Link between aging and the nucleolus." Genes Dev 11(19): 2449-2455.

Gupta, V. and J. R. Warner (2014). "Ribosome-omics of the human ribosome." RNA 20(7): 1004-1013.

Guttinger, S., E. Laurell and U. Kutay (2009). "Orchestrating nuclear envelope disassembly and reassembly during mitosis." Nat Rev Mol Cell Biol 10(3): 178-191.

Hansen, M., S. Taubert, D. Crawford, N. Libina, S. J. Lee and C. Kenyon (2007). "Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans." Aging Cell 6(1): 95-110.

Hariharan, N. and M. A. Sussman (2014). "Stressing on the nucleolus in cardiovascular disease." Biochim Biophys Acta 1842(6): 798-801.

Harms, K. L. and X. Chen (2006). "The functional domains in p53 family proteins exhibit both common and distinct properties." Cell Death Differ 13(6): 890-897.

Hayflick, L. (1965). "The Limited in Vitro Lifetime of Human Diploid Cell Strains." Exp Cell Res 37: 614-636.

Heiss, N. S., S. W. Knight, T. J. Vulliamy, S. M. Klauck, S. Wiemann, P. J. Mason, A. Poustka and I. Dokal (1998). "X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions." Nat Genet 19(1): 32-38.

Heix, J., A. Vente, R. Voit, A. Budde, T. M. Michaelidis and I. Grummt (1998). "Mitotic silencing of human rRNA synthesis: inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation." EMBO J 17(24): 7373-7381.

Henras, A. K., C. Plisson-Chastang, M. F. O'Donohue, A. Chakraborty and P. E. Gleizes (2015). "An overview of pre-ribosomal RNA processing in eukaryotes." Wiley Interdiscip Rev RNA 6(2): 225-242.

Hernandez-Verdun, D. (2006). "The nucleolus: a model for the organization of nuclear functions." Histochem Cell Biol 126(2): 135-148.

Hernandez-Verdun, D. (2006). "Nucleolus: from structure to dynamics." Histochem Cell Biol 125(1-2): 127-137.

Hernandez-Verdun, D. (2011). "Assembly and disassembly of the nucleolus during the cell cycle." Nucleus 2(3): 189-194.

Hernandez-Verdun, D., P. Roussel and T. Gautier (1993). "Nucleolar proteins during mitosis." Chromosomes Today: 79-90.

Hernandez-Verdun, D., P. Roussel and J. Gebrane-Younes (2002). "Emerging concepts of nucleolar assembly." J Cell Sci 115(Pt 11): 2265-2270.

Hetman, M. and M. Pietrzak (2012). "Emerging roles of the neuronal nucleolus." Trends Neurosci 35(5): 305-314.

Holmberg Olausson, K., M. Nister and M. S. Lindstrom (2012). "p53 -Dependent and -Independent Nucleolar Stress Responses." Cells 1(4): 774-798.

Hyman, A. A., C. A. Weber and F. Julicher (2014). "Liquid-liquid phase separation in biology." Annu Rev Cell Dev Biol 30: 39-58.

Iacono, D., R. O'Brien, S. M. Resnick, A. B. Zonderman, O. Pletnikova, G. Rudow, Y. An, M. J. West, B. Crain and J. C. Troncoso (2008). "Neuronal hypertrophy in asymptomatic Alzheimer disease." J Neuropathol Exp Neurol 67(6): 578-589.

Iadevaia, V., S. Caldarola, L. Biondini, A. Gismondi, S. Karlsson, I. Dianzani and F. Loreni (2010). "PIM1 kinase is destabilized by ribosomal stress causing inhibition of cell cycle progression." Oncogene 29(40): 5490-5499.

Itahana, K., H. Mao, A. Jin, Y. Itahana, H. V. Clegg, M. S. Lindstrom, K. P. Bhat, V. L. Godfrey, G. I. Evan and Y. Zhang (2007). "Targeted inactivation of Mdm2 RING finger

E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation." Cancer Cell 12(4): 355-366.

Jack, K., C. Bellodi, D. M. Landry, R. O. Niederer, A. Meskauskas, S. Musalgaonkar, N. Kopmar, O. Krasnykh, A. M. Dean, S. R. Thompson, D. Ruggero and J. D. Dinman (2011). "rRNA pseudouridylation defects affect ribosomal ligand binding and translational fidelity from yeast to human cells." Mol Cell 44(4): 660-666.

James, A., Y. Wang, H. Raje, R. Rosby and P. DiMario (2014). "Nucleolar stress with and without p53." Nucleus 5(5): 402-426.

Jarvelin, A. I., M. Noerenberg, I. Davis and A. Castello (2016). "The new (dis)order in RNA regulation." Cell Commun Signal 14: 9.

Kalmarova, M., E. Smirnov, M. Masata, K. Koberna, A. Ligasova, A. Popov and I. Raska (2007). "Positioning of NORs and NOR-bearing chromosomes in relation to nucleoli." J Struct Biol 160(1): 49-56.

Kannappan, R., S. Mattapally, P. A. Wagle and J. Zhang (2018). "Transactivation domain of p53 regulates DNA repair and integrity in human iPS cells." Am J Physiol Heart Circ Physiol 315(3): H512-H521.

Kapahi, P., D. Chen, A. N. Rogers, S. D. Katewa, P. W. Li, E. L. Thomas and L. Kockel (2010). "With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging." Cell Metab 11(6): 453-465.

Kappeler, L., C. De Magalhaes Filho, J. Dupont, P. Leneuve, P. Cervera, L. Perin, C. Loudes, A. Blaise, R. Klein, J. Epelbaum, Y. Le Bouc and M. Holzenberger (2008). "Brain IGF-1 receptors control mammalian growth and lifespan through a neuroendocrine mechanism." PLoS Biol 6(10): e254.

Kasai, H., D. Nadano, E. Hidaka, K. Higuchi, M. Kawakubo, T. A. Sato and J. Nakayama (2003). "Differential expression of ribosomal proteins in human normal and neoplastic colorectum." J Histochem Cytochem 51(5): 567-574.

Kawamura, K., F. Qi, Q. Meng, I. Hayashi and J. Kobayashi (2019). "Nucleolar protein nucleolin functions in replication stress-induced DNA damage responses." J Radiat Res 60(3): 281-288.

Kenyon, C., J. Chang, E. Gensch, A. Rudner and R. Tabtiang (1993). "A C. elegans mutant that lives twice as long as wild type." Nature 366(6454): 461-464.

Knibiehler, B., C. Mirre and R. Rosset (1982). "Nucleolar organizer structure and activity in a nucleolus without fibrillar centres: the nucleolus in an established Drosophila cell line." J Cell Sci 57: 351-364.

Kobayashi, J., H. Fujimoto, J. Sato, I. Hayashi, S. Burma, S. Matsuura, D. J. Chen and K. Komatsu (2012). "Nucleolin participates in DNA double-strand break-induced damage response through MDC1-dependent pathway." PLoS One 7(11): e49245.

Koerte, A., T. Chong, X. Li, K. Wahane and M. Cai (1995). "Suppression of the yeast mutation rft1-1 by human p53." J Biol Chem 270(38): 22556-22564.

Koh, C. M., B. Gurel, S. Sutcliffe, M. J. Aryee, D. Schultz, T. Iwata, M. Uemura, K. I. Zeller, U. Anele, Q. Zheng, J. L. Hicks, W. G. Nelson, C. V. Dang, S. Yegnasubramanian and A. M. De Marzo (2011). "Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene." Am J Pathol 178(4): 1824-1834.

Komar, A. A. and M. Hatzoglou (2011). "Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states." Cell Cycle 10(2): 229-240.

Kondrashov, N., A. Pusic, C. R. Stumpf, K. Shimizu, A. C. Hsieh, J. Ishijima, T. Shiroishi and M. Barna (2011). "Ribosome-mediated specificity in Hox mRNA translation and vertebrate tissue patterning." Cell 145(3): 383-397.

Kounatidis, I., S. Chtarbanova, Y. Cao, M. Hayne, D. Jayanth, B. Ganetzky and P. Ligoxygakis (2017). "NF-kappaB Immunity in the Brain Determines Fly Lifespan in Healthy Aging and Age-Related Neurodegeneration." Cell Rep 19(4): 836-848.

Kreiner, G., A. Sonmez, B. Liss and R. Parlato (2019). "Integration of the Deacetylase SIRT1 in the Response to Nucleolar Stress: Metabolic Implications for Neurodegenerative Diseases." Front Mol Neurosci 12: 106.

Kuai, L., F. Fang, J. S. Butler and F. Sherman (2004). "Polyadenylation of rRNA in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A 101(23): 8581-8586.

Kufel, J. and P. Grzechnik (2019). "Small Nucleolar RNAs Tell a Different Tale." Trends Genet 35(2): 104-117.

Kuilman, T., C. Michaloglou, W. J. Mooi and D. S. Peeper (2010). "The essence of senescence." Genes Dev 24(22): 2463-2479.

Kumazawa, T., K. Nishimura and Y. Saito (2017). "Nucleolus Mediated Cellular Senescence is Involved in Cardiac Hypertrophy." Circulation 136(suppl_1): Abstract 20208.

Kushner, S. R. (2004). "mRNA decay in prokaryotes and eukaryotes: different approaches to a similar problem." IUBMB Life 56(10): 585-594.

Kwon, I., S. Xiang, M. Kato, L. Wu, P. Theodoropoulos, T. Wang, J. Kim, J. Yun, Y. Xie and S. L. McKnight (2014). "Poly-dipeptides encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill cells." Science 345(6201): 1139-1145.

Lafontaine, D. L. (2015). "Noncoding RNAs in eukaryotic ribosome biogenesis and function." Nat Struct Mol Biol 22(1): 11-19.

Lafontaine, D. L. and D. Tollervey (2001). "The function and synthesis of ribosomes." Nat Rev Mol Cell Biol 2(7): 514-520.

Lewinska, A., B. Miedziak, K. Kulak, M. Molon and M. Wnuk (2014). "Links between nucleolar activity, rDNA stability, aneuploidy and chronological aging in the yeast Saccharomyces cerevisiae." Biogerontology 15(3): 289-316.

Lindstrom, M. S., D. Jurada, S. Bursac, I. Orsolic, J. Bartek and S. Volarevic (2018). "Nucleolus as an emerging hub in maintenance of genome stability and cancer pathogenesis." Oncogene 37(18): 2351-2366.

Lipton, J. M. and S. R. Ellis (2009). "Diamond-Blackfan anemia: diagnosis, treatment, and molecular pathogenesis." Hematol Oncol Clin North Am 23(2): 261-282.

Liu, J. M. and S. R. Ellis (2006). "Ribosomes and marrow failure: coincidental association or molecular paradigm?" Blood 107(12): 4583-4588.

Lopes, A. M., R. N. Miguel, C. A. Sargent, P. J. Ellis, A. Amorim and N. A. Affara (2010). "The human RPS4 paralogue on Yq11.223 encodes a structurally conserved ribosomal protein and is preferentially expressed during spermatogenesis." BMC Mol Biol 11: 33.

Lopez-Otin, C., M. A. Blasco, L. Partridge, M. Serrano and G. Kroemer (2013). "The hallmarks of aging." Cell 153(6): 1194-1217.

Loughery, J., M. Cox, L. M. Smith and D. W. Meek (2014). "Critical role for p53-serine 15 phosphorylation in stimulating transactivation at p53-responsive promoters." Nucleic Acids Res 42(12): 7666-7680.

Lu, H. (2011). "A ribosomal tactic to halt cancer." Nat Med 17(8): 930-931.

Ma, N., S. Matsunaga, H. Takata, R. Ono-Maniwa, S. Uchiyama and K. Fukui (2007). "Nucleolin functions in nucleolus formation and chromosome congression." J Cell Sci 120(Pt 12): 2091-2105.

Maggi, L. B., Jr., M. Kuchenruether, D. Y. Dadey, R. M. Schwope, S. Grisendi, R. R. Townsend, P. P. Pandolfi and J. D. Weber (2008). "Nucleophosmin serves as a rate-limiting nuclear export chaperone for the Mammalian ribosome." Mol Cell Biol 28(23): 7050-7065.

Mahlaoui, N., V. Minard-Colin, C. Picard, A. Bolze, C. L. Ku, O. Tournilhac, B. Gilbert-Dussardier, B. Pautard, P. Durand, D. Devictor, E. Lachassinne, B. Guillois, M. Morin, F. Gouraud, F. Valensi, A. Fischer, A. Puel, L. Abel, D. Bonnet and J. L. Casanova (2011). "Isolated congenital asplenia: a French nationwide retrospective survey of 20 cases." J Pediatr 158(1): 142-148, 148 e141.

Malkin, D., F. P. Li, L. C. Strong, J. F. Fraumeni, Jr., C. E. Nelson, D. H. Kim, J. Kassel, M. A. Gryka, F. Z. Bischoff, M. A. Tainsky and et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms." Science 250(4985): 1233-1238.

Mann, D. M. and P. O. Yates (1982). "Pathogenesis of Parkinson's disease." Arch Neurol 39(9): 545-549.

Marcel, V., S. E. Ghayad, S. Belin, G. Therizols, A. P. Morel, E. Solano-Gonzalez, J. A. Vendrell, S. Hacot, H. C. Mertani, M. A. Albaret, J. C. Bourdon, L. Jordan, A. Thompson, Y. Tafer, R. Cong, P. Bouvet, J. C. Saurin, F. Catez, A. C. Prats, A. Puisieux and J. J. Diaz (2013). "p53 acts as a safeguard of translational control by regulating fibrillarin and rRNA methylation in cancer." Cancer Cell 24(3): 318-330.

Marinho, J., F. Casares and P. S. Pereira (2011). "The Drosophila Nol12 homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth." Development 138(2): 349-357.

Marinho, J., T. Martins, M. Neto, F. Casares and P. S. Pereira (2013). "The nucleolar protein Viriato/Nol12 is required for the growth and differentiation progression activities of the Dpp pathway during Drosophila eye development." Dev Biol 377(1): 154-165.

Martin, R. M., G. Ter-Avetisyan, H. D. Herce, A. K. Ludwig, G. Lattig-Tunnemann and M. C. Cardoso (2015). "Principles of protein targeting to the nucleolus." Nucleus 6(4): 314-325.

McStay, B. (2016). "Nucleolar organizer regions: genomic 'dark matter' requiring illumination." Genes Dev 30(14): 1598-1610.

Meng, L., T. Lin, G. Peng, J. K. Hsu, S. Lee, S. Y. Lin and R. Y. Tsai (2013). "Nucleostemin deletion reveals an essential mechanism that maintains the genomic stability of stem and progenitor cells." Proc Natl Acad Sci U S A 110(28): 11415-11420.

Meyer, N. and L. Z. Penn (2008). "Reflecting on 25 years with MYC." Nat Rev Cancer 8(12): 976-990.

Mills, E. W. and R. Green (2017). "Ribosomopathies: There's strength in numbers." Science 358(6363).

Mitrea, D. M., J. A. Cika, C. S. Guy, D. Ban, P. R. Banerjee, C. B. Stanley, A. Nourse, A. A. Deniz and R. W. Kriwacki (2016). "Nucleophosmin integrates within the nucleolus via multi-modal interactions with proteins displaying R-rich linear motifs and rRNA." Elife 5.

Moll, U. M. and O. Petrenko (2003). "The MDM2-p53 interaction." Mol Cancer Res 1(14): 1001-1008.

Montanaro, L., D. Trere and M. Derenzini (2008). "Nucleolus, ribosomes, and cancer." Am J Pathol 173(2): 301-310.

Moreno, E. and K. Basler (2004). "dMyc transforms cells into super-competitors." Cell 117(1): 117-129.

Morris, S. W., M. N. Kirstein, M. B. Valentine, K. G. Dittmer, D. N. Shapiro, D. L. Saltman and A. T. Look (1994). "Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma." Science 263(5151): 1281-1284.

Moskalev, A. and M. Shaposhnikov (2011). "Pharmacological inhibition of NF-kappaB prolongs lifespan of Drosophila melanogaster." Aging (Albany NY) 3(4): 391-394.

Mukudai, Y., S. Kubota, H. Kawaki, S. Kondo, T. Eguchi, K. Sumiyoshi, T. Ohgawara, T. Shimo and M. Takigawa (2008). "Posttranscriptional regulation of chicken ccn2 gene expression by nucleophosmin/B23 during chondrocyte differentiation." Mol Cell Biol 28(19): 6134-6147.

Murano, K., M. Okuwaki, M. Hisaoka and K. Nagata (2008). "Transcription regulation of the rRNA gene by a multifunctional nucleolar protein, B23/nucleophosmin, through its histone chaperone activity." Mol Cell Biol 28(10): 3114-3126.

Muro, E., J. Gebrane-Younis, A. Jobart-Malfait, E. Louvet, P. Roussel and D. Hernandez-Verdun (2010). "The traffic of proteins between nucleolar organizer regions and prenucleolar bodies governs the assembly of the nucleolus at exit of mitosis." Nucleus 1(2): 202-211.

Narla, A. and B. L. Ebert (2010). "Ribosomopathies: human disorders of ribosome dysfunction." Blood 115(16): 3196-3205.

Natan, E., C. Baloglu, K. Pagel, S. M. Freund, N. Morgner, C. V. Robinson, A. R. Fersht and A. C. Joerger (2011). "Interaction of the p53 DNA-binding domain with its n-terminal extension modulates the stability of the p53 tetramer." J Mol Biol 409(3): 358-368.

Neuburger, M., G. W. Herget, L. Plaumann, A. Falk, H. Schwalb and C. P. Adler (1998). "Change in size, number and morphology of the nucleoli in human hearts as a result of hyperfunction." Pathol Res Pract 194(6): 385-389.

Newton, K., E. Petfalski, D. Tollervey and J. F. Caceres (2003). "Fibrillarin is essential for early development and required for accumulation of an intron-encoded small nucleolar RNA in the mouse." Mol Cell Biol 23(23): 8519-8527.

Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devilee and et al. (1989). "Mutations in the p53 gene occur in diverse human tumour types." Nature 342(6250): 705-708.

Nishimura, K., T. Kumazawa, T. Kuroda, N. Katagiri, M. Tsuchiya, N. Goto, R. Furumai, A. Murayama, J. Yanagisawa and K. Kimura (2015). "Perturbation of ribosome biogenesis drives cells into senescence through 5S RNP-mediated p53 activation." Cell Rep 10(8): 1310-1323.

Nunez Villacis, L., M. S. Wong, L. L. Ferguson, N. Hein, A. J. George and K. M. Hannan (2018). "New Roles for the Nucleolus in Health and Disease." Bioessays 40(5): e1700233.

O'Donohue, M. F., V. Choesmel, M. Faubladier, G. Fichant and P. E. Gleizes (2010). "Functional dichotomy of ribosomal proteins during the synthesis of mammalian 40S ribosomal subunits." J Cell Biol 190(5): 853-866.

O'Sullivan, A. C., G. J. Sullivan and B. McStay (2002). "UBF binding in vivo is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat." Mol Cell Biol 22(2): 657-668.

Oeffinger, M., D. Zenklusen, A. Ferguson, K. E. Wei, A. El Hage, D. Tollervey, B. T. Chait, R. H. Singer and M. P. Rout (2009). "Rrp17p is a eukaryotic exonuclease required for 5' end processing of Pre-60S ribosomal RNA." Mol Cell 36(5): 768-781.

Ogawa, L. M. and S. J. Baserga (2017). "Crosstalk between the nucleolus and the DNA damage response." Mol Biosyst 13(3): 443-455.

Okuwaki, M., K. Matsumoto, M. Tsujimoto and K. Nagata (2001). "Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone." FEBS Lett 506(3): 272-276.

Okuwaki, M., M. Tsujimoto and K. Nagata (2002). "The RNA binding activity of a ribosome biogenesis factor, nucleophosmin/B23, is modulated by phosphorylation with a cell cycle-dependent kinase and by association with its subtype." Mol Biol Cell 13(6): 2016-2030.

Olson, M. O. (2004). "Sensing cellular stress: another new function for the nucleolus?" Sci STKE 2004(224): pe10.

Olson, M. O., M. Dundr and A. Szebeni (2000). "The nucleolus: an old factory with unexpected capabilities." Trends Cell Biol 10(5): 189-196.

Orihara-Ono, M., E. Suzuki, M. Saito, Y. Yoda, T. Aigaki and C. Hama (2005). "The slender lobes gene, identified by retarded mushroom body development, is required for proper nucleolar organization in Drosophila." Dev Biol 281(1): 121-133.

Orsolic, I., D. Jurada, N. Pullen, M. Oren, A. G. Eliopoulos and S. Volarevic (2016). "The relationship between the nucleolus and cancer: Current evidence and emerging paradigms." Semin Cancer Biol 37-38: 36-50.

Osorio, F. G., C. Soria-Valles, O. Santiago-Fernandez, J. M. Freije and C. Lopez-Otin (2016). "NF-kappaB signaling as a driver of ageing." Int Rev Cell Mol Biol 326: 133-174.

Palaniswamy, V., K. C. Moraes, C. J. Wilusz and J. Wilusz (2006). "Nucleophosmin is selectively deposited on mRNA during polyadenylation." Nat Struct Mol Biol 13(5): 429-435.

Parlato, R. and G. Kreiner (2013). "Nucleolar activity in neurodegenerative diseases: a missing piece of the puzzle?" J Mol Med (Berl) 91(5): 541-547.

Partridge, L. and M. Mangel (1999). "Messages from mortality: the evolution of death rates in the old." Trends Ecol Evol 14(11): 438-442.

Pavletich, N. P., K. A. Chambers and C. O. Pabo (1993). "The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots." Genes Dev 7(12B): 2556-2564.

Pederson, T. (2011). "The nucleolus." Cold Spring Harb Perspect Biol 3(3).

Pederson, T. and R. Y. Tsai (2009). "In search of nonribosomal nucleolar protein function and regulation." J Cell Biol 184(6): 771-776.

Pertschy, B., C. Schneider, M. Gnadig, T. Schafer, D. Tollervey and E. Hurt (2009). "RNA helicase Prp43 and its co-factor Pfa1 promote 20 to 18 S rRNA processing catalyzed by the endonuclease Nob1." J Biol Chem 284(50): 35079-35091.

Peter, M., J. Nakagawa, M. Doree, J. C. Labbe and E. A. Nigg (1990). "Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase." Cell 60(5): 791-801.

Pfister, A. S. (2019). "Emerging Role of the Nucleolar Stress Response in Autophagy." Front Cell Neurosci 13: 156.

Pianese, G. and R. Teuscher (1896). Beitrag zur Histologie und Aetiologie des Carcinoms : histologische und experimentelle Untersuchungen. Jena, G. Fischer.

Pollard, T. D., W. C. Earnshaw, J. Lippincott-Schwartz and G. T. Johnson (2017). "Mitosis and Cytokinesis." Cell Biology (3rd edition): 755-778.

Poortinga, G., K. M. Hannan, H. Snelling, C. R. Walkley, A. Jenkins, K. Sharkey, M. Wall, Y. Brandenburger, M. Palatsides, R. B. Pearson, G. A. McArthur and R. D. Hannan (2004). "MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation." EMBO J 23(16): 3325-3335.

Proudfoot, N. J. (2016). "Transcriptional termination in mammals: Stopping the RNA polymerase II juggernaut." Science 352(6291): aad9926.

Quin, J. E., J. R. Devlin, D. Cameron, K. M. Hannan, R. B. Pearson and R. D. Hannan (2014). "Targeting the nucleolus for cancer intervention." Biochim Biophys Acta 1842(6): 802-816.

Raj, N. and L. D. Attardi (2017). "The Transactivation Domains of the p53 Protein." Cold Spring Harb Perspect Med 7(1).

Ranaweera, R. S. and X. Yang (2013). "Auto-ubiquitination of Mdm2 enhances its substrate ubiquitin ligase activity." J Biol Chem 288(26): 18939-18946.

Raska, I., P. J. Shaw and D. Cmarko (2006). "New insights into nucleolar architecture and activity." Int Rev Cytol 255: 177-235.

Redner, R. L., E. A. Rush, S. Faas, W. A. Rudert and S. J. Corey (1996). "The t(5;17) variant of acute promyelocytic leukemia expresses a nucleophosmin-retinoic acid receptor fusion." Blood 87(3): 882-886.

Rieker, C., D. Engblom, G. Kreiner, A. Domanskyi, A. Schober, S. Stotz, M. Neumann, X. Yuan, I. Grummt, G. Schutz and R. Parlato (2011). "Nucleolar disruption in dopaminergic neurons leads to oxidative damage and parkinsonism through repression of mammalian target of rapamycin signaling." J Neurosci 31(2): 453-460.

Romanova, L., A. Grand, L. Zhang, S. Rayner, N. Katoku-Kikyo, S. Kellner and N. Kikyo (2009). "Critical role of nucleostemin in pre-rRNA processing." J Biol Chem 284(8): 4968-4977.

Rosete, M., M. R. Padros and O. Vindrola (2007). "[The nucleolus as a regulator of cellular senescence]." Medicina (B Aires) 67(2): 183-194.

Roussel, P., C. Andre, L. Comai and D. Hernandez-Verdun (1996). "The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs." J Cell Biol 133(2): 235-246.

Ruggero, D. (2009). "The role of Myc-induced protein synthesis in cancer." Cancer Res 69(23): 8839-8843.

Ruggero, D. (2012). "Revisiting the nucleolus: from marker to dynamic integrator of cancer signaling." Sci Signal 5(241): pe38.

Russell, J. and J. C. Zomerdijk (2005). "RNA-polymerase-I-directed rDNA transcription, life and works." Trends Biochem Sci 30(2): 87-96.

Salminen, A., J. Huuskonen, J. Ojala, A. Kauppinen, K. Kaarniranta and T. Suuronen (2008). "Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging." Ageing Res Rev 7(2): 83-105.

Salminen, A., J. Ojala, J. Huuskonen, A. Kauppinen, T. Suuronen and K. Kaarniranta (2008). "Interaction of aging-associated signaling cascades: inhibition of NF-kappaB signaling by longevity factors FoxOs and SIRT1." Cell Mol Life Sci 65(7-8): 1049-1058.

Salvetti, A., Y. Coute, A. Epstein, L. Arata, A. Kraut, V. Navratil, P. Bouvet and A. Greco (2016). "Nuclear Functions of Nucleolin through Global Proteomics and Interactomic Approaches." J Proteome Res 15(5): 1659-1669.

Savas, J. N., A. Makusky, S. Ottosen, D. Baillat, F. Then, D. Krainc, R. Shiekhattar, S. P. Markey and N. Tanese (2008). "Huntington's disease protein contributes to RNA-mediated gene silencing through association with Argonaute and P bodies." Proc Natl Acad Sci U S A 105(31): 10820-10825.

Savino, T. M., J. Gebrane-Younes, J. De Mey, J. B. Sibarita and D. Hernandez-Verdun (2001). "Nucleolar assembly of the rRNA processing machinery in living cells." J Cell Biol 153(5): 1097-1110.

Savkur, R. S. and M. O. Olson (1998). "Preferential cleavage in pre-ribosomal RNA byprotein B23 endoribonuclease." Nucleic Acids Res 26(19): 4508-4515.

Schlesinger, S., S. Selig, Y. Bergman and H. Cedar (2009). "Allelic inactivation of rDNA loci." Genes Dev 23(20): 2437-2447.

Schlosser, I., M. Holzel, M. Murnseer, H. Burtscher, U. H. Weidle and D. Eick (2003). "A role for c-Myc in the regulation of ribosomal RNA processing." Nucleic Acids Res 31(21): 6148-6156.

Schmidt, E. V. (1999). "The role of c-myc in cellular growth control." Oncogene 18(19): 2988-2996.

Scott, D. D. and M. Oeffinger (2016). "Nucleolin and nucleophosmin: nucleolar proteins with multiple functions in DNA repair." Biochem Cell Biol 94(5): 419-432.

Scott, D. D., C. Trahan, P. J. Zindy, L. C. Aguilar, M. Y. Delubac, E. L. Van Nostrand, S. Adivarahan, K. E. Wei, G. W. Yeo, D. Zenklusen and M. Oeffinger (2017). "Nol12 is a multifunctional RNA binding protein at the nexus of RNA and DNA metabolism." Nucleic Acids Res 45(21): 12509-12528.

Scott, M. S., F. M. Boisvert, M. D. McDowall, A. I. Lamond and G. J. Barton (2010). "Characterization and prediction of protein nucleolar localization sequences." Nucleic Acids Res 38(21): 7388-7399.

Selman, C., S. Lingard, A. I. Choudhury, R. L. Batterham, M. Claret, M. Clements, F. Ramadani, K. Okkenhaug, E. Schuster, E. Blanc, M. D. Piper, H. Al-Qassab, J. R. Speakman, D. Carmignac, I. C. Robinson, J. M. Thornton, D. Gems, L. Partridge and D. J. Withers (2008). "Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice." FASEB J 22(3): 807-818.

Shaw, P. and J. Doonan (2005). "The nucleolus. Playing by different rules?" Cell Cycle 4(1): 102-105.

Sheaffer, K. L., D. L. Updike and S. E. Mango (2008). "The Target of Rapamycin pathway antagonizes pha-4/FoxA to control development and aging." Curr Biol 18(18): 1355-1364.

Shi, Z., K. Fujii, K. M. Kovary, N. R. Genuth, H. L. Rost, M. N. Teruel and M. Barna (2017). "Heterogeneous Ribosomes Preferentially Translate Distinct Subpools of mRNAs Genome-wide." Mol Cell 67(1): 71-83 e77.

Shimizu, H., L. R. Burch, A. J. Smith, D. Dornan, M. Wallace, K. L. Ball and T. R. Hupp (2002). "The conformationally flexible S9-S10 linker region in the core domain of p53 contains a novel MDM2 binding site whose mutation increases ubiquitination of p53 in vivo." J Biol Chem 277(32): 28446-28458.

Shwachman, H., L. K. Diamond, F. A. Oski and K. T. Khaw (1964). "The Syndrome of Pancreatic Insufficiency and Bone Marrow Dysfunction." J Pediatr 65: 645-663.

Singh, N., F. A. Ebrahimi, A. A. Gimelbrant, A. W. Ensminger, M. R. Tackett, P. Qi, J. Gribnau and A. Chess (2003). "Coordination of the random asynchronous replication of autosomal loci." Nat Genet 33(3): 339-341.

Sirri, V., P. Roussel and D. Hernandez-Verdun (1999). "The mitotically phosphorylated form of the transcription termination factor TTF-1 is associated with the repressed rDNA transcription machinery." J Cell Sci 112 (Pt 19): 3259-3268.

Sloan, K. E., M. T. Bohnsack and N. J. Watkins (2013). "The 5S RNP couples p53 homeostasis to ribosome biogenesis and nucleolar stress." Cell Rep 5(1): 237-247.

Sloan, K. E., S. Mattijssen, S. Lebaron, D. Tollervey, G. J. Pruijn and N. J. Watkins (2013). "Both endonucleolytic and exonucleolytic cleavage mediate ITS1 removal during human ribosomal RNA processing." J Cell Biol 200(5): 577-588.

Sloan, K. E., A. S. Warda, S. Sharma, K. D. Entian, D. L. J. Lafontaine and M. T. Bohnsack (2017). "Tuning the ribosome: The influence of rRNA modification on eukaryotic ribosome biogenesis and function." RNA Biol 14(9): 1138-1152.

Slomovic, S., E. Fremder, R. H. Staals, G. J. Pruijn and G. Schuster (2010). "Addition of poly(A) and poly(A)-rich tails during RNA degradation in the cytoplasm of human cells." Proc Natl Acad Sci U S A 107(16): 7407-7412.

Slomovic, S., D. Laufer, D. Geiger and G. Schuster (2005). "Polyadenylation and degradation of human mitochondrial RNA: the prokaryotic past leaves its mark." Mol Cell Biol 25(15): 6427-6435.

Slomovic, S., D. Laufer, D. Geiger and G. Schuster (2006). "Polyadenylation of ribosomal RNA in human cells." Nucleic Acids Res 34(10): 2966-2975.

Slomovic, S., V. Portnoy, V. Liveanu and G. Schuster (2006). "RNA Polyadenylation in Prokaryotes and Organelles; Different Tails Tell Different Tales." Critical Reviews in Plant Sciences 25(1): 65-77.

Sorensen, P. D. and S. Frederiksen (1991). "Characterization of human 5S rRNA genes." Nucleic Acids Res 19(15): 4147-4151.

Spector, D. L. (2001). "Nuclear domains." J Cell Sci 114(Pt 16): 2891-2893.

Srivastava, M., O. W. McBride, P. J. Fleming, H. B. Pollard and A. L. Burns (1990). "Genomic organization and chromosomal localization of the human nucleolin gene." J Biol Chem 265(25): 14922-14931.

Steffen, K. K., V. L. MacKay, E. O. Kerr, M. Tsuchiya, D. Hu, L. A. Fox, N. Dang, E. D. Johnston, J. A. Oakes, B. N. Tchao, D. N. Pak, S. Fields, B. K. Kennedy and M. Kaeberlein (2008). "Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4." Cell 133(2): 292-302.

Stoneley, M., F. E. Paulin, J. P. Le Quesne, S. A. Chappell and A. E. Willis (1998). "C-Myc 5' untranslated region contains an internal ribosome entry segment." Oncogene 16(3): 423-428.

Sulic, S., L. Panic, I. Dikic and S. Volarevic (2005). "Deregulation of cell growth and malignant transformation." Croat Med J 46(4): 622-638.

Sulima, S. O., I. J. F. Hofman, K. De Keersmaecker and J. D. Dinman (2017). "How Ribosomes Translate Cancer." Cancer Discov 7(10): 1069-1087.

Suzuki, S., T. Fujiwara and M. Kanno (2007). "Nucleolar protein Nop25 is involved in nucleolar architecture." Biochem Biophys Res Commun 358(4): 1114-1119.

Suzuki, S., M. Kanno, T. Fujiwara, H. Sugiyama, A. Yokoyama, H. Takahashi and J. Tanaka (2006). "Molecular cloning and characterization of Nop25, a novel nucleolar RNA binding protein, highly conserved in vertebrate species." Exp Cell Res 312(7): 1031-1041.

Tajrishi, M. M., R. Tuteja and N. Tuteja (2011). "Nucleolin: The most abundant multifunctional phosphoprotein of nucleolus." Commun Integr Biol 4(3): 267-275.

Thiry, M. and D. L. Lafontaine (2005). "Birth of a nucleolus: the evolution of nucleolar compartments." Trends Cell Biol 15(4): 194-199.

Thomas, G. (2000). "An encore for ribosome biogenesis in the control of cell proliferation." Nat Cell Biol 2(5): E71-72.

Thomson, E., S. Ferreira-Cerca and E. Hurt (2013). "Eukaryotic ribosome biogenesis at a glance." J Cell Sci 126(Pt 21): 4815-4821.

Tiku, V. and A. Antebi (2018). "Nucleolar Function in Lifespan Regulation." Trends Cell Biol 28(8): 662-672.

Tiku, V., C. Jain, Y. Raz, S. Nakamura, B. Heestand, W. Liu, M. Spath, H. E. D. Suchiman, R. U. Muller, P. E. Slagboom, L. Partridge and A. Antebi (2017). "Small nucleoli are a cellular hallmark of longevity." Nat Commun 8: 16083.

Tilstra, J. S., C. L. Clauson, L. J. Niedernhofer and P. D. Robbins (2011). "NF-κB in Aging and Disease." Aging and disease 2(6): 449-465.

Tilstra, J. S., A. R. Robinson, J. Wang, S. Q. Gregg, C. L. Clauson, D. P. Reay, L. A. Nasto, C. M. St Croix, A. Usas, N. Vo, J. Huard, P. R. Clemens, D. B. Stolz, D. C. Guttridge, S. C. Watkins, G. A. Garinis, Y. Wang, L. J. Niedernhofer and P. D. Robbins (2012). "NF-kappaB inhibition delays DNA damage-induced senescence and aging in mice." J Clin Invest 122(7): 2601-2612.

Toledo, F., K. A. Krummel, C. J. Lee, C. W. Liu, L. W. Rodewald, M. Tang and G. M. Wahl (2006). "A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network." Cancer Cell 9(4): 273-285.

Torres-Machorro, A. L., R. Hernandez, A. M. Cevallos and I. Lopez-Villasenor (2010). "Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny?" FEMS Microbiol Rev 34(1): 59-86.

Tsai, R. Y. (2014). "Turning a new page on nucleostemin and self-renewal." J Cell Sci 127(Pt 18): 3885-3891.

Tseng, H., W. Chou, J. Wang, X. Zhang, S. Zhang and R. M. Schultz (2008). "Mouse ribosomal RNA genes contain multiple differentially regulated variants." PLoS One 3(3): e1843.

Turner, A. J., A. A. Knox, J. L. Prieto, B. McStay and N. J. Watkins (2009). "A novel small-subunit processome assembly intermediate that contains the U3 snoRNP, nucleolin, RRP5, and DBP4." Mol Cell Biol 29(11): 3007-3017.

Ugrinova, I., K. Monier, C. Ivaldi, M. Thiry, S. Storck, F. Mongelard and P. Bouvet (2007). "Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication." BMC Mol Biol 8: 66.

Umekawa, H., J. H. Chang, J. J. Correia, D. Wang, P. T. Wingfield and M. O. Olson (1993). "Nucleolar protein B23: bacterial expression, purification, oligomerization and secondary structures of two isoforms." Cell Mol Biol Res 39(7): 635-645.

Valdez, B. C., L. Perlaky, D. Henning, Y. Saijo, P. K. Chan and H. Busch (1994). "Identification of the nuclear and nucleolar localization signals of the protein p120. Interaction with translocation protein B23." J Biol Chem 269(38): 23776-23783.

van Hoof, A., P. Lennertz and R. Parker (2000). "Three conserved members of the RNase D family have unique and overlapping functions in the processing of 5S, 5.8S, U4, U5, RNase MRP and RNase P RNAs in yeast." EMBO J 19(6): 1357-1365.

van Riggelen, J., A. Yetil and D. W. Felsher (2010). "MYC as a regulator of ribosome biogenesis and protein synthesis." Nat Rev Cancer 10(4): 301-309.

Venema, J. and D. Tollervey (1999). "Ribosome synthesis in Saccharomyces cerevisiae." Annu Rev Genet 33: 261-311.

Vernon, R. M. and J. D. Forman-Kay (2019). "First-generation predictors of biological protein phase separation." Curr Opin Struct Biol 58: 88-96.

Vogelstein, B., S. Sur and C. Prives (2010). "p53: The most frequently altered gene in human cancers." Nature Education 3(9): 6.

Walne, A. J. and I. Dokal (2008). "Dyskeratosis Congenita: a historical perspective." Mech Ageing Dev 129(1-2): 48-59.

Wang, H., L. N. Zhao, K. Z. Li, R. Ling, X. J. Li and L. Wang (2006). "Overexpression of ribosomal protein L15 is associated with cell proliferation in gastric cancer." BMC Cancer 6: 91.

Wang, M. and D. G. Pestov (2011). "5'-end surveillance by Xrn2 acts as a shared mechanism for mammalian pre-rRNA maturation and decay." Nucleic Acids Res 39(5): 1811-1822.

Wang, S., J. Huang, J. He, A. Wang, S. Xu, S. F. Huang and S. Xiao (2010). "RPL41, a small ribosomal peptide deregulated in tumors, is essential for mitosis and centrosome integrity." Neoplasia 12(3): 284-293.

Warner, J. R. (1977). "In the absence of ribosomal RNA synthesis, the ribosomal proteins of HeLa cells are synthesized normally and degraded rapidly." J Mol Biol 115(3): 315-333.

Watanabe-Susaki, K., H. Takada, K. Enomoto, K. Miwata, H. Ishimine, A. Intoh, M. Ohtaka, M. Nakanishi, H. Sugino, M. Asashima and A. Kurisaki (2014). "Biosynthesis of ribosomal RNA in nucleoli regulates pluripotency and differentiation ability of pluripotent stem cells." Stem Cells 32(12): 3099-3111.

Watson, K. L., K. D. Konrad, D. F. Woods and P. J. Bryant (1992). "Drosophila homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system." Proc Natl Acad Sci U S A 89(23): 11302-11306.

Weeks, S. E., B. J. Metge and R. S. Samant (2019). "The nucleolus: a central response hub for the stressors that drive cancer progression." Cell Mol Life Sci 76(22): 4511-4524.

West, S., N. Gromak and N. J. Proudfoot (2004). "Human 5' --> 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites." Nature 432(7016): 522-525.

Wilker, E. W., M. A. van Vugt, S. A. Artim, P. H. Huang, C. P. Petersen, H. C. Reinhardt, Y. Feng, P. A. Sharp, N. Sonenberg, F. M. White and M. B. Yaffe (2007). "14-3-3sigma controls mitotic translation to facilitate cytokinesis." Nature 446(7133): 329-332.

Wong, C. C., D. Traynor, N. Basse, R. R. Kay and A. J. Warren (2011). "Defective ribosome assembly in Shwachman-Diamond syndrome." Blood 118(16): 4305-4312.

Wu, A., X. Tu, M. Prisco and R. Baserga (2005). "Regulation of upstream binding factor 1 activity by insulin-like growth factor I receptor signaling." J Biol Chem 280(4): 2863-2872.

Xie, H., S. Vucetic, L. M. Iakoucheva, C. J. Oldfield, A. K. Dunker, V. N. Uversky and Z. Obradovic (2007). "Functional anthology of intrinsic disorder. 1. Biological processes and functions of proteins with long disordered regions." J Proteome Res 6(5): 1882-1898.

Xirodimas, D., M. K. Saville, C. Edling, D. P. Lane and S. Lain (2001). "Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 in vivo." Oncogene 20(36): 4972-4983.

Xue, S. and M. Barna (2012). "Specialized ribosomes: a new frontier in gene regulation and organismal biology." Nat Rev Mol Cell Biol 13(6): 355-369.

Yadavilli, S., L. D. Mayo, M. Higgins, S. Lain, V. Hegde and W. A. Deutsch (2009). "Ribosomal protein S3: A multi-functional protein that interacts with both p53 and MDM2 through its KH domain." DNA Repair (Amst) 8(10): 1215-1224.

Yamashita, M., E. Nitta, G. Nagamatsu, Y. M. Ikushima, K. Hosokawa, F. Arai and T. Suda (2013). "Nucleostemin is indispensable for the maintenance and genetic stability of hematopoietic stem cells." Biochem Biophys Res Commun 441(1): 196-201.

Yang, K., J. Yang and J. Yi (2018). "Nucleolar Stress: hallmarks, sensing mechanism and diseases." Cell Stress 2(6): 125-140.

Yoneda-Kato, N., A. T. Look, M. N. Kirstein, M. B. Valentine, S. C. Raimondi, K. J. Cohen, A. J. Carroll and S. W. Morris (1996). "The t(3;5)(q25.1;q34) of myelodysplastic syndrome and acute myeloid leukemia produces a novel fusion gene, NPM-MLF1." Oncogene 12(2): 265-275.

Yong, W. H., M. Shabihkhani, D. Telesca, S. Yang, J. L. Tso, J. C. Menjivar, B. Wei, G. M. Lucey, S. Mareninov, Z. Chen, L. M. Liau, A. Lai, S. F. Nelson, T. F. Cloughesy and C. L. Tso (2015). "Ribosomal Proteins RPS11 and RPS20, Two Stress-Response Markers of Glioblastoma Stem Cells, Are Novel Predictors of Poor Prognosis in Glioblastoma Patients." PLoS One 10(10): e0141334.

Yoon, I. K., H. K. Kim, Y. K. Kim, I. H. Song, W. Kim, S. Kim, S. H. Baek, J. H. Kim and J. R. Kim (2004). "Exploration of replicative senescence-associated genes in human dermal fibroblasts by cDNA microarray technology." Exp Gerontol 39(9): 1369-1378.

Young, A. R., M. Narita, M. Ferreira, K. Kirschner, M. Sadaie, J. F. Darot, S. Tavare, S. Arakawa, S. Shimizu, F. M. Watt and M. Narita (2009). "Autophagy mediates the mitotic senescence transition." Genes Dev 23(7): 798-803.

Yu, X. and J. R. Warner (2001). "Expression of a micro-protein." J Biol Chem 276(36): 33821-33825.

Yu, Y., L. B. Maggi, Jr., S. N. Brady, A. J. Apicelli, M. S. Dai, H. Lu and J. D. Weber (2006). "Nucleophosmin is essential for ribosomal protein L5 nuclear export." Mol Cell Biol 26(10): 3798-3809.

Yuan, F., C. Xu, G. Li and T. Tong (2018). "Nucleolar TRF2 attenuated nucleolus stress-induced HCC cell-cycle arrest by altering rRNA synthesis." Cell Death Dis 9(5): 518.

Zhang, J., P. Harnpicharnchai, J. Jakovljevic, L. Tang, Y. Guo, M. Oeffinger, M. P. Rout, S. L. Hiley, T. Hughes and J. L. Woolford, Jr. (2007). "Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes." Genes Dev 21(20): 2580-2592.

Zhang, M., L. Yu, Y. Xin, P. Hu, Q. Fu, C. Yu and S. Zhao (1999). "Cloning and mapping of the XRN2 gene to human chromosome 20p11.1-p11.2." Genomics 59(2): 252-254.

Zhu, L., T. M. Richardson, L. Wacheul, M. T. Wei, M. Feric, G. Whitney, D. L. J. Lafontaine and C. P. Brangwynne (2019). "Controlling the material properties and rRNA processing function of the nucleolus using light." Proc Natl Acad Sci U S A 116(35): 17330-17335.

Zimmermann, R. A. (2003). "The double life of ribosomal proteins." Cell 115(2): 130-132.

CHAPTER 2

NOL12 repression induces nucleolar stress-driven cellular senescence and is associated with normative aging

In this study, I participated in the experimental design, cell culture handling, immunostaining and western blotting experiments, sample preparation and acquisition for transmission electron microscopy and flow cytometry, sample preparation for qPCR, lentiviral production and infection and I also performed image analysis and quantification. Additionally, I participated in the writing of the manuscript and in the revision process.

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1. ABSTRACT

The nucleolus is a subnuclear compartment with key roles in rRNA synthesis and ribosome biogenesis, complex processes that require hundreds of proteins and factors. Alterations in nucleolar morphology and protein content have been linked to the control of cell proliferation and stress responses and, recently, further implicated in cell senescence and ageing. In this study, we report the functional role of NOL12 in the nucleolar homeostasis of human primary fibroblasts. NOL12 repression induces specific changes in nucleolar morphology, with increased nucleolar area but reduced nucleolar number, along with nucleolar accumulation and increased levels of fibrillarin and nucleolin. Moreover, NOL12 repression leads to stabilization and activation of p53 in an RPL11-dependent manner, which arrests cells at G2 phase and ultimately leads to senescence. Importantly, we found NOL12 repression in association with nucleolar stress-like responses in human fibroblasts from elderly donors, disclosing it as a biomarker in human chronological aging.

2. INTRODUCTION

The nucleolus is a subnuclear multifunctional compartment with a central role in ribosome biogenesis and RNA-processing events, and it is also involved in the sensing of cellular stress and in cell cycle regulation (Boisvert et al., 2007; Boulon et al., 2010; Tsai and Pederson, 2014). The main function of the nucleolus is the synthesis of rRNA and its complex processing and co assembly with ribosomal proteins into ribosome subunits (Henras et al., 2015). RNA polymerase I (RNA Pol I) synthesizes a precursor rRNA transcript (47S pre-rRNA in humans) that is processed and modified (including 2'O-methylation and pseudouridylation) into 28S, 18S, and 5.8S rRNAs. These three rRNAs, together with the 5S rRNA synthesized by RNA Pol III in the nucleoplasm, are assembled with ribosomal proteins into large and small ribosomal subunits to be exported independently to the cytoplasm (Henras et al., 2015). Ribosome biogenesis is one of the most energy-consuming processes in a cell, and it is highly regulated, so that protein synthesis potential (ribosome levels) matches energy supply to ensure proper cellular proliferation and cell growth (Lempiainen and Shore, 2009). Mammalian cells have the ability to sense extreme variations in their internal and external environments and frequently respond accordingly with cell cycle arrest or apoptosis. p53 is a tumor suppressor protein, with crucial functions in protecting genome integrity upon cellular stress (Woods et al., 2015). Direct and localized micropore UV irradiation of cell nucleoli was shown to induce p53 stabilization. However, cells could tolerate a large amount of DNA damage without inducing a p53 response if this DNA damage was not localized and if the nucleoli were not

disrupted (Rubbi and Milner, 2003). Furthermore, in addition to DNA damage, several other stresses, including chemotherapeutic drugs and inhibition of RNA Pol I transcription by low doses of actinomycin D (ActD), interfere with nucleolar homeostasis and promote a p53-dependent nucleolar stress response (Tsai and Pederson, 2014). Under normal conditions, MDM2, an E3 ubiquitin ligase, prevents p53 stabilization through proteasome-mediated degradation. If nucleolar stress occurs or ribosome biogenesis is perturbed by ribosomal protein deficiency or oncogenic overstimulation, the intermediate ribosomal assembly complex 5S RNP, comprised of RPL11, RPL5, and 5S rRNA, directly binds and represses MDM2 to stabilize p53 (Bursac et al., 2012; Donati et al., 2013; Horn and Vousden, 2008; Sloan et al., 2013a). Also, both replicative stress (with delayed rRNA processing) and oncogenic stress (with accelerated rRNA transcription) were shown to promote the association of the 5S RNP complex with MDM2, leading to p53 stabilization (Nishimura et al., 2015). Nucleoli have been shown to behave like liquid droplets (Brangwynne et al., 2011; Feric et al., 2016), where proteins and other molecules that participate in ribosome biogenesis dynamically self-segregate into the three microscopically recognized nucleolar regions: dense fibrillar component (DFC), fibrillar center (FC), and granular component (GC). Therefore, alterations in nucleolar structure are expected to closely reflect changes in the nature or dynamic partition of enzymatic nucleolar reactions. For decades, pathologists have recognized the correlation between changes in nucleolar size and/or number and tumor aggressiveness (Derenzini et al., 2009). In our previous studies, we identified viriato, the single *Drosophila* member of the NOL12/Nop25 gene family, as a crucial regulator of nucleolar architecture (Marinho et al., 2011), as also described for rat Nop25 (Suzuki et al., 2007). The yeast NOL12 homologue Rrp17 was shown to function as a 5'-to-3' RNA exonuclease for processing of the internal transcribed spacer 1 (ITS1) region of pre-rRNA during ribosome biogenesis (Oeffinger et al., 2009; Sahasranaman et al., 2011). Human NOL12 was shown to be required for pre-rRNA ITS1 processing, in particular for cleavage of site 2 (Scott et al., 2017; Sloan et al., 2013b), but its putative 5'-to-3' RNA exonucleolytic activity has not yet been ascertained. Interestingly, NOL12 colocalized with DNA repair proteins, such as Dhx9 and TOPBP1, and was required for HCT116 cells to recover from DNA stress (Scott et al., 2017). In this colon cancer cell line, p53 stabilization was observed, but it was not required for cell cycle arrest or apoptosis (Scott et al., 2017). We also previously found that viriato is a novel transcriptional target of *Drosophila* Myc with a crucial function in ensuring a coordinated nucleolar response to dMyc-induced tissue growth (Marinho et al., 2011). Furthermore, through a retina-targeted double RNA interference (RNAi) screen, we identified a genetic interaction between viriato and several *Drosophila* transforming growth factor β (TGF-β) signaling gene members (Marinho et al., 2013). This led us to study and implicate TGF-β/activin signaling in the regulation of

nucleolar biogenesis and cell growth in *Drosophila* salivary glands (Martins et al., 2017). Furthermore, we also disclosed that, during retina development, viriato knockdown induced an increase of p53-independent, caspase-mediated apoptotic cell death (Marinho et al., 2011). Overall, our analysis of *Drosophila* Viriato suggested a potential novel link between structural/functional changes in the nucleolus and cell proliferation and apoptosis. Nevertheless, the putative role of p53 activation in response to nucleolar stress induced by Viriato/NOL12 knockdown awaited further analysis. Using primary human fibroblasts to investigate the functional role of human NOL12, here we show that NOL12 is important for nucleolar homeostasis, regulating its structure and the nucleolar levels of the multifunctional fibrillarin and nucleolin proteins. Moreover, we found NOL12 depletion to induce strong p53 activation, which at the mechanistic level requires the function of MDM2 inhibitor 60S ribosomal protein L11 and which causes G2 arrest. Importantly, we show that NOL12 repression, either experimental or age-associated, leads to p53-driven senescence, suggesting an important role for NOL12 in replicative and chronological aging and its potential as aging biomarker.

3. MATERIALS AND METHODS

Cell culture. Human dermal fibroblasts retrieved from skin biopsies of Caucasian males reported as healthy, were acquired from cell biobanks as summarized in Supplementary Table 1. Cells were seeded at 1-1.5 x 10^4 cells per cm^2 of growth area in minimal essential medium Eagle-Earle (MEM) supplemented with 15% fetal bovine serum (FBS), 2.5 mM L-glutamine and antibiotic-antimycotic (1:100) (all from Gibco®; Life Technologies, Thermo Scientific, CA, USA). Only early passage dividing fibroblasts (up to passage 3-5) with cumulative population doubling level PDL<24, well below replicative senescence, were used in all experiments. Cells were grown at 37ºC and humidified atmosphere with 5% CO_2. For immunostaining experiments, cells were cultured in coverslips coated with 50 µg/ml fibronectin (Sigma-Aldrich; MO, USA).

Drug treatments. For RNA Polymerase I inhibition, fibroblasts were incubated for 4h in medium containing 8 nM Actinomycin D (Sigma-Aldrich, MO, USA). For siRNA-depleted cells, Actinomycin was added 4 hours before cell harvesting or fixation. For apoptosis induction, cells were incubated for 4h in medium with 5 µM staurosporine (LC Laboratories). For the puromycin assay, puromycin was added to cell culture at 10 µg/mL for 10 min at 37ºC, before cell harvesting.

Senescence-associated β-galactosidase assay. Cells were incubated in medium with 100 nM Bafilomycin A1 (Sigma-Aldrich, MO, USA) for 90 min to induce lysosomal

alkalinization. 33 µM of the fluorogenic substrate for β-galactosidase, fluorescein di-β-D-galactopyranoside (Sigma-Aldrich, MO, USA), was then added to the cell culture medium, and incubation carried out for 90 min. Cells were fixed in 4% paraformaldehyde for 15 min, rinsed with PBS and permeabilized for 15 min with 0.1% Triton-X100 in PBS. Finally, cells were counterstained with 1 µg/ml DAPI (Sigma-Aldrich; MO, USA).

siRNA transfection. For NOL12 depletion, we tested 3 different siRNAs from Sigma (SASI_Hs01_00047858, SASI_Hs01_00047859 and SASI_Hs01_00047860). SASI_Hs01_00047859 depleted NOL12 the most efficiently. Sequences of all siRNAs used in this study (from Sigma-Aldrich) are summarized in Supplementary Table 2. For XRN2 efficient depletion, we used a mixture of two previously reported siRNAs (Sloan et al., 2013b; West et al., 2004). One hour after their plating, cells were transfected with a final concentration of 45nM siRNA using Lipofectamine RNAiMAX transfection reagent (Thermo Scientific, CA, USA) accordingly to the manufacturer's instructions. Cells treated under the same conditions with transfection reagent without siRNA were used as controls (mock-depleted cells). 6h after transfection, medium was replaced by fresh medium supplemented with 5% FBS, and the next day, by complete MEM medium. Phenotypes were analyzed and quantified 72h posttransfection.

Immunostaining. Fibroblasts were grown on sterilized glass coverslips coated with 50 µg/ml fibronectin (Sigma-Aldrich; MO, USA). Cells were fixed in freshly prepared 2% paraformaldehyde in PBS for 20 minutes or in 4% paraformaldehyde in PBS for 15 minutes in the case of 53BP1 and p21 markers. Following fixation, cells were rinsed in PBS, permeabilized with 0.3% Triton-X100 in PBS for 7 min and blocked in 0.05% Tween-20 in PBS (PBS-T) supplemented with 10% fetal bovine serum (FBS) for 1h at room temperature. Cells were then incubated overnight at 4ºC with primary antibodies diluted in PBS-T supplemented with 5% FBS as follows: mouse anti-NOL12 (sc-374257, Santa Cruz Biotechnology, CA, USA), 1:800; rabbit anti-fibrillarin (ab5821, Abcam, Shanghai, China), 1:1000; rabbit anti-p53 (sc-6243, Santa Cruz Biotechnology, CA, USA), 1:1000; mouse anti-fibrillarin (ab4566, Abcam, Shanghai, China), 1:3000; rabbit anti-nucleolin (sc-13057, Santa Cruz Biotechnology, CA, USA) 1:500; rabbit anti-53BP1 (#4937, Cell Signaling Technology, MA, USA), 1:100; mouse anti-p21 (sc-6246, Santa Cruz Biotechnology, CA, USA), 1:1000; rat anti-α-tubulin (Sigma-Aldrich; MO, USA), 1:100; rabbit anti-Ki67 (ab15580, Abcam, Shanghai, China), 1:1500; rabbit anti-cyclin B1 (#4138, Cell Signaling Technology, MA, USA), 1:100. Secondary antibodies AlexaFluor®-488, 568 and 647 were diluted 1:1500 in PBS-T supplemented with 5% FBS and coverslips were incubated for 45 minutes at room temperature. DAPI (Sigma-Aldrich, MO, USA) was used at 1 µg/ml for nuclei staining and coverslips were then mounted in slides with mounting solution (90% glycerol, 0.5% N-propyl gallate and 20 nM Tris pH 8).

FUrd incorporation assay. To assess rDNA transcription in human primary fibroblasts, 5-Fluorouridine (FUrd; F5130, Sigma) was added to cell culture for 20 min at 2 mM. Coverslips were then rapidly washed in cold PBS, fixed in 1% PFA for 10 min, washed in cold PBS and permeabilized with 0.5% Triton-X100 in PBS for 10 min on ice. Coverslips were then immunostained as described above.

EdU incorporation assay. To assess the proliferative activity of human primary fibroblasts, 5-ethynyl-2′-deoxyuridine (EdU) (Invitrogen, Darmstadt, Germany; 20 μM/well) was added to cells for 3 hours before each time point monitored (24, 48 and 72 hours). Coverslips were fixed in 4% PFA for 15 minutes, washed with 10% FBS and permeabilized with 0,3% Triton-X100 in PBS for 7 min. Instructions from Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) were then followed.

Microscopy and image analysis. Slides were analyzed under HC PL APO CS 63x/1.30 Glycerine 21ºC objective in a Laser Scanning confocal microscope Leica TCS SP5 II (Leica Microsystems, Germany). Images were acquired at 1024x1024 pixel resolution and edited using Adobe Photoshop CS4 Extended Version 11.0. All image fields used for quantitative analyses were acquired in IN Cell Analyzer 2000® (GE Healthcare, UK), equipped with a Photometrics CoolSNAP K4 camera. IN Cell Investigator software was used for measuring nucleolus and nucleus parameters (number and areas); nuclear area was measured in the DAPI channel whereas the total nucleolar area and the number of nucleoli per nucleus were measured in the fibrillarin channel. The mean pixel intensity of fibrillarin, nucleolin and p53 (referred as “nuclear protein levels”) were measured based on mask defined by the DAPI channel. In senescence associated-β-galactosidase activity assay, cells displaying >5 fluorescent granules were considered positive.

Western blotting. Cell pellets were resuspended in lysis buffer (150 nM NaCl, 10 nM Tris-HCl pH=7.4, 1 nM EDTA, 1 nM EGTA, 0.5% IGEPAL) and instantaneously frozen in liquid nitrogen. Clarified lysates were quantified for protein content by the Lowry Method (DC™ Protein Assay; BioRad, CA, USA). 20 μg of total extract (except for the detection of cleaved caspase-3, in which we used 40 μg of total extract) were loaded in SDS-PAGE gels and transferred into nitrocellulose membranes for western blot analysis. Membranes were blocked with 0.05% Tween-20 in TBS (TBS-T) containing 5% low fat milk, during 1h at RT. Primary antibodies were diluted in TBS-T containing 2% low fat milk as follows: mouse anti-NOL12 (sc-374257), 1:1000; rabbit anti-p53 (sc-6243), 1:1500; mouse anti-p21 (sc-6246), 1:1000; mouse anti-cyclin B1 (#4135, Cell Signaling Technology, MA, USA) 1:1500; rabbit anti-RPL11 (ab79352, Abcam) 1:1000; rabbit anti-nucleolin (sc-13057) 1:3000; rabbit anti-cleaved caspase-3 (#9661, Cell Signaling Technology, MA, USA) 1:1000; mouse anti-fibrillarin (ab4566) 1:1000; rabbit anti-XRN2 (A301-103A-T, Bethyl Laboratories, Montgomery, TX) 1:1000; mouse anti-α-tubulin (Sigma-Aldrich, CA, USA), 1:100000;

mouse anti-GAPDH (Protein Tech Group Inc, IL, USA), 1:30000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies (sc-2005 and sc-2004, Santa Cruz Biotechnology, CA, USA, respectively) were used at a dilution of 1:3000 in TBS-T containing 2% low fat milk. Signal was detected using Clarity Western ECL Substrate reagent (BioRad) according to manufacturer's instructions. A GS-800 calibrated densitometer (Bio-Rad Laboratories, CA, USA) was used for quantitative analysis of protein levels.

Phase-contrast live-cell imaging. Fibroblasts were cultured in glass-bottom 35mm µ-dishes (Ibidi GmbH, Germany) coated with 50 µg/ml fibronectin (Sigma-Aldrich, MO, USA) and transfected with siRNAs. 24h post-transfection, cell cultures were imaged on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss, Germany) equipped with a CoolSnap camera (Photometrics Tucson, USA), XY motorized stage and NanoPiezo Z stage, under controlled temperature, atmosphere and humidity. Neighbor fields (20-25) were imaged every 2.5 min for 42h, using a 20x/0.3 NA A-Plan objective. Stitching of neighboring fields was done using the plugin "Stitch Grid" (Stephan Preibisch) from ImageJ/Fiji software. The time between two consecutive mitoses (referred as cell cycle duration) was manually quantified using ImageJ/Fiji software.

Flow cytometry. Cells were trypsinized, washed twice in cold PBS, and fixed in 70% ethanol at -20ºC overnight. Cells were washed twice in cold PBS and then incubated at 37ºC for 6 hours in propidium iodide (20 µg/mL) plus RNase (40 µg/mL) solution containing. Finally, cells were filtrated into a FACS tube. Data was recorded in FACS Canto II and analyzed with the FlowJo software using the Watson-Pragmatic algorithm to generate the cell cycle profiles.

Transmission Electron Microscopy. Cells were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, USA) and 2% paraformaldehyde (Merck, Darmstadt, Germany) in cacodylate buffer 0.1M (pH 7.4), dehydrated and embedded in Epon resin (TAAB, Berks, England). 40–60 nm ultrathin sections were prepared on a RMC Ultramicrotome (PowerTome, USA) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan).

qRT-PCR. Total RNA was isolated from cultured cells using the RNeasy Mini Kit (Qiagen) accordingly to the manufacturer's instructions. 1 µg of RNA was reverse transcribed (RT) using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). Quantitative real-time PCR analysis was performed in duplicate in 20 µl reactions containing iQ SYBR Green Supermix (BioRad), each gene-specific primer at 125

nM (100 nM for *G6PD*), and 1 µl of cDNA template previously diluted at 1:5. Cycling conditions in the iCycler iQ5 (Bio-Rad Laboratories) were 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing for 20 seconds at 60ºC, and a final extension step at 55ºC for 10 seconds. The $2-\Delta\Delta Ct$ method was used to quantify the transcript levels of *NOL12, CDKN1A, MMP1, CXCL8* and *TSPAN13* against the transcript levels of the housekeeping genes (*TBP* and *G6PD*). Primers were designed to span at least one exon–intron junction (Supplementary Table 3). Data was analyzed using Bio-Rad iQ5 – Standard Edition (Bio-Rad Laboratories).

Lentiviral plasmids. NOL12 was amplified from pENTR223.1-NOL12 (clone OCABo5050F031D, ImaGenes) as a BamHI-NOL12-NotI fragment, by using the following primers: 5'-CGGGATCCATGGGCCGCAACAAGAAG-3' and 5'-ATAAGAATGCGGCCGCTCACTCCCCGCTGTGC-3', forward and reverse, respectively. This BamHI-NOL12-NotI fragment was cloned into pLVX–Tight-Puro (Clontech, CA, USA) digested with BamHI + NotI, to generate pLVX–Tight-Puro-NOL12.

Lentiviral production and infection. Lentiviruses were produced according to the protocol described in Lenti-X Tet-ON Advanced Inducible Expression System (Clontech). Lentiviruses carrying pLVX–Tight-Puro–NOL12 and lentiviruses carrying pLVX–Tet-On Advanced (which expresses rtTA) were generated in HEK293T helper cells transfected with packaging plasmids (pMd2.G and psPAX2) using Lipofectamine 2000 (Life Technologies, Thermo Scientific, CA, USA). Human fibroblasts were co-infected for 12–16 h with responsive and *trans*-activator lentiviruses at 2:1 ratio, in the presence of 8 µg/ml polybrene (AL-118, Sigma-Aldrich, MO, USA). 375 ng/ml doxycycline (D9891, Sigma-Aldrich, MO, USA) was added for 1 day to induce co-transduction, and washed out for two days. This cyclic induction scheme was repeated 7 times before phenotype quantitative analysis. Western blot was used to monitor the efficiency of transduction.

Statistical Analysis. P-values were obtained using GraphPad Prism version 7.00 (GraphPad, San Diego, CA, USA). Data were tested for parametric vs. non-parametric distribution using D'Agostino & Pearson omnibus normality test. Mann-Whitney, paired t-test, one-way ANOVA, Kruskal-Wallis or two-tailed $\chi 2$-square were then applied accordingly to the experiment.

4. RESULTS

NOL12 regulates nucleolar structure and the protein levels of fibrillarin and nucleolin. To investigate the functional role of NOL12 at the nucleolus, we started by evaluating the NOL12 localization pattern in human primary dermal fibroblasts (HDFs) from

neonatal foreskin by immunostaining (Fig. 2.1A; Fig. S2.1A). We observed that NOL12 localization is mainly restricted to the nucleolus, partially colocalizing with the fibrillarin RNA methyltransferase at the DFC compartment and with the nucleolin RNA-binding protein that also localizes to the GC (Fig. 2.1A) (Ochs et al., 1985; Ugrinova et al., 2007). To gain insight into the functional role of NOL12 in neonatal HDF, we efficiently depleted NOL12 by about 80% at both transcript and protein levels (Fig. S2.1B and C). Importantly, the NOL12 nucleolar immunolocalization pattern observed was specific, as it was abolished following NOL12 small interfering RNA (siRNA [siNOL12])-mediated depletion (Fig. S2.1A).

Interestingly, we found that NOL12 repression increases both nucleolin and fibrillarin protein levels (Fig. 2.1B, F-H; Fig. S2.1D, E). These results are distinct from previous reports showing that fibrillarin, nucleolin, and nucleophosmin repressions do not interfere with the levels of other nucleolar proteins involved in ribosome biogenesis (Amin et al., 2007; Amin et al., 2008; Ugrinova et al., 2007). NOL12 knockdown also interfered with nucleolar organization, as evidenced by an increased ratio between nucleolar and nuclear areas (6.2%), along with a decrease in the average number of nucleoli per nucleus (from 3.4 to 2.6) (Fig. 2.1C, D; Fig. S2.1F, G). Using transmission electron microscopy (TEM), we observed that, whereas mock-depleted fibroblasts exhibited highly defined nucleoli with the typical amniote tripartite organization (Thiry and Lafontaine, 2005), nucleoli in NOL12-depleted fibroblasts presented poorly defined FC/DFC units (Fig. 2.1E).

We then asked if the observed nucleolar phenotype is specific to NOL12 repression or if other regulators of pre-rRNA processing might reveal a similar phenotype. To answer that, we depleted XRN2, a well-characterized nucleolar 5'-to-3' RNA exonuclease (Coccia et al., 2017; Memet et al., 2017; West et al., 2004). However, fibrillarin and nucleolin nuclear levels (Fig. 2.1F-H), as well as the average number of nucleoli per nucleus (Fig. 2.1I) were only marginally affected. Unlike the results of NOL12 depletion, XRN2-depleted fibroblasts actually displayed reduced nucleolar-/nuclear-area ratios (Fig. 2.1J; Fig. S2.1F, G). No cross-regulation between NOL12 and XRN2 protein levels was observed; therefore, the effects of each repression were specific (Fig. 2.1H).

Lastly, FBL and NCL were reported as being involved in ribosome biogenesis: while FBL is mainly involved in the 2'-O-methylation of rRNA (Sloan et al., 2017), NCL plays different roles in rDNA transcription, rRNA processing and ribosome assembly (Salvetti et al., 2016; Turner et al., 2009). Moreover, NOL12 depletion was shown to impair rRNA processing (Sloan et al., 2013b). Therefore, we addressed nucleolar function, particularly rDNA transcription, by measuring the incorporation of FUrd into newly synthesized rRNA transcripts. Relevantly, we found a decrease of immunofluorescence levels of incorporated FUrd in NOL12-depleted HDFs (Fig. S2.2), suggesting that accumulation of unprocessed rRNA transcripts in NOL12-depleted HDFs disturbs rDNA transcription. Based on this, we

hypothesized that NOL12 depletion distresses ribosome biogenesis and subsequently protein synthesis. Therefore, we measured the incorporation of puromycin into newly synthesized proteins and we found decreased global protein synthesis upon NOL12 repression (Fig. S2.2).

Altogether, our results showed that human NOL12 is a nucleolar protein specifically required for the maintenance of nucleolar structure, as well as for the regulation of nucleolar levels of fibrillarin and nucleolin, key players in pre-rRNA processing and ribosome assembly. Consistently, the dysregulation of nucleolar proteins seems to impair nucleolar function, reducing rDNA transcription and protein synthesis.

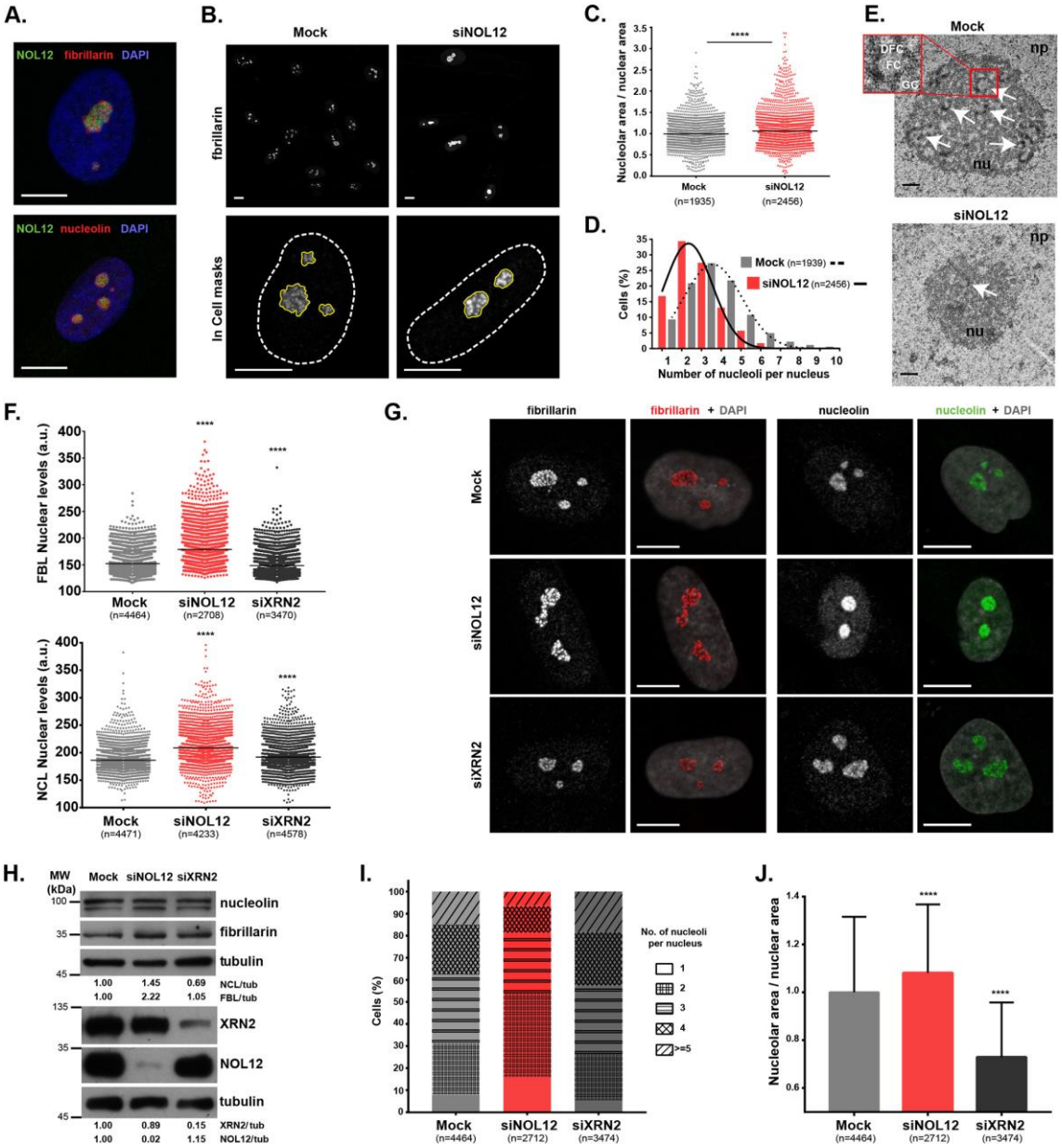

Figure 2.1. NOL12 repression induces a specific nucleolar stress response in human untransformed cells. (A) NOL12 immunolocalization pattern in neonatal dermal fibroblasts (green) and co-localization with fibrillarin and nucleolin nucleolar markers (red). DAPI was used for DNA staining (blue). **(B)** fibrillarin immunostaining (greyscale) in control (mock) and NOL12 siRNA-depleted (siNOL12). In the nuclei

magnifications (63x; lower panels), the white dashed and the yellow solid lines represent the masks used to define and measure nuclear and nucleolar areas, respectively. **(C)** Ratio between nucleolar and nuclear areas. Each dot represents a single cell. Horizontal lines represent the mean values normalized to mock controls. **(D)** Histogram and respective distribution curves for the percentage of mock- and siNOL12-depleted cells exhibiting a total number of nucleoli as indicated. **(E)** Ultrastructure of mock- and siNOL12-depleted nucleoli accessed by transmission electron microscopy. Representative micrographs, at 20000x magnification, are shown. Arrows indicate FC/DFC units. Inset in red is a 6.6x magnification of a nucleolar unit containing well defined FC, DFC and GC compartments. Scale bars, 0.5 µm. np: nucleoplasm, nu: nucleolus, FC: fibrillar center, DFC: dense fibrillar component, GC: granular component. **(F)** Scatter plots of the mean pixel intensities of fibrillarin (FBL) and nucleolin (NCL) nuclear levels in mock-, siNOL12- and siXRN2-depleted cells. Each dot represents a single cell, and horizontal lines represent the mean values. **(G)** Immunostaining of fibrillarin and nucleolin (greyscale/red and greyscale/green, respectively) in mock-, siNOL12- and siXRN2-depleted cells. Nuclei were stained with DAPI (grey). **(H)** Cell extracts from mock-, siNOL12- and siXRN2-depleted neonatal fibroblasts were immunoblotted for nucleolin, fibrillarin, XRN2 and NOL12. Tubulin levels were used for loading control. Protein levels were normalized to mock control. **(I)** Stacked bars for the distribution of the total number of nucleoli per nucleus in mock-, siNOL12- and siXRN2-depleted cells. Different patterns in bars represent different number of nucleoli per nucleus. **(J)** Ratio between nucleolar and nuclear areas in mock-, siNOL12 and siXRN2-depleted cells. Values are mean ± SD normalized to control mean. Scale bars are 10µm in A), B) and G). n= **t**otal number of cells analyzed. ****$p \leq 0.0001$ by Mann-Whitney and Kruskal-Wallis statistical tests in C) and J).

NOL12 repression activates the p53-signaling pathway in an RPL11-dependent manner. Next, we asked whether NOL12 knockdown and the associated disruption of nucleolar structure could induce p53 stabilization, which is elicited by a variety of cellular stresses (Joerger and Fersht, 2016), including RNA Pol I inhibition-driven nucleolar stress (Choong et al., 2009). Both immunoblot and immunofluorescence quantitative analyses of p53 levels revealed its stabilization upon NOL12 and XRN2 knockdowns, although the level was significantly higher in NOL12 repression (Fig. 2.2A-C).

At low doses, actinomycin D (ActD) specifically inhibits RNA Pol I, preventing the transcription of rDNA into a 47S pre-rRNA primary transcript (Hadjiolova et al., 1995) and inducing alterations in nucleolar structure (Boulon et al., 2010). Mechanistically, it was previously shown in U2OS cells that p53 activation by ActD treatment is dependent on the tripartite complex RPL11-RPL5-5S rRNA binding to MDM2, preventing the MDM2-mediated p53 degradation (Sloan et al., 2013a). We confirmed that RPL11 is also required for ActD-induced p53 stabilization in HDF (Fig. 2.2D, F, G; Fig. S2.3). Therefore, we explored the contribution of RPL11 to the p53 activation induced by NOL12 repression. Both immunofluorescence and immunoblot analyses revealed RPL11 to be required for p53 activation upon NOL12 knockdown (Fig. 2.2E-G). Accordingly, upon NOL12 knockdown, we detected a significant induction of the p53 downstream target p21/CDKN1A (Wang and El-Deiry, 2005), which was abolished in the double depletion of NOL12 and RPL11 using

siRNAs siNOL12 and siRPL11 (Fig. 2.2E-G). Altogether, our data suggest that nucleolar stress caused by NOL12 knockdown activates the p53-dependent signaling pathway in an RPL11-dependent manner.

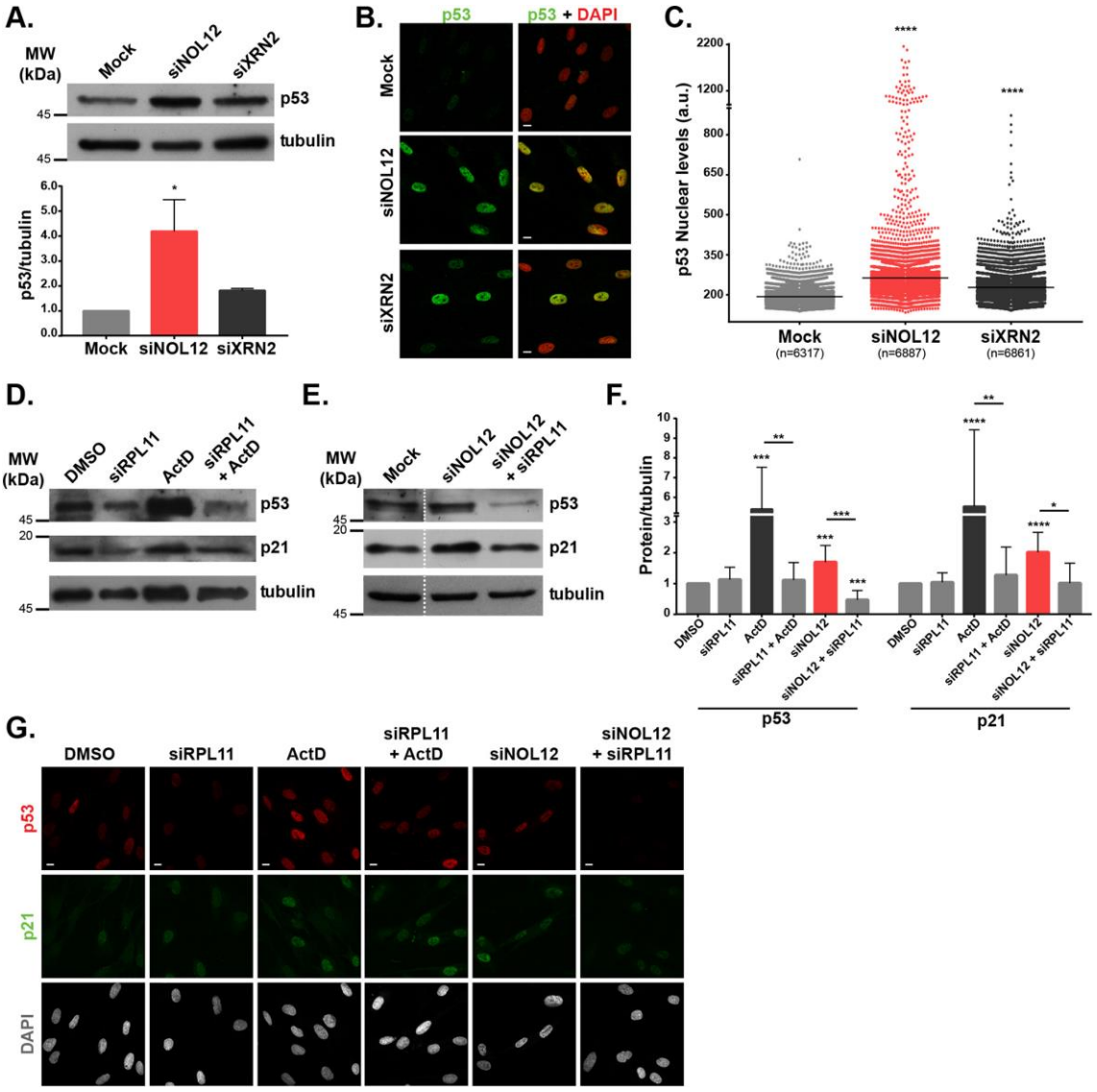

Figure 2.2. NOL12 repression activates the p53-signaling pathway in an RPL11-dependent manner. (A) Western blotting analysis of p53 levels in cell extracts from mock-, siNOL12- and siXRN2-depleted neonatal dermal fibroblasts. Tubulin protein levels were used as loading control. In the graph, bars are mean ± SD values from three independent experiments and normalized to mock control. * $p \leq 0.05$ by Kruskal-Wallis statistical test. **(B)** p53 immunostaining (green) of mock-, siNOL12- and siXRN2-depleted human fibroblasts. DNA was stained with DAPI (red). Scale bars, 10µm. **(C)** Scatter plot of p53 nuclear mean pixel intensity levels in mock-, siNOL12- and siXRN2-depleted cells. Each dot represents a single cell and horizontal lines represent mean values. n= total number of cells analyzed. **** $p \leq 0.0001$ by Kruskal-Wallis statistical test. **(D)** Western blotting analysis of p53 and p21 protein levels in cell extracts from control or siRpL11-depleted fibroblasts, untreated or treated with 8nM Actinomycin D for 4 hours, as indicated. Tubulin was used as loading control. **(E)** Western blotting analysis of p53 and p21 protein levels in cell extracts from mock, siNOL12- and siNOL12+siRpL11-depleted fibroblasts. Tubulin was used as loading control. **(F)** p53 and p21 protein levels measured by western blotting analysis (as

shown in D and E) from four independent experiments. Bars represent mean ± s.d values normalized to tubulin loading control and to DMSO control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$, by Mann-Whitney statistical test. **(G)** Immunostaining of p53 (red) and p21 (green) in control, siRpL11-, siNOL12- and siNOL12+siRpL11-depleted fibroblasts, untreated and treated with 8nM Actinomycin D for 4 hours. DNA was stained with DAPI (grey). Scale bars, 10µm.

NOL12 repression induces p53-dependent G2 arrest and cellular senescence.

As a consequence of p53 activation, cells can undergo marked phenotypic changes, ranging from increased DNA repair to senescence and apoptosis (Joerger and Fersht, 2016). Independently of the cell fate, p53 activation typically inhibits the cell cycle by inducing the expression of p21/CDKN1A (Wang and El-Deiry, 2005). To gain insight into the contribution of stabilized p53 in cell proliferation, we performed time-lapse phase-contrast microscopy of mock-, NOL12-, p53-, and NOL12-and-p53-depleted primary fibroblast cultures (Fig. 2.3A; Movies S1 to S4). Live-cell imaging revealed a clear reduction in the proliferation rate of NOL12-depleted cell cultures, confirmed by a decreased percentage of living cells that incorporated 5-ethynyl-2'-deoxyuridine (EdU) (Fig. 2.3B), with cycling cells from NOL12-depleted cell cultures exhibiting a cell cycle delay in comparison to the cycling of the control (Fig. 2.3C). This cell cycle delay was consistent along three cell generations (from a grandmother cell to the granddaughter cells), although there was a progressive decrease in the number of cycling cells over the recorded period (Fig. S2.4B). Reduced proliferative capacity was further confirmed by an increased percentage of fixed cells staining negative for the proliferation marker Ki67 (66.5% ± 6.9% [mean ± standard deviation] versus 10.7% ± 5.5% in controls) (Fig. 2.3D; Fig. S2.4A). Importantly, the data showed that the proliferation rate of NOL12-depleted cells was rescued by p53 depletion (Fig. 2.3A-D).

To determine which cell cycle phase primarily contributes to the cell cycle delay observed following NOL12 repression, we performed flow cytometry cell cycle profiling. We found a significant increase (3.7-fold) in the G2/M cell subpopulation in NOL12-depleted cell cultures, which was rescued by p53 depletion (Fig. 2.3E; Fig. S2.4C). Moreover, cell cultures treated both with siRNA against p53 (siP53) and with siNOL12 and siP53 together (siNOL12 + siP53) exhibited an accumulation of S-phase cells, which is likely due to the absence of the breaker role of p53 in cell cycle progression (Fig. 2.3E). In addition, we measured the levels of cyclin B1, which specifically accumulates at the G2/M transition (Pines and Hunter, 1989), and surprisingly, we found them to be significantly reduced in NOL12-depleted cell extracts (Fig. 2.3F). In agreement with the role of p53 as a cell cycle suppressor, p53 repression induced increased levels of cyclin B1 and restored the cyclin B1 levels in NOL12-depleted cell extracts (Fig. 2.3F).

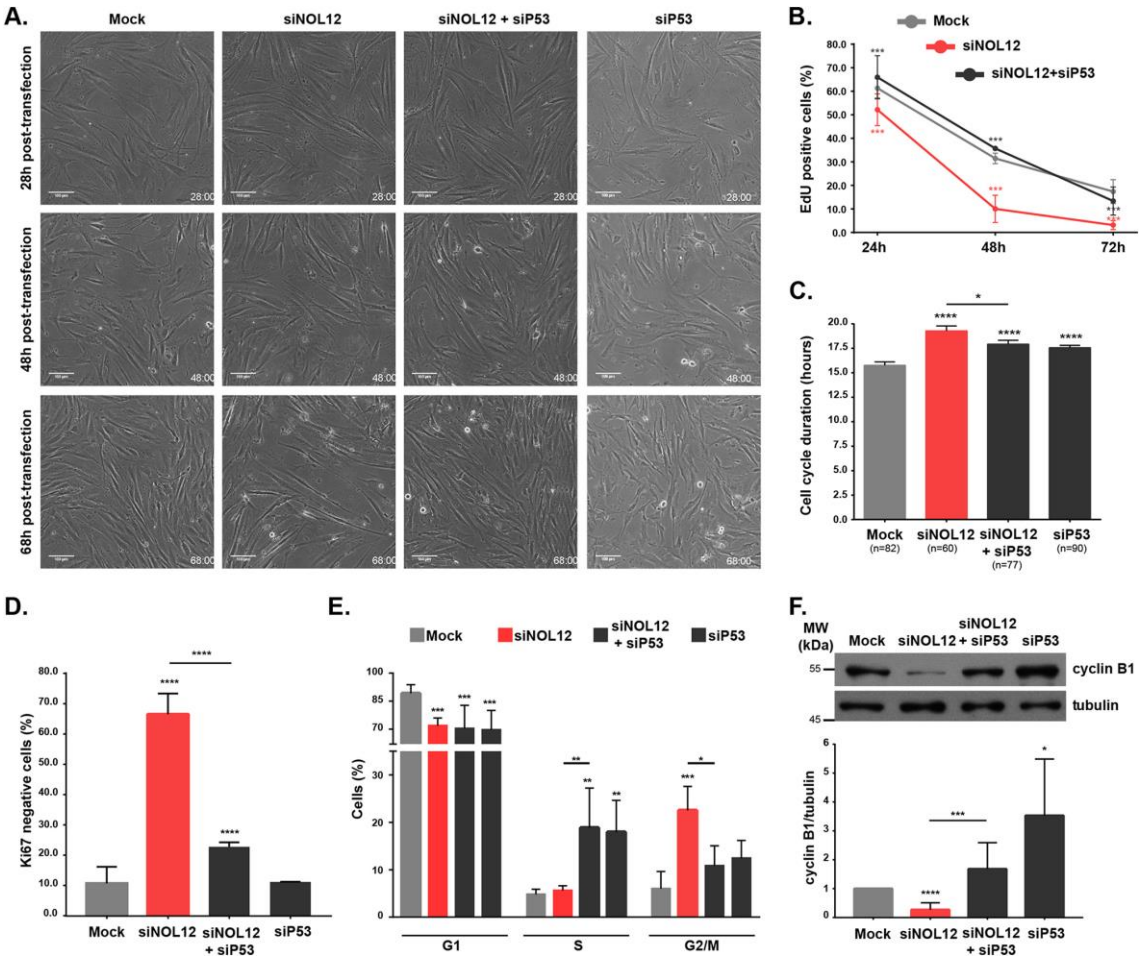

Figure 2.3. NOL12 repression induces p53-dependent G2 arrest. (A) Long-term phase-contrast live-cell imaging of mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cell cultures. Movie representative images captured at 28, 48 and 68 hours post-transfection are shown. Scale bars, 100µm. **(B)** Percentage of mock-, siNOL12- and siNOL12+siP53-depleted cells staining positive upon EdU incorporation assay at the 24, 48 and 72 hours post-transfection. Data are mean ± SEM from three independent experiments. *** $p \leq 0.001$ by χ2-square statistic test. **(C)** Cell cycle duration, measured as the interval between mother cell mitosis and daughter cell mitosis, in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cell cultures. Values are mean ± SEM from n= total number of cells analyzed. * $p \leq 0.05$ and **** $p \leq 0.0001$ by Mann-Whitney statistical test. **(D)** Percentage of cells staining negative for the proliferation marker Ki67 in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted cell cultures. Values are mean ± SD from at least two independent experiments. **** $p \leq 0.0001$ by χ2-square statistical test. **(E)** Percentage of cells in G1, S and G2/M cell cycle phases in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cultures as determined by flow cytometer cell cycle profiling. Values are mean ± SD from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ by two-way ANOVA statistical test. **(F)** Western blot analysis of Cyclin B1 levels in cell extracts from mock-, siNOL12-, siP53- and siNOL12+siP53-depleted neonatal fibroblasts. Tubulin levels were used as loading control and Cyclin B1 levels were normalized to mock controls. Values are mean ± SD from at least seven independent experiments. * $p \leq 0.05$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ by Mann-Whitney statistical test.

Since the proliferation rate and cyclin B1 levels were significantly decreased following NOL12 repression, we hypothesized that the G2/M subpopulation in the cell cycle profiling most likely reflects a G2 arrest. Interestingly, transient induction of p53 in G2 has been shown to act as the first irreversible step in the onset of senescence, a state of permanent withdrawal from the cell cycle without undergoing cell death (Krenning et al., 2014). Indeed, both live-cell imaging (Fig. 2.3A; Movies S1, S2) and immunoblot analysis of cleaved caspase-3 (Fig. S2.4D) excluded apoptosis as a significant contributor to the decreased proliferation rate in NOL12-depleted cell cultures. Thus, we asked whether NOL12 repression was inducing an accumulation of G2-arrested senescent cells. This permanent arrest is dependent on induction of p53 and p21, resulting in transient nuclear retention of cyclin B1 in the presence of high nuclear p21 levels (Krenning et al., 2014). Additionally, a study in primary fibroblasts showed p21 as being able to retain inactive cyclin B1/Cdk1 complexes in the nucleus (Charrier-Savournin et al., 2004). Therefore, we stained NOL12-depleted cells for both p21 and cyclin B1 and investigated the presence of double-positive staining. Albeit scarce due to the transient nature of nuclear cyclin B1 retention, we found a 6-fold enrichment of p21/cyclin B1 double-positive nuclei in NOL12 depletion (Fig. 2.4A). p53 depletion was sufficient to prevent nuclear retention of cyclin B1 in NOL12-depleted cells (Fig. 2.4A). Furthermore, we quantified the percentages of cells exhibiting senescence-associated markers, namely, β-galactosidase (β-Gal) activity and double positivity for p21 and 53BP1 (a DNA damage response marker) (Macedo et al., 2018). We found significant increases in these markers following NOL12 repression (p21 positive [$p21^+$]/$53BP1^+$, 10.68% ± 3.46% versus 1.55% ± 0.09% in controls; senescence-associated β-Gal positive [SA–β-Gal^+], 16.42% ± 4.28% versus 5.38% ± 0.52% in controls), which was rescued upon p53 depletion (Fig. 2.4B, C). Accordingly, we also found p53-dependent upregulation of the senescence-associated secretome by assessing the RNA expression of selected genes (Fig. 2.4D) (Macedo et al., 2018).

Even though p53 activation was reported to impact rDNA transcription and ribosome biogenesis (Golomb et al., 2014; Zhai and Comai, 2000), we did not observe any significant alteration in the number of nucleoli per nucleus or the nucleolar size in siNOL12+siP53 double-depleted cells in comparison to siNOL12-depleted cells based on FBL staining (Fig. S2.5A-C). siP53-depleted cells also did not exhibit reduced number of nuclei per nucleus, and the nucleolar/nuclear size ratio was lower rather than higher (Fig. S2.5B, C). Similarly, FBL upregulation in NOL12-depleted cells is not dependent on P53 activation, as we found that P53 activation does not account for FBL upregulation in NOL12-depleted cells (Fig. S2.5D). Interestingly, siP53-depleted cells showed upregulation of FBL, which is in accordance with previous reports (Fig. S2.5D) (Chen et al., 2020; Marcel et al., 2013).

Taken together, our data strongly support that cellular senescence induced by NOL12 knockdown is p53-dependent. Notably, we found senescence as the primary cell fate of untransformed cells in response to nucleolar stress, contrary to the induction of p53-dependent and -independent apoptosis previously reported in transformed cells (James et al., 2014).

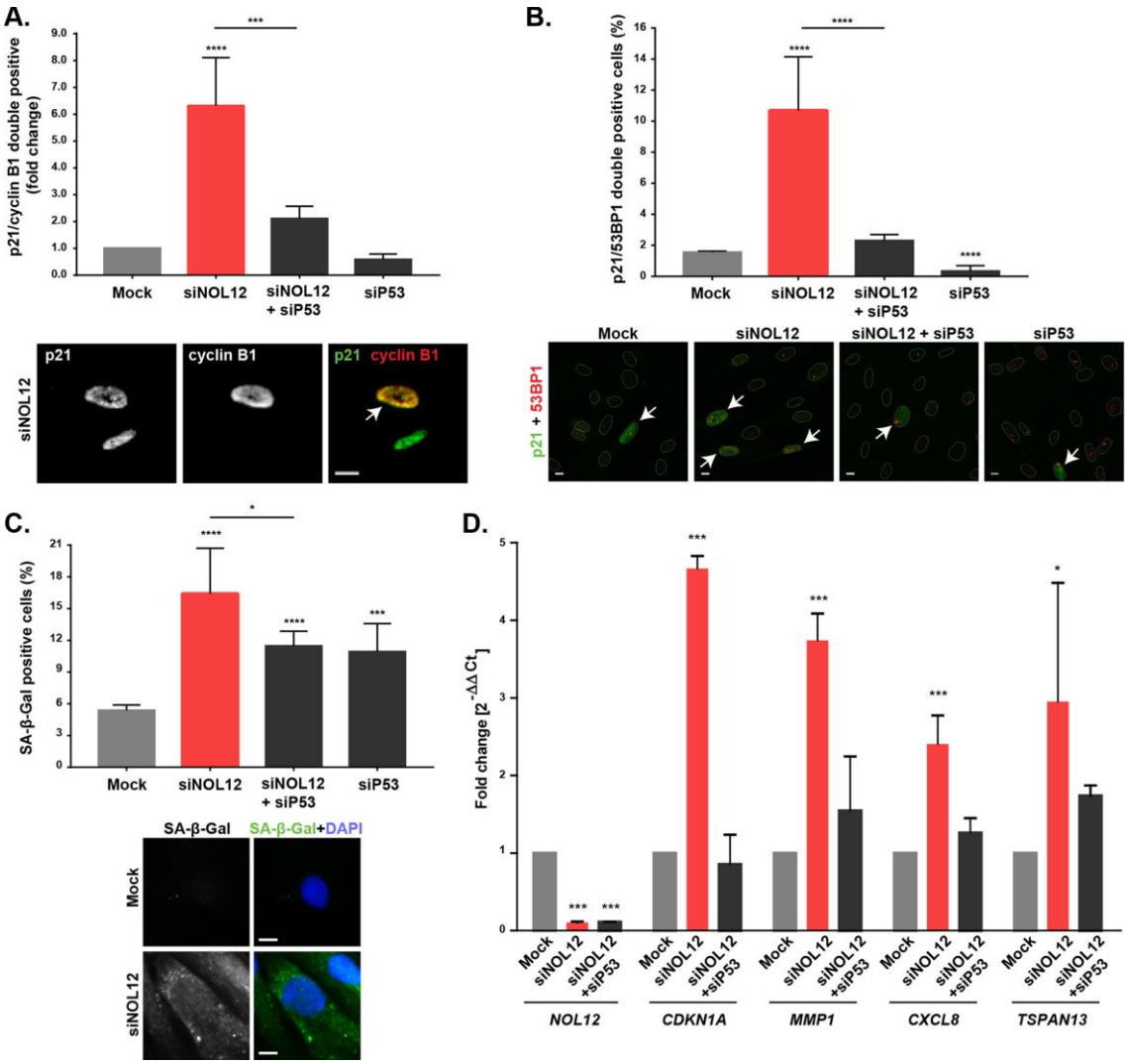

Figure 2.4. NOL12 repression induces p53-dependent senescence. (A) Percentage of cells staining positive for p21/Cyclin B1 immunofluorescence in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cultures. In the graph, values are mean ± SD from n>50000 cells, and values are shown as fold change in comparison to mock controls. *** $p \leq 0.001$ and **** $p \leq 0.0001$ by χ2-square statistical test. Panel below shows a representative image of a siNOL12-depleted cell staining double positive for p21/Cyclin B1 (white arrow). Scale bar, 10µm. **(B)** Percentage of cells double positive for p21/53BP1 immunostaining in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cultures. Values are mean ± SD from two independent experiments. **** $p \leq 0.0001$ by χ2-square statistical test. Panel below shows representative images for each experimental condition. Nuclei masking is shown in white. Arrows indicate p21/53BP1 double positive cells. Scale bars, 10µm. **(C)** Percentage of SA-β-galactosidase (SA-β-Gal) positive cells in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cultures. Values are mean ± SD from two independent experiments. *

$p \leq 0.05$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ by $\chi 2$-square statistical test. Panel below shows representative images of mock- and siNOL12-depleted cells staining negative and positive for SA-β-gal, respectively. DNA was stained with DAPI (blue). Scale bars, 10μm. **(D)** qPCR analysis of transcript levels of *NOL12*, *CDKN1A*, *MMP1*, *CXCL8* and *TSPAN13* in mock-, siNOL12-, and siNOL12+siP53-depleted fibroblast cultures. *G6PD* and T*BP* were used as housekeeping genes. Values are mean ± SD from four independent experiments and normalized to controls. * $p \leq 0.05$ and *** $p \leq 0.001$ by ordinary one-way ANOVA statistical test.

NOL12 repression and nucleolar stress during chronological aging. Our findings showing a link between NOL12 repression and cellular senescence led us to ask whether regulation of NOL12 plays a role in naturally aged cells and in replicative senescence. In agreement with the widely reported accumulation of senescent cells during chronological and replicative aging (Baker et al., 2011; Dimri et al., 1995), we found significantly higher levels of senescence-associated biomarkers in the proliferating fibroblast cultures from elderly than from neonatal donors and in high- versus low-passage-number cells, thus validating their suitability as models of aging (Fig. S2.6A-C).

Importantly, we found downregulation of NOL12 protein levels in primary fibroblasts from 77-, 84-, 85-, and 87-year-old donors (<5 early cell culture passages), as well as in replicatively aged cells (Fig. 2.5A, B). Next, we investigated whether naturally and replicatively aged primary cells with decreased NOL12 levels were under nucleolar stress. Remarkably, nucleoli in elderly donor cells displayed undefined and/or reduced numbers of FC/DFC units (Fig. 2.5C). Also, both naturally and replicatively senescent cells exhibited alterations in the nucleolar organization similar to those observed in NOL12-depleted cells, namely, upregulation of fibrillarin nuclear levels, decreased numbers of nucleoli per nucleus, and increased ratios between nucleolar/nuclear areas (Fig. 2.5D-F; Fig. S2.6D, E). Since NOL12 depletion induces nucleolar stress, ultimately leading to a decreased proliferation rate and accumulation of senescent cells in human primary neonatal fibroblast cultures, we hypothesized that NOL12 overexpression in elderly donor cells should counteract this stress response. Using lentiviral infection, we efficiently overexpressed NOL12 in fibroblasts retrieved from an 84-year-old donor (Fig. S2.7A). We found NOL12 overexpression to partially rescue the proliferation rate and fibrillarin nuclear levels (Fig. S2.7B, C). However, other nucleolar-stress-associated phenotypes, such as the ratio between nucleolar/nuclear area and the number of nucleoli per nucleus, were not significantly altered (Fig. S2.7D, E). Also, the number of senescent cells remained unchanged, as evidenced by quantification of SA–β-Gal-positive cells (Fig. S2.7F).

Thus, by restoring NOL12 protein levels in elderly donor cells, we partially rescued the proliferation rate but not the accumulation of senescent cells. Still, our data disclosed a

correlation between NOL12 repression, nucleolar stress, and chronological aging in human primary fibroblasts.

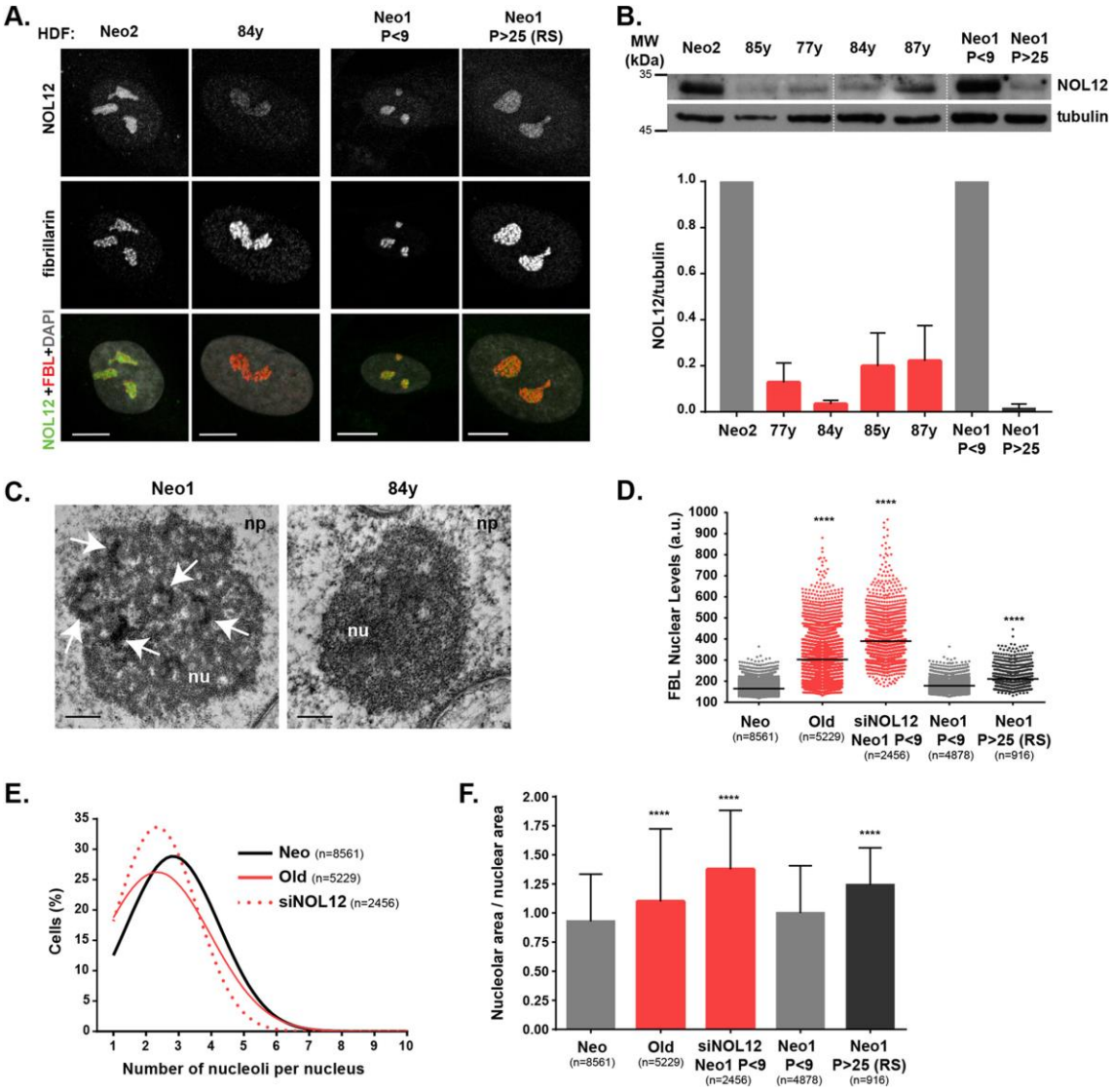

Figure 2.5. NOL12 repression and nucleolar stress in human elderly fibroblasts. (A) Immunofluorescence analysis of NOL12 (grey/green) and fibrillarin (grey/red) levels in neonatal (Neo2) vs. 84-year-old (84y) HDFs, and low passage (P<9) vs. high passage (P>25; RS- replicative senescence) neonatal (Neo1) fibroblasts. DNA was stained with DAPI (grey). Scale bars, 10µm. **(B)** Western blot analysis of NOL12 protein levels in cell extracts from neonatal (Neo2), 85y, 77y, 84y and 87y HDFs, as well as from low and high passage neonatal fibroblasts (Neo1 P<9 and Neo1 P>25). Tubulin was used as loading control. In the graph, values are mean ± SD from two independent experiments and normalized to Neo2 (all red bars) or Neo1 (dark grey bar). **(C)** Ultrastructural analysis of neonatal and 84y nucleoli by transmission electron microscopy. Representative micrographs, at 30000x and 50000x magnification respectively, are shown. Arrows indicate FC/DFC units. Scale bars, 0.5µm. np: nucleoplasm, nu: nucleolus. **(D)** Scatter plot shows the mean pixel intensity of fibrillarin nuclear levels in neonatal (Neo1 and Neo2), elderly (Old; 77y, 84y, 85y and 87y), NOL12 repressed (siNOL12) and neonatal low and high passage (Neo1 P<9 and Neo1 P>25) cells. Each dot represents a single cell. Horizontal lines represent the mean. **** $p \leq 0.0001$ by Kruskal-Wallis statistical test. **(E)** Distribution curves for the total number of nucleoli per cell in neonatal (Neo1 and Neo2), elderly (Old; 77y, 84y, 85y and 87y) and NOL12

repressed (siNOL12) fibroblast cultures. **(F)** Ratio between nucleolar and nuclear areas in neonatal (Neo1 and Neo2 were), elderly (Old; 77y, 84y, 85y and 87y), NOL12 repressed (siNOL12) and neonatal low and high passage (Neo1 P<9 and Neo1 P>25) cells. Values are mean ± SD normalized to Neo (red bars) or Neo1 (dark grey bar). **** $p \leq 0.0001$ by Kruskal-Wallis statistical test. RS, replicative senescence. P, cell passage.

5. DISCUSSION

We previously showed that *Drosophila* Viriato/NOL12 is a nucleolar protein crucial for proper nucleolar structure and dMyc-induced cell growth (Marinho et al., 2011). In this work, we used human primary fibroblasts to further investigate the cellular functions of NOL12.

We examined the nucleolar localization of NOL12 in human primary fibroblasts, finding that it co-localizes with fibrillarin and nucleolin, in accordance with a recent study performed in the HCT116 cell line (Scott et al., 2017). To investigate the largely elusive role of NOL12 in nucleolar homeostasis, we knocked down NOL12 in neonatal primary fibroblasts, which revealed that NOL12 is required for proper nucleolar organization due to a role in limiting nucleolar size and regulating nucleolar number. Interestingly, repression of NOL11 or hUTP4 in human MCF10A cells was also found to cause a strong reduction in nucleolar number, in correlation with defects in pre-rRNA processing (Freed et al., 2012). Moreover, a reduction in nucleolar number was recently used in a genome-wide siRNA screen to identify 16 regulators of ribosome biogenesis (Farley-Barnes et al., 2018).

In addition, we found NOL12 acting to restrain nucleolar accumulation of nucleolin and fibrillarin. To evaluate whether this pattern of nucleolar stress was specific for NOL12, we knocked down XRN2, another nucleolar 5'-to-3' RNA exoribonuclease able to localize to the nucleolus (Coccia et al., 2017; Memet et al., 2017), which has multiple roles in pre-rRNA cleavage and rRNA maturation (Sloan et al., 2014; Sloan et al., 2013b). We found that in contrast to NOL12 repression, XRN2 depletion actually decreased nucleolar size without modifying the number of nucleoli per cell. Moreover, whereas NOL12 depletion caused significant increases in fibrillarin and nucleolin cellular protein levels, XRN2 did not have that role. Repression of other nucleolar proteins, such as nucleolin (Ma et al., 2007), fibrillarin (Amin et al., 2007), and nucleophosmin (Amin et al., 2008), also did not change the expression levels of different nucleolar proteins.

Stabilization and/or activation of p53 is central in the nucleolar stress response to defects in rRNA synthesis/processing and ribosomal subunit assembly (Deisenroth et al., 2016), even though p53-independent mechanisms have also been suggested (Holmberg Olausson et al., 2012; James et al., 2014; Jayaraman et al., 2017). Thus, we assessed p53 protein levels upon NOL12 or XRN2 repression in human fibroblasts. Interestingly, we found

significantly increased p53 protein levels in NOL12-depleted cells compared with the levels in mock- or XRN2-depleted cells. The transcriptional activity of p53 also increased, as p21 target protein levels were upregulated. p53 has been described to repress fibrillarin expression at the transcriptional level (Marcel et al., 2013), so we were intrigued by the concomitant increase of both p53 and fibrillarin upon NOL12 knockdown. Nevertheless, we cannot exclude the possibility that in the context of NOL12 repression, p53 activation actually helps to limit an otherwise stronger upregulation of nucleolar fibrillarin. Given that high fibrillarin expression levels are associated with poor prognosis in breast cancer (Marcel et al., 2013), more studies are needed to clarify the mechanisms behind fibrillarin upregulation.

Mechanistically, our results show that activation of the p53 signaling pathway by NOL12 repression is dependent on RPL11 expression. In response to nucleolar stress, RPL11 interacts with MDM2 (or HDM2 in human), inhibiting its ability to regulate p53 degradation. This has been reported for several stress conditions, including the inhibition of the activity of RNA Pol I by low-level actinomycin D treatment (Lohrum et al., 2003; Zhang et al., 2003) or the perturbation of ribosome biogenesis by RPL29 and RPL30 knockdown (Sun et al., 2010).

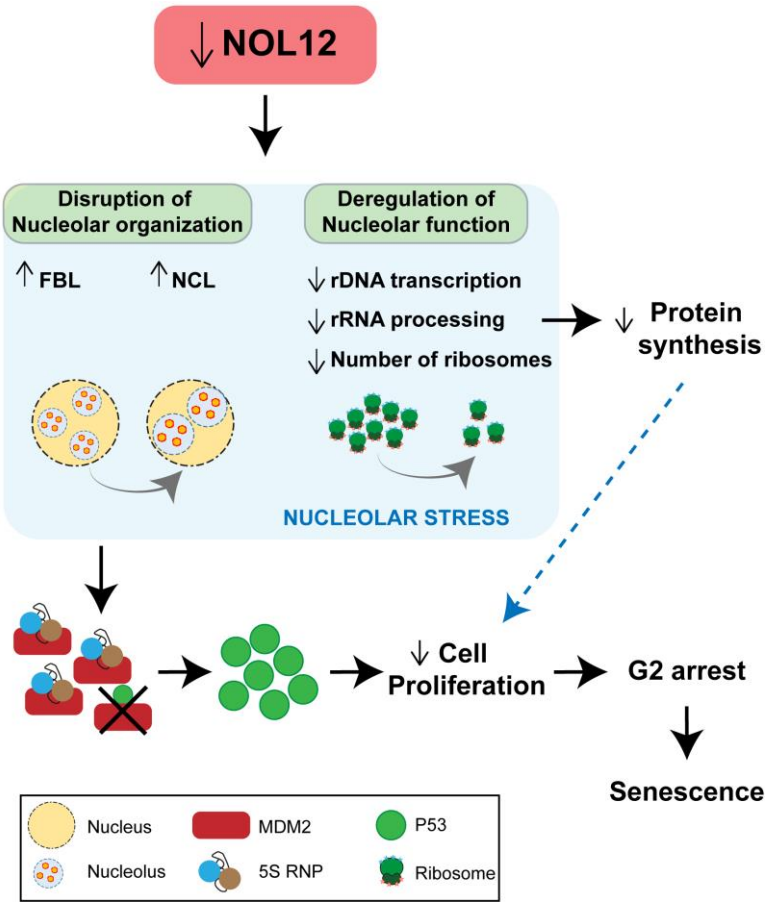

Figure 2.6. Proposed model for the impact of NOL12 downregulation in nucleolar homeostasis and senescence in HDFs. NOL12 repression changed the nucleolar architecture by increasing nucleolar size, decreasing the number of nucleoli per nucleus and upregulating both FBL and NCL protein levels. Regarding nucleolar function, NOL12 downregulation decreased rDNA transcription and rRNA processing, thus likely reducing ribosome biogenesis, which supports the decreased global protein synthesis observed. These nucleolar alterations led to the accumulation of the 5S RNP bound to MDM2, leading to P53 activation. This, in turn, decreased the proliferative capacity, arrested cells at the G2 phase and ultimately led to senescence. Similarly to P53 activation, decreased protein synthesis could also contribute for decreased cell proliferation (dashed arrow).

We next explored the cellular consequences of p53 activation in NOL12-depleted cells. Phase-contrast live-cell-imaging analysis first indicated enlarged cell morphology and reduced proliferative capacity in NOL12-depleted cell cultures. These effects were rescued by NOL12 and p53 codepletion, suggesting defective cell proliferation, as previously described for *Drosophila* vito RNAi (Marinho et al., 2011). In agreement with previous studies showing direct correlation between cellular amount of proteins and proliferative capacity (Polymenis and Aramayo, 2015), we also found an overall decrease in protein synthesis. Therefore, although our data clearly disclosed p53 activation as a major contributor for decreased cell proliferation, we cannot exclude defective protein synthesis driven by faulty nucleolar function as an additional contributor. In addition, we excluded apoptosis as potential cause for reduced cell number as we did not detect cleaved caspase-3 or an increased sub-G1 population by flow cytometry. This is in contrast to nucleolar stress caused by depletion of TIF-IA, a basal transcription initiation factor for RNA Pol I, found to induce p53-dependent apoptosis in embryonic fibroblasts (Yuan et al., 2005). One possibility is that the increase in fibrillarin nucleolar levels in NOL12-depleted cells contributes to biased translation for internal ribosomal entry site (IRES)-containing mRNAs encoding important antiapoptotic proteins, as previously described (Marcel et al., 2015; Marcel et al., 2013). With apoptosis excluded in NOL12-depleted cell cultures, we next evaluated senescence. Cell cycle profiling and immunodetection of cyclin B1 levels allowed us to conclude that NOL12-depleted cells are arrested in G2 phase in a p53-dependent manner. Interestingly, the increased nucleolar area observed in NOL12-depleted cells is consistent with cells at the G2 phase (Hernandez-Verdun, 2011; Junera et al., 1995; Maszewski and Kwiatkowska, 1984). Previous studies also found the repression of nucleolin and nucleostemin to induce G2 arrest (Huang et al., 2015; Ugrinova et al., 2007), even though p53 activation was not investigated. Remarkably, an irreversible G2 arrest, characterized by a transient nuclear accumulation of p21 and cyclin B1, was shown to be the first step in the onset of senescence (Krenning et al., 2014). Accordingly, we found that NOL12-depleted cell cultures are enriched in p21/cyclin B1 double-positive cells. Moreover,

Nol12-depleted cell cultures displayed increased numbers of cells positive for senescence-associated biomarkers, SA–β-galactosidase activity, and p21/53BP1 immunostaining and exhibited the senescence-associated secretory phenotype. Therefore, our results show that senescence, rather than apoptosis, is the outcome for untransformed cells in response to NOL12 depletion-driven nucleolar stress. Interestingly, therapeutic inhibition of Pol I transcription by treatment with CX-5461, a specific small-molecule inhibitor of RNA Pol I, was shown to induce cell death in malignant B cell lymphomas but not in normal B cells (Bywater et al., 2012). This bias was also reported in mouse epidermis upon p53 activation induced by MDM2 deletion (Gannon et al., 2011). In contrast, in transformed cells, it was reported that apoptosis mainly configures the response to nucleolar stress (James et al., 2014). During preparation of our manuscript, increased apoptosis upon NOL12 depletion in colon cancer HCT116 cells was reported (Scott et al., 2017). In contrast to our results, apoptosis was associated with p53-independent G1 arrest and ATR-Chk1 activation (Scott et al., 2017). It has been shown that, in response to nucleostemin depletion, embryonic fibroblasts and HCT116 cells have different p53-regulated responses (Huang et al., 2015). At the mechanistic level, whereas transient induction of p53 in untransformed G2 cells is sufficient to induce the onset of senescence (Krenning et al., 2014), HCT116 cancer cells can overcome the G2 arrest 18 h after a DNA-damaging insult, upon checkpoint recovery mediated by the function of the Wip1 phosphatase (Lindqvist et al., 2009). Therefore, we hypothesize that a similar differential response to p53 activation could explain the distinct phenotypes induced by NOL12 knockdown in primary fibroblasts and HCT116 cells.

After establishing a connection between NOL12 repression and increased senescence, we next asked whether regulation of NOL12 expression plays a role in aging-associated senescence. Remarkably, we found that both low-passage-number fibroblast cultures retrieved from elderly donors or high-passage-number human neonatal fibroblasts (replicative senescence) exhibit significant downregulation of NOL12 expression. Similar to the nucleolar phenotypes observed following NOL12 repression, both replicatively and naturally aged cells exhibited increased nucleolar area, lower numbers of nucleoli per nucleus, higher nuclear fibrillarin levels, and altered nucleolar ultrastructure. In parallel, recent studies characterized fibrillarin upregulation and increased nucleolar size as hallmarks of aging across species (Buchwalter and Hetzer, 2017; Tiku et al., 2017). In *Caenorhabditis elegans*, fibrillarin knockdown reduced nucleolar size and ribosome biogenesis, and these alterations correlated with extended lifespan (Tiku et al., 2017). Furthermore, prematurely aged cells from HGPS (Hutchinson-Gilford progeria syndrome) donors exhibited bigger but fewer nucleoli (Buchwalter and Hetzer, 2017). By restoring NOL12 protein levels in fibroblasts from elderly donors, we expected to rescue their senescent phenotype. Although we found a partial rescue in the number of proliferative cells

and a mild reduction of fibrillarin levels, the number of senescent cells and the nucleolar organization remained unchanged, likely due to the complexity of the senescence process. Eventually, cells would need more time in culture in order to allow the dilution of the senescent cells and their paracrine effects by the more fit proliferating cells expressing NOL12. While the recovery of NOL12 protein levels was not sufficient to revert senescence in cell cultures from elderly donors, FoxM1, a transcription factor involved in G2/M transition (Fischer et al., 2016; Sadasivam et al., 2012), was recently reported as able to do it (Macedo et al., 2018). Interestingly, RNA sequencing profiling of neonatal FoxM1-depleted cells and of octogenarian FoxM1-overexpressing cells showed that NOL12 expression is responsive to FoxM1 modulation (Macedo et al., 2018), suggesting that cell proliferation is closely linked to nucleolar homeostasis. Further work will be needed to address the functions of NOL12 during aging, in particular the mechanism responsible for its aging-associated repression and its contribution to ribosome biogenesis and p53-dependent senescence.

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7. REFERENCES

Amin, M.A., Matsunaga, S., Ma, N., Takata, H., Yokoyama, M., Uchiyama, S., Fukui, K., 2007. Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells. Biochemical and biophysical research communications 360, 320-326.

Amin, M.A., Matsunaga, S., Uchiyama, S., Fukui, K., 2008. Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. The Biochemical journal 415, 345-351.

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., van Deursen, J.M., 2011. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature 479, 232-236.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J., Lamond, A.I., 2007. The multifunctional nucleolus. Nature reviews. Molecular cell biology 8, 574-585.

Boulon, S., Westman, B.J., Hutten, S., Boisvert, F.M., Lamond, A.I., 2010. The nucleolus under stress. Molecular cell 40, 216-227.

Brangwynne, C.P., Mitchison, T.J., Hyman, A.A., 2011. Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes. Proceedings of the National Academy of Sciences of the United States of America 108, 4334-4339.

Buchwalter, A., Hetzer, M.W., 2017. Nucleolar expansion and elevated protein translation in premature aging. Nature communications 8, 328.

Bursac, S., Brdovcak, M.C., Pfannkuchen, M., Orsolic, I., Golomb, L., Zhu, Y., Katz, C., Daftuar, L., Grabusic, K., Vukelic, I., Filic, V., Oren, M., Prives, C., Volarevic, S., 2012. Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. Proceedings of the National Academy of Sciences of the United States of America 109, 20467-20472.

Bywater, M.J., Poortinga, G., Sanij, E., Hein, N., Peck, A., Cullinane, C., Wall, M., Cluse, L., Drygin, D., Anderes, K., Huser, N., Proffitt, C., Bliesath, J., Haddach, M., Schwaebe, M.K., Ryckman, D.M., Rice, W.G., Schmitt, C., Lowe, S.W., Johnstone, R.W., Pearson, R.B., McArthur, G.A., Hannan, R.D., 2012. Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53. Cancer cell 22, 51-65.

Charrier-Savournin, F.B., Chateau, M.T., Gire, V., Sedivy, J., Piette, J., Dulic, V., 2004. p21-Mediated nuclear retention of cyclin B1-Cdk1 in response to genotoxic stress. Molecular biology of the cell 15, 3965-3976.

Chen, P.H., Chen, Y.T., Chu, T.Y., Ma, T.H., Wu, M.H., Lin, H.H., Chang, Y.S., Tan, B.C., Lo, S.J., 2020. Nucleolar control by a non-apoptotic p53-caspases-deubiquitinylase

axis promotes resistance to bacterial infection. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 34, 1107-1121.

Choong, M.L., Yang, H., Lee, M.A., Lane, D.P., 2009. Specific activation of the p53 pathway by low dose actinomycin D: a new route to p53 based cyclotherapy. Cell cycle 8, 2810-2818.

Coccia, M., Rossi, A., Riccio, A., Trotta, E., Santoro, M.G., 2017. Human NF-kappaB repressing factor acts as a stress-regulated switch for ribosomal RNA processing and nucleolar homeostasis surveillance. Proceedings of the National Academy of Sciences of the United States of America 114, 1045-1050.

Deisenroth, C., Franklin, D.A., Zhang, Y., 2016. The Evolution of the Ribosomal Protein-MDM2-p53 Pathway. Cold Spring Harbor perspectives in medicine 6.

Derenzini, M., Montanaro, L., Trere, D., 2009. What the nucleolus says to a tumour pathologist. Histopathology 54, 753-762.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al., 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proceedings of the National Academy of Sciences of the United States of America 92, 9363-9367.

Donati, G., Peddigari, S., Mercer, C.A., Thomas, G., 2013. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. Cell reports 4, 87-98.

Farley-Barnes, K.I., McCann, K.L., Ogawa, L.M., Merkel, J., Surovtseva, Y.V., Baserga, S.J., 2018. Diverse Regulators of Human Ribosome Biogenesis Discovered by Changes in Nucleolar Number. Cell reports 22, 1923-1934.

Feric, M., Vaidya, N., Harmon, T.S., Mitrea, D.M., Zhu, L., Richardson, T.M., Kriwacki, R.W., Pappu, R.V., Brangwynne, C.P., 2016. Coexisting Liquid Phases Underlie Nucleolar Subcompartments. Cell 165, 1686-1697.

Fischer, M., Grossmann, P., Padi, M., DeCaprio, J.A., 2016. Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. Nucleic acids research 44, 6070-6086.

Freed, E.F., Prieto, J.L., McCann, K.L., McStay, B., Baserga, S.J., 2012. NOL11, implicated in the pathogenesis of North American Indian childhood cirrhosis, is required for pre-rRNA transcription and processing. PLoS genetics 8, e1002892.

Gannon, H.S., Donehower, L.A., Lyle, S., Jones, S.N., 2011. Mdm2-p53 signaling regulates epidermal stem cell senescence and premature aging phenotypes in mouse skin. Developmental biology 353, 1-9.

Golomb, L., Volarevic, S., Oren, M., 2014. p53 and ribosome biogenesis stress: the essentials. FEBS letters 588, 2571-2579.

Hadjiolova, K.V., Hadjiolov, A.A., Bachellerie, J.P., 1995. Actinomycin D stimulates the transcription of rRNA minigenes transfected into mouse cells. Implications for the in vivo hypersensitivity of rRNA gene transcription. European journal of biochemistry 228, 605-615.

Henras, A.K., Plisson-Chastang, C., O'Donohue, M.F., Chakraborty, A., Gleizes, P.E., 2015. An overview of pre-ribosomal RNA processing in eukaryotes. Wiley interdisciplinary reviews. RNA 6, 225-242.

Hernandez-Verdun, D., 2011. Assembly and disassembly of the nucleolus during the cell cycle. Nucleus 2, 189-194.

Holmberg Olausson, K., Nister, M., Lindstrom, M.S., 2012. p53 -Dependent and -Independent Nucleolar Stress Responses. Cells 1, 774-798.

Horn, H.F., Vousden, K.H., 2008. Cooperation between the ribosomal proteins L5 and L11 in the p53 pathway. Oncogene 27, 5774-5784.

Huang, G., Meng, L., Tsai, R.Y., 2015. p53 Configures the G2/M Arrest Response of Nucleostemin-Deficient Cells. Cell death discovery 1.

James, A., Wang, Y., Raje, H., Rosby, R., DiMario, P., 2014. Nucleolar stress with and without p53. Nucleus 5, 402-426.

Jayaraman, S., Chittiboyina, S., Bai, Y., Abad, P.C., Vidi, P.A., Stauffacher, C.V., Lelievre, S.A., 2017. The nuclear mitotic apparatus protein NuMA controls rDNA transcription and mediates the nucleolar stress response in a p53-independent manner. Nucleic acids research 45, 11725-11742.

Joerger, A.C., Fersht, A.R., 2016. The p53 Pathway: Origins, Inactivation in Cancer, and Emerging Therapeutic Approaches. Annual review of biochemistry 85, 375-404.

Junera, H.R., Masson, C., Geraud, G., Hernandez-Verdun, D., 1995. The three-dimensional organization of ribosomal genes and the architecture of the nucleoli vary with G1, S and G2 phases. Journal of cell science 108 (Pt 11), 3427-3441.

Krenning, L., Feringa, F.M., Shaltiel, I.A., van den Berg, J., Medema, R.H., 2014. Transient activation of p53 in G2 phase is sufficient to induce senescence. Molecular cell 55, 59-72.

Lempiainen, H., Shore, D., 2009. Growth control and ribosome biogenesis. Current opinion in cell biology 21, 855-863.

Lindqvist, A., de Bruijn, M., Macurek, L., Bras, A., Mensinga, A., Bruinsma, W., Voets, O., Kranenburg, O., Medema, R.H., 2009. Wip1 confers G2 checkpoint recovery competence by counteracting p53-dependent transcriptional repression. The EMBO journal 28, 3196-3206.

Lohrum, M.A., Ludwig, R.L., Kubbutat, M.H., Hanlon, M., Vousden, K.H., 2003. Regulation of HDM2 activity by the ribosomal protein L11. Cancer cell 3, 577-587.

Ma, N., Matsunaga, S., Takata, H., Ono-Maniwa, R., Uchiyama, S., Fukui, K., 2007. Nucleolin functions in nucleolus formation and chromosome congression. Journal of cell science 120, 2091-2105.

Macedo, J.C., Vaz, S., Bakker, B., Ribeiro, R., Bakker, P.L., Escandell, J.M., Ferreira, M.G., Medema, R., Foijer, F., Logarinho, E., 2018. FoxM1 repression during human aging leads to mitotic decline and aneuploidy-driven full senescence. Nature communications 9, 2834.

Marcel, V., Catez, F., Diaz, J.J., 2015. Ribosome heterogeneity in tumorigenesis: the rRNA point of view. Molecular & cellular oncology 2, e983755.

Marcel, V., Ghayad, S.E., Belin, S., Therizols, G., Morel, A.P., Solano-Gonzalez, E., Vendrell, J.A., Hacot, S., Mertani, H.C., Albaret, M.A., Bourdon, J.C., Jordan, L., Thompson, A., Tafer, Y., Cong, R., Bouvet, P., Saurin, J.C., Catez, F., Prats, A.C., Puisieux, A., Diaz, J.J., 2013. p53 acts as a safeguard of translational control by regulating fibrillarin and rRNA methylation in cancer. Cancer cell 24, 318-330.

Marinho, J., Casares, F., Pereira, P.S., 2011. The Drosophila Nol12 homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth. Development 138, 349-357.

Marinho, J., Martins, T., Neto, M., Casares, F., Pereira, P.S., 2013. The nucleolar protein Viriato/Nol12 is required for the growth and differentiation progression activities of the Dpp pathway during Drosophila eye development. Developmental biology 377, 154-165.

Martins, T., Eusebio, N., Correia, A., Marinho, J., Casares, F., Pereira, P.S., 2017. TGFbeta/Activin signalling is required for ribosome biogenesis and cell growth in Drosophila salivary glands. Open biology 7.

Maszewski, J., Kwiatkowska, M., 1984. Number, size, and transcriptional activity of nucleoli during different periods of interphase in antheridial filaments of Chara vulgaris L. Folia histochemica et cytobiologica 22, 9-19.

Memet, I., Doebele, C., Sloan, K.E., Bohnsack, M.T., 2017. The G-patch protein NF-kappaB-repressing factor mediates the recruitment of the exonuclease XRN2 and activation of the RNA helicase DHX15 in human ribosome biogenesis. Nucleic acids research 45, 5359-5374.

Nishimura, K., Kumazawa, T., Kuroda, T., Katagiri, N., Tsuchiya, M., Goto, N., Furumai, R., Murayama, A., Yanagisawa, J., Kimura, K., 2015. Perturbation of ribosome biogenesis drives cells into senescence through 5S RNP-mediated p53 activation. Cell reports 10, 1310-1323.

Ochs, R.L., Lischwe, M.A., Spohn, W.H., Busch, H., 1985. Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biology of the cell 54, 123-133.

Oeffinger, M., Zenklusen, D., Ferguson, A., Wei, K.E., El Hage, A., Tollervey, D., Chait, B.T., Singer, R.H., Rout, M.P., 2009. Rrp17p is a eukaryotic exonuclease required for 5' end processing of Pre-60S ribosomal RNA. Molecular cell 36, 768-781.

Pines, J., Hunter, T., 1989. Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell 58, 833-846.

Polymenis, M., Aramayo, R., 2015. Translate to divide: control of the cell cycle by protein synthesis. Microbial cell 2, 94-104.

Rubbi, C.P., Milner, J., 2003. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. The EMBO journal 22, 6068-6077.

Sadasivam, S., Duan, S., DeCaprio, J.A., 2012. The MuvB complex sequentially recruits B-Myb and FoxM1 to promote mitotic gene expression. Genes & development 26, 474-489.

Sahasranaman, A., Dembowski, J., Strahler, J., Andrews, P., Maddock, J., Woolford, J.L., Jr., 2011. Assembly of Saccharomyces cerevisiae 60S ribosomal subunits: role of factors required for 27S pre-rRNA processing. The EMBO journal 30, 4020-4032.

Salvetti, A., Coute, Y., Epstein, A., Arata, L., Kraut, A., Navratil, V., Bouvet, P., Greco, A., 2016. Nuclear Functions of Nucleolin through Global Proteomics and Interactomic Approaches. Journal of proteome research 15, 1659-1669.

Scott, D.D., Trahan, C., Zindy, P.J., Aguilar, L.C., Delubac, M.Y., Van Nostrand, E.L., Adivarahan, S., Wei, K.E., Yeo, G.W., Zenklusen, D., Oeffinger, M., 2017. Nol12 is a multifunctional RNA binding protein at the nexus of RNA and DNA metabolism. Nucleic acids research 45, 12509-12528.

Sloan, K.E., Bohnsack, M.T., Schneider, C., Watkins, N.J., 2014. The roles of SSU processome components and surveillance factors in the initial processing of human ribosomal RNA. Rna 20, 540-550.

Sloan, K.E., Bohnsack, M.T., Watkins, N.J., 2013a. The 5S RNP couples p53 homeostasis to ribosome biogenesis and nucleolar stress. Cell reports 5, 237-247.

Sloan, K.E., Mattijssen, S., Lebaron, S., Tollervey, D., Pruijn, G.J., Watkins, N.J., 2013b. Both endonucleolytic and exonucleolytic cleavage mediate ITS1 removal during human ribosomal RNA processing. The Journal of cell biology 200, 577-588.

Sloan, K.E., Warda, A.S., Sharma, S., Entian, K.D., Lafontaine, D.L.J., Bohnsack, M.T., 2017. Tuning the ribosome: The influence of rRNA modification on eukaryotic ribosome biogenesis and function. RNA biology 14, 1138-1152.

Sun, X.X., Wang, Y.G., Xirodimas, D.P., Dai, M.S., 2010. Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation. The Journal of biological chemistry 285, 25812-25821.

Suzuki, S., Fujiwara, T., Kanno, M., 2007. Nucleolar protein Nop25 is involved in nucleolar architecture. Biochemical and biophysical research communications 358, 1114-1119.

Thiry, M., Lafontaine, D.L., 2005. Birth of a nucleolus: the evolution of nucleolar compartments. Trends in cell biology 15, 194-199.

Tiku, V., Jain, C., Raz, Y., Nakamura, S., Heestand, B., Liu, W., Spath, M., Suchiman, H.E.D., Muller, R.U., Slagboom, P.E., Partridge, L., Antebi, A., 2017. Small nucleoli are a cellular hallmark of longevity. Nature communications 8, 16083.

Tsai, R.Y., Pederson, T., 2014. Connecting the nucleolus to the cell cycle and human disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 28, 3290-3296.

Turner, A.J., Knox, A.A., Prieto, J.L., McStay, B., Watkins, N.J., 2009. A novel small-subunit processome assembly intermediate that contains the U3 snoRNP, nucleolin, RRP5, and DBP4. Molecular and cellular biology 29, 3007-3017.

Ugrinova, I., Monier, K., Ivaldi, C., Thiry, M., Storck, S., Mongelard, F., Bouvet, P., 2007. Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. BMC molecular biology 8, 66.

Wang, S., El-Deiry, W.S., 2005. P53, Cell Cycle Arrest and Apoptosis, in: Hainaut, P., Wiman, K.G. (Eds.), 25 Years of p53 Research. Springer Netherlands, Dordrecht, pp. 141-163.

West, S., Gromak, N., Proudfoot, N.J., 2004. Human 5' --> 3' exonuclease Xrn2 promotes transcription termination at co-transcriptional cleavage sites. Nature 432, 522-525.

Woods, S.J., Hannan, K.M., Pearson, R.B., Hannan, R.D., 2015. The nucleolus as a fundamental regulator of the p53 response and a new target for cancer therapy. Biochimica et biophysica acta 1849, 821-829.

Yuan, X., Zhou, Y., Casanova, E., Chai, M., Kiss, E., Grone, H.J., Schutz, G., Grummt, I., 2005. Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. Molecular cell 19, 77-87.

Zhai, W., Comai, L., 2000. Repression of RNA polymerase I transcription by the tumor suppressor p53. Molecular and cellular biology 20, 5930-5938.

Zhang, Y., Wolf, G.W., Bhat, K., Jin, A., Allio, T., Burkhart, W.A., Xiong, Y., 2003. Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Molecular and cellular biology 23, 8902-8912.

8. SUPPLEMENTAL MATERIAL

Supplemental material for this article can be found at https://doi.org/10.1128/MCB .00099-19 (except Figures S2.2 and S2.5).

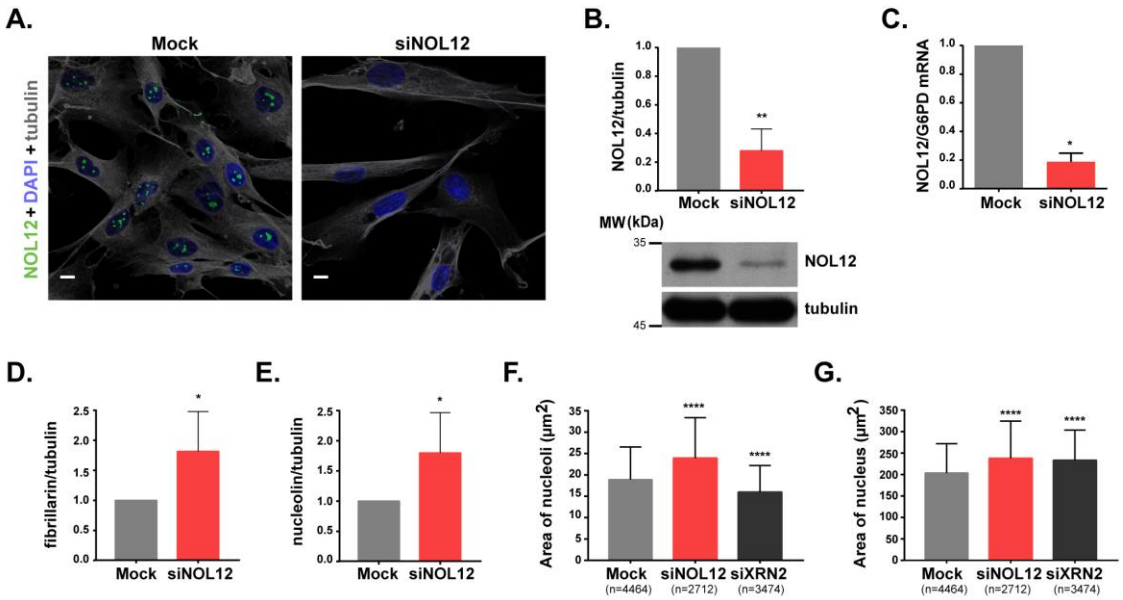

Figure S2.1. NOL12 regulates fibrillarin and nucleolin protein levels, and nucleolar area. (A) NOL12 (green) and tubulin (grey) immunostaining of mock- and siNOL12-depleted fibroblasts. DNA was stained with DAPI (blue). Scale bars, 10µm. **(B)** Western blot analysis of NOL12 protein levels in cell extracts from mock- and siNOL12-depleted fibroblasts. Tubulin levels were used as loading control. Values are mean ± SD from three independent experiments and normalized to mock controls. ** $p \leq 0.01$ by Mann-Whitney statistical test. **(C)** qPCR analysis of *NOL12* transcript levels in mock- and siNOL12-depleted fibroblasts. *G6PD* was used as housekeeping gene. Values are mean ± SD from four independent experiments and normalized to mock controls. * $p \leq 0.05$ by Mann-Whitney statistical analysis. **(D)** Western blot analysis of fibrillarin protein levels. Tubulin levels were used as loading control. Values are mean ± SD from four independent experiments and normalized to mock controls. * $p \leq 0.05$ by Mann-Whitney statistical test. **(E)** Western blot analysis of nucleolin protein levels. Tubulin levels were used as loading control. Values are mean ± SD from four independent experiments and normalized to mock controls. * $p \leq 0.05$ by Mann-Whitney statistical test. **(F)** Nucleolar area (µm^2) in mock-, siNOL12- and siXRN2-depleted cells. Values are mean ± SD of n=total number of cells. **** $p \leq 0.0001$ by Kruskal-Wallis statistical test. **(G)** Nuclear area (µm^2) in mock-, siNOL12- and siXRN2-depleted cells. Values are mean ± SD of n= total number of cells. **** $p \leq 0.0001$ by Kruskal-Wallis statistical test.

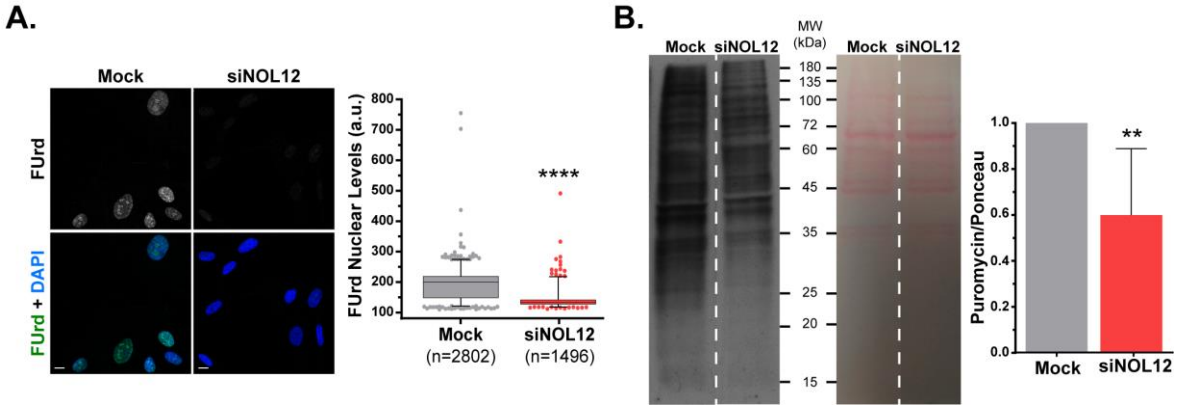

Figure S2.2. NOL12 downregulation decreases ribosome biogenesis. (A) Immunostaining of incorporated FUrd (grayscale/green) in mock- and siNOL12-treated cells. Scale bars, 10µm. Box-and-whiskers plot of median nuclear immunofluorescence levels of incorporated FUrd. Horizontal line within box represents the median fluorescence intensity; whiskers-representing boundaries of the box, 1st and 99th percentiles. a.u., arbitrary units. n = total number of cells analysed. **(B)** Cell extracts from mock- and siNOL12-treated cells were immunoblotted for puromycin. Ponceau S levels were used for the loading control. In bar graphs, values are means ± SD from four independent experiments, normalized to the values for the mock-treated control. MW, molecular weight. ** $p \leq 0.01$ and **** and $p \leq 0.0001$ by Mann-Whitney statistical test.

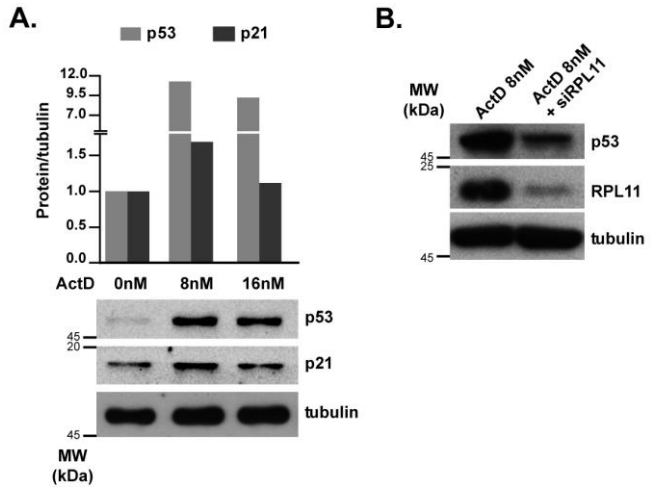

Figure S2.3. RPL11 is required for Actinomycin D-induced p53 stabilization in human dermal fibroblasts. (A) p53 and p21 levels in cell extracts from neonatal fibroblasts incubated with 0nM, 8nM and 16nM Actinomycin D (ActD) for 4 hours. Tubulin was used as loading control in the immunoblotting. In the graph, bars are the protein levels normalized to the untreated control from a single experiment. **(B)** Western blot analysis of p53 and RPL11 protein levels in cell extracts from control and siRpL11-depleted cells treated with 8nM Actinomycin D for 4 hours. Tubulin was used as the loading control.

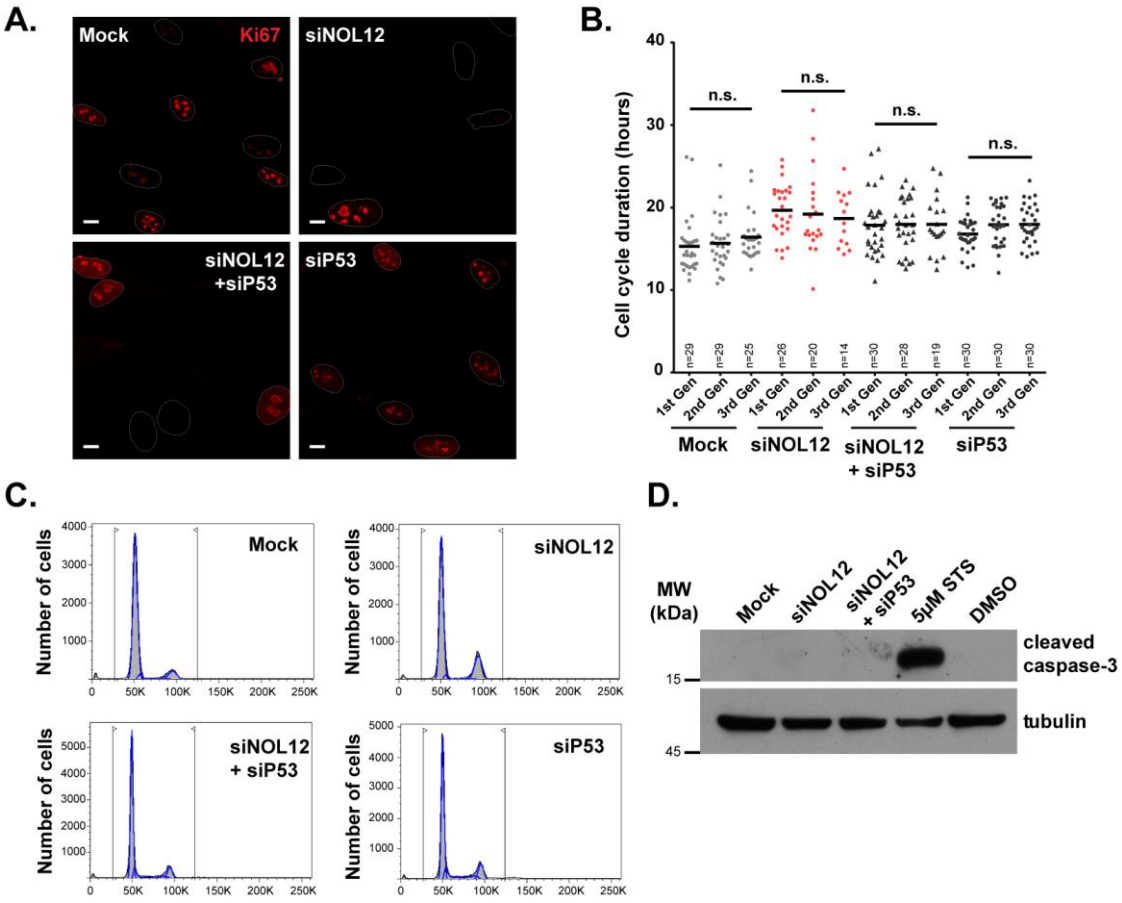

Figure S2.4. NOL12 repression inhibits cell proliferation in an apoptosis-independent manner. (A) Immunostaining of Ki67 cell proliferation marker (red) in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted human dermal fibroblasts. DAPI staining was used for nuclei masking (white solid lines). Scale bars, 10μm. **(B)** Cell cycle duration was measured as the interval between mother cell mitosis and daughter cell mitosis over three generations (1st – 3rd Gen), in mock-, siNOL12-, siP53- and siNOL12+siP53-depleted fibroblast cultures. Each dot represents a single cell. n= total number of cells analyzed. n.s., not significant. **(C)** Representative flow cytometry cell cycle profiles from control mock-, siNOL12-, siP53- and siNOL12+siP53-depleted cell cultures. **(D)** Western blot analysis of the apoptotic marker cleaved-caspase 3 in cell extracts from mock-, siNOL12- and siNOL12+siP53-depleted fibroblasts. Extracts from cells treated with DMSO or 5μM staurosporine (STS) for 4 hours were used as negative and positive controls, respectively. Tubulin was used as the loading control.

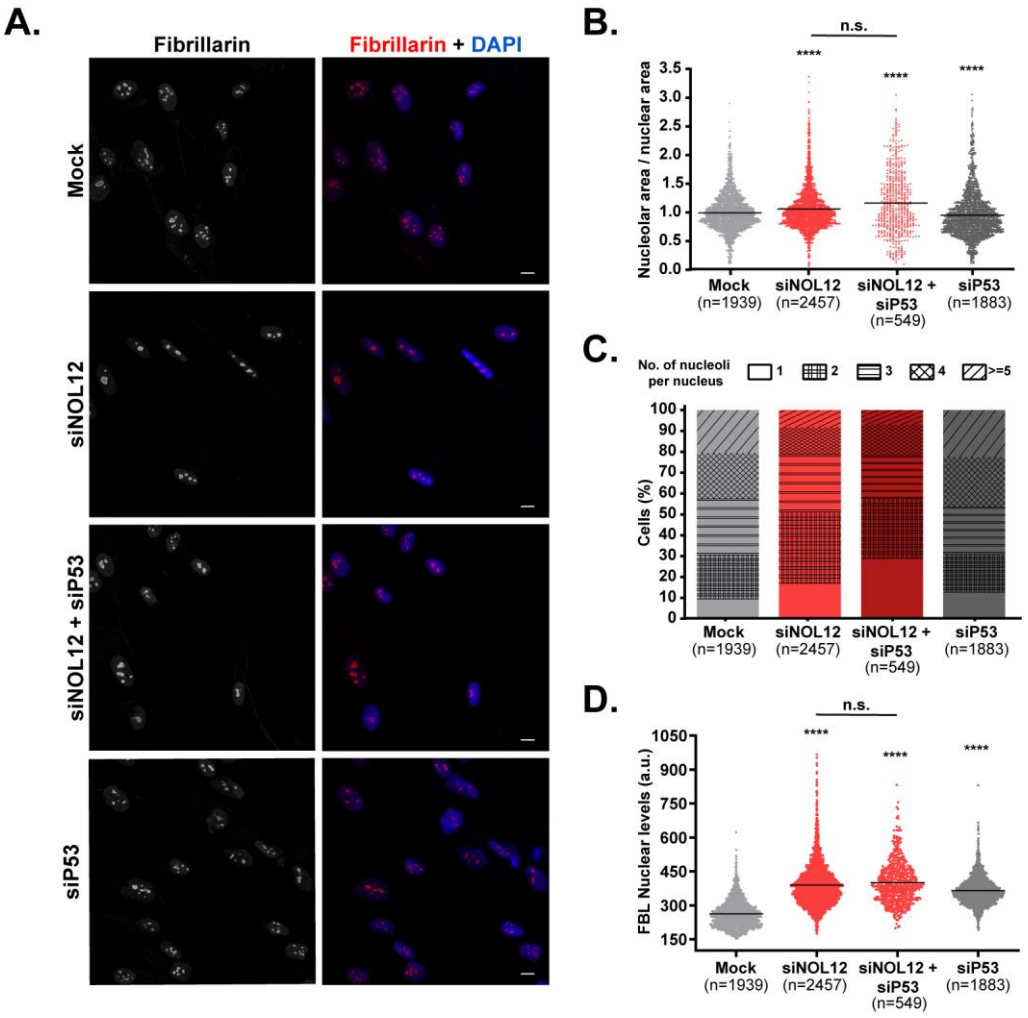

Figure S2.5. Nucleolar alterations induced by NOL12 downregulation occur in a p53-independent manner. **(A)** Fibrillarin (FBL, grayscale/red) immunostaining in mock-, siNOL12-, siNOL12+siP53- and siP53-depleted cells. Scale bars are 10µm. **(B)** Ratio between nucleolar and nuclear areas in mock-, siNOL12-, siNOL12+siP53- and siP53-depleted cells. Each dot represents a single cell. Horizontal lines represent the mean values normalized to mock controls. **(C)** Stacked bars for the distribution of the total number of nucleoli per nucleus in mock-, siNOL12-, siNOL12+siP53- and siP53-depleted cells. Different patterns in bars represent different number of nucleoli per nucleus. **(D)** Scatter plots of the mean pixel intensities of FBL nuclear levels in mock-, siNOL12-, siNOL12+siP53- and siP53-depleted cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. a.u., arbitrary units. n= total number of cells analyzed in panels B, C and D. n.s., not significant. **** $p \leq 0.0001$ by Kruskal-Wallis statistical tests.

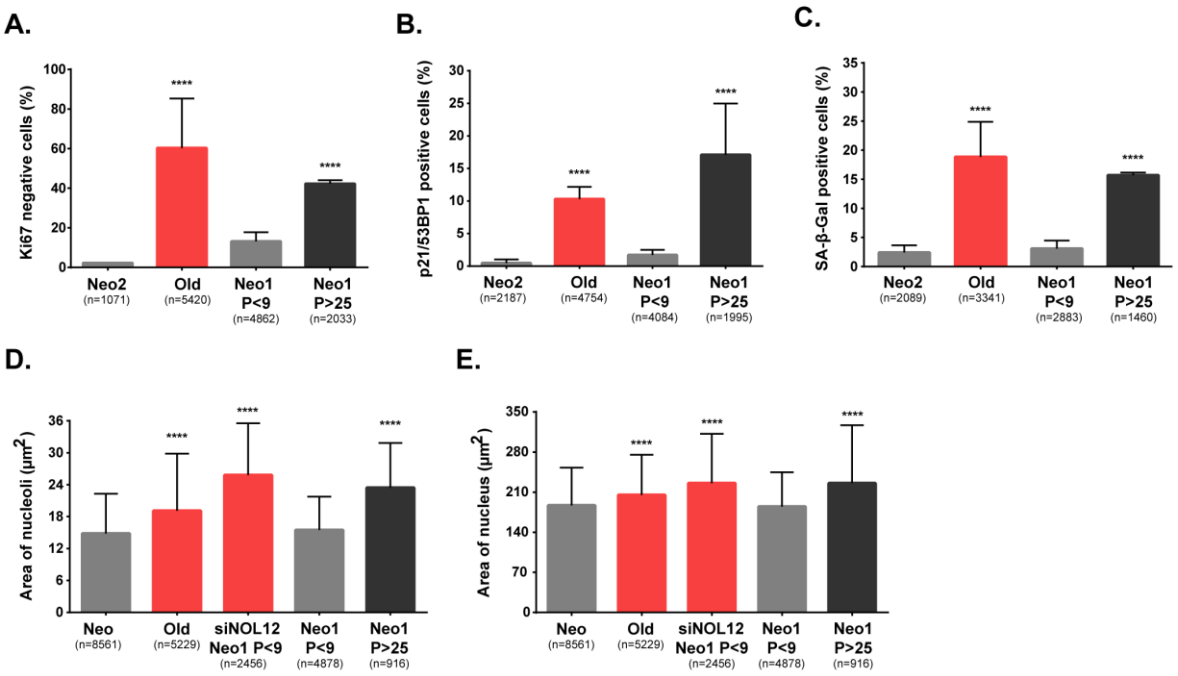

Figure S2.6. Quantification of senescence markers in naturally aged and replicative senescent fibroblasts. (A) Percentage of Ki67 negative cells in neonatal (Neo2), vs. elderly (Old; 77y, 84y, 85y and 87y average), as well as in neonatal low vs. high passage (Neo1 P<9 and Neo1 P>25) fibroblast cultures. Values are mean ± SD from at least two independent experiments. ****$p \leq 0.0001$ by χ2-square statistical test. **(B)** Percentage of cells double positive for p21/53BP1 staining. Values are mean ± SD from two independent experiments. ****$p \leq 0.0001$ by χ2-square statistical test. **(C)** Percentage of SA-β-galactosidase (SA-β-Gal) positive cells. Values are mean ± SD from two independent experiments. ****$p \leq 0.0001$ by χ2-square statistical test. **(D)** Nucleolar area (µm^2). Values are mean ± SD. ****$p \leq 0.0001$ by Kruskal-Wallis statistical test. **(E)** Nuclear area (µm^2). Values are mean ± SD. ****$p \leq 0.0001$ by Kruskal-Wallis statistical test. In all graphs (A-E), values were normalized to Neo (red bars) or Neo1 (dark grey bar), and n= total number of cells analyzed.

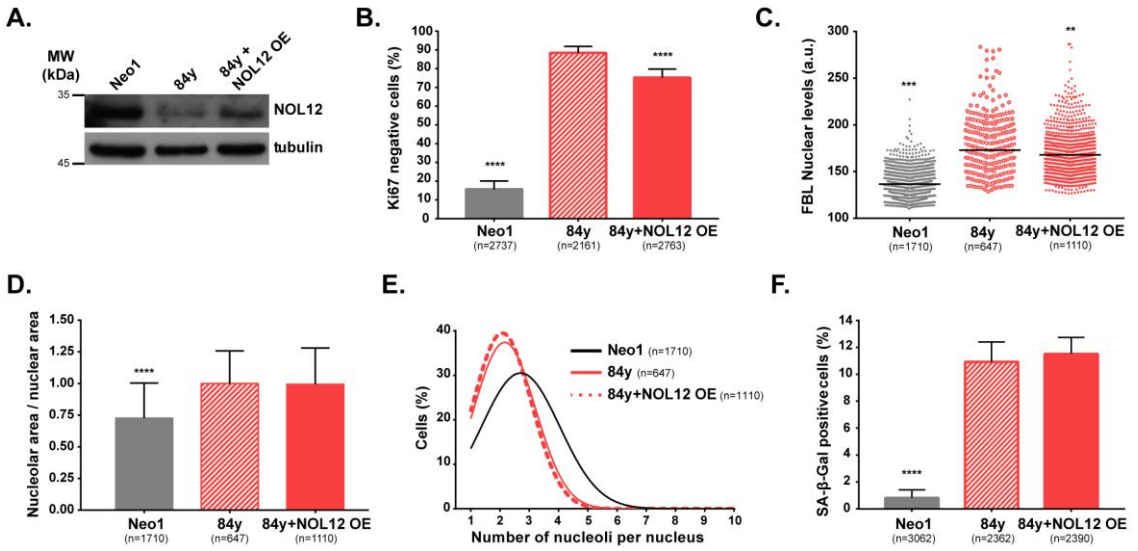

Figure S2.7. Overexpression of NOL12 in elderly cells negligibly impacts on nucleolar stress and senescence phenotypes. (A) NOL12 immunoblotting in cell extracts from neonatal (Neo1), 84-year-old (84y) and 84-year-old fibroblasts overexpressing NOL12 (84y+NOL12 OE). Tubulin was used as the loading control. **(B)** Percentage of Ki67 negative cells in Neo1, 84y and 84y+NOL12OE cell cultures. Values are mean ± s.d from two independent experiments. ****$p \leq 0.0001$ by χ2-square statistical test. **(C)** Scatter plot of the mean pixel intensity of fibrillarin nuclear levels in Neo1, 84y and 84y+NOL12OE nuclei. Each dot represents a single cell. Horizontal lines represent the mean. **$p \leq 0.01$ and ***$p \leq 0.001$ by Kruskal-Wallis statistical test. **(D)** Ratio between nucleolar and nuclear areas in neonatal Neo1, 84y and 84y+NOL12OE cells. Values are mean ± SD and normalized to 84y mean value. ***$p \leq 0.001$ by Kruskal-Wallis statistical test. **(E)** Distribution curves of the percentage of Neo1, 84y and 84y+NOL12OE cells exhibiting a total number of nucleoli as indicated. **(F)** Percentage of SA-β-galactosidase (SA-β-Gal) positive cells in Neo1, 84y and 84y+NOL12OE cell cultures. Values are mean ± SD from two independent experiments. ****$p \leq 0.0001$ by χ2-square statistical test. n=total number of cells analyzed in each experiment.

Table S2.1. Human dermal fibroblasts (HDFs) used in this study.

HDFs (age)	***Reference, Repository***	***Referred as***
Neonatal	DFM021711A, Zen Bio	Neo2
1 day	GM21811, Coriell Cell Repository	Neo1
77 years	AG07135, Coriell Cell Repository	77y
84 years	AG11488, Coriell Cell Repository	84y
85 years	AG09271, Coriell Cell Repository	85y
87 years	AG10884, Coriell Cell Repository	87y

Table S2.2. Sequences of siRNAs used in this study.

mRNA targets	***Sequence 5'-3'***
NOL12	(SASI_Hs01_00047859) CAGAUGAGCUGGACCGGUU[dT][dT] (sense) AACCGGUCCAGCUCAUCUG[dT][dT] (antisense)
P53	(SASI_Hs02_00302766) GAGGUUGGCUCUGACUGUA[dT][dT] (sense) UACAGUCAGAGCCAACCUC[dT][dT] (antisense)
RPL11	CGCGAGCAGCCAAGGUGUUGGAGCA[dT][dT] (sense) UGCUCCAACACCUUGGCUGCUCGCG[dT][dT] (antisense)
XRN2	siRNA 1: AAGAGUACAGAUGAUCAUG[dT][dT] (sense) CAUGAUCAUCUGUACUCUU[dT][dT] (antisense) siRNA 2: GGGAAGAAAUAUUGGCAAA[dT][dT (sense) UUUGCCAAUAUUUCUUCCC[dT][dT (antisense)

Table S2.3. Sequences of primers used in qPCR experiments.

Gene	**Sequence (5'-3')**	
	Forward	Reverse
G6PD	AACATCGCCTGCGTTATCCTC	ACGTCCCGGATGATCCCAA
NOL12	GGCCGAGGCTCGTTCTTAG	TGCCTTCTTTCGCTCGACC
CDKN1A	TGGACCTGGAGACTCTCAGG	CGGATTAGGGCTTCCTCTTGG
MMP1	AGCGTGTGACAGTAAGCTAACC	AACTTCCGGGTAGAAGGGATTTG
CXCL8	GCCTTCCTGATTTCTGCAGCT	GCACTGACATCTAAGTTCTTTAGCA
TSPAN13	CGCCATGTGCTCCAATCATAG	GTAGGTCAGCCAAACACCCA
TBP	GAGCCAAGAGTGAAGAACAGTC	GCTCCCCACCATATTCTGAATCT

Supplementary Movies 1-4 (online available). Related to Figure 2.3. Long-term phase-contrast live-cell imaging (Movies 1-4) of mock-, siNOL12-, siP53- and siNOL12+siP53-depleted cell cultures, respectively. Movie records started 28 hours and ended 70 hours after post-transfection post-transfection. Images were acquired every 2.5 min. Scale bars, 100µm.

CHAPTER 3

Forkhead box M1 transcriptional activity regulates nucleolar homeostasis

in preparation

In this study, I participated in the experimental design, cell culture handling, immunostaining and western blotting experiments and I also performed image analysis and quantification. Additionally, I contributed to the writing of the manuscript.

Keywords:
FoxM1, nucleolar stress, senescence

1. ABSTRACT

The nucleolus is the subnuclear compartment previously identified as the ribosome factory. Notably, studies have increasingly emphasized the nucleolus as a sensor of cellular stress and a coordinator of stress responses leading to cell cycle arrest and apoptosis. Previously, we disclosed an association between nucleolar structure disruption, senescence and aging. In addition, we found that repression of transcription factor FoxM1 during aging primarily accounts for senescence accrual during aging. Here, we investigated the impact of FoxM1 transcriptional activity in nucleolar homeostasis. We demonstrate FoxM1 as being required for regulated expression levels of different nucleolar proteins and for proper nucleolar morphology and function. Interestingly, FoxM1 repression leads to increased pre-rRNA transcription, but uncoordinated synthesis of mature 18S and 28S rRNA and reduced global protein synthesis. Moreover, we show that nucleolar stress induced by FoxM1 repression activates P53 in a FBL- and RPL11-dependent manner. Finally, we identified PIM1 and PARP1 as transcriptional targets of FoxM1 that potentially account for loss of nucleolar homeostasis during aging.

2. INTRODUCTION

The nucleolus is a phase-separated subnuclear compartment (Feric et al., 2016) in which ribosome biogenesis takes place (Leary and Huang, 2001). Briefly, this complex process starts with the transcription of the ribosomal DNA (rDNA) by RNA Polymerase I into a precursor rRNA transcript – the 47S pre-rRNA (Goodfellow and Zomerdijk, 2013). This primary transcript is then processed and modified, including 2'-O-methylation and pseudouridylation, which are carried out by the methyl transferase fibrillarin (FBL) and dyskerin, respectively (Sloan et al., 2017). This processing step leads to the synthesis of the three rRNA species – 18S, 28S and 5.8S – which are then assembled with the 5S rRNA (transcribed by RNA Polymerase III) and several ribosomal proteins to generate the small and large ribosome subunits (Fromont-Racine et al., 2003). Although mostly recognized by this main function, the nucleolus has emerged as a multifunctional and dynamic structure (Boisvert et al., 2007). Particularly, its capacity to sense intra- and extracellular alterations and to build a cellular response, makes the nucleolus a central hub for stress responses (Lindstrom and Latonen, 2013). Generally, any disruption in the nucleolar structure or function put cells under the so-called nucleolar stress (Yang et al., 2018). Mammalian cells often respond to this stress by activating P53, a tumour suppressor protein (James et al., 2014). For instance, low dose actinomycin D specifically inhibits RNA Polymerase I, decreases rDNA transcription and activates the P53-dependent signaling pathway (Tsai

and Pederson, 2014). Under normal conditions, the 5S RNP complex, which comprises RPL5, RPL11 and 5S rRNA, assembles with the pre-60S rRNA allowing the proper formation of a mature ribosome (Donati et al., 2013). However, under nucleolar stress, this complex binds MDM2, a negative regulator of P53, inhibiting its binding to P53 and subsequently P53 proteasomal degradation (Donati et al., 2013). Relevantly, this mechanism was shown to mediate P53 stabilization in cells with accelerated rDNA transcription, delayed rRNA processing or ribosomal protein deficiency (Dutt et al., 2011; Nishimura et al., 2015; Sloan et al., 2013a). Moreover, we recently showed that repression of NOL12, a nucleolar protein involved in rRNA processing (Scott et al., 2017; Sloan et al., 2013b), activates P53 through this 5S RNP mechanism and induces senescence (Pinho et al., 2019). Additionally, we found NOL12 downregulation in dermal fibroblasts from elderly donors in association with nucleolar features similar to those observed in NOL12-depleted neonatal fibroblasts (Pinho et al., 2019), namely reduced number but increased area of nucleoli and fibrillarin upregulation (Pinho et al., 2019). This is in line with increasing evidence implicating nucleolar stress in senescence (Chen and Stark, 2019) and aging (Tiku and Antebi, 2018). Particularly, premature aged cells from Hutchinson-Gilford progeria syndrome (HGPS) patients were shown to exhibit bigger but fewer nucleoli (Buchwalter and Hetzer, 2017), and a longer lifespan has been associated with smaller nucleoli in *Caenorhabditis elegans*, *Drosophila melanogaster*, mice and human cells (Tiku et al., 2017). Recently, repression of FoxM1 transcriptional activity was found in human fibroblasts from elderly donors (Macedo et al., 2018). FoxM1 belongs to the forkhead box (FOX) protein family that comprises several transcription regulators (Myatt and Lam, 2007). In particular, FoxM1 is a pro-oncogenic factor found overexpressed in several different human tumors (Lee et al., 2016). Mechanistically, its role in promoting proliferation stands from its capacity to form a complex with Myb-MuvB – the MMB-FoxM1 complex – which promotes the transcription of several cell cycle genes, allowing cells to progress into mitosis (Fischer et al., 2016; Sadasivam et al., 2012). FoxM1 repression during aging was shown to increase the incidence of lagging chromosomes during mitosis, leading to the emergence of aneuploid senescent cells (Macedo et al., 2018).

Given the physiological relevance of both FoxM1 and nucleolar function in aging, here we investigated the functional link between FoxM1 transcriptional activity, nucleolar homeostasis and cellular senescence. We found FoxM1 repression to impact on nucleolar function and we identified putative transcriptional targets that account for this functional link.

3. MATERIALS AND METHODS

Cell culture. Human dermal fibroblasts retrieved from neonatal skin biopsy of a healthy Caucasian male (DFM021711A) were acquired from Zen Bio repository. Cells were seeded at 1-1.5 x 104 cells per cm2 of growth area in minimal essential medium Eagle-Earle (MEM) supplemented with 15% fetal bovine serum (FBS), 2.5 mM L-glutamine and antibiotic-antimycotic (1:100) (all from Gibco®; Life Technologies, Thermo Scientific, CA, USA). Only early passage dividing fibroblasts (up to passage 3-5) with cumulative population doubling level PDL<24, well below replicative senescence, were used in all experiments. Cells were grown at 37ºC and humidified atmosphere with 5% CO2. For immunostaining experiments, cells were cultured in coverslips coated with 50 µg/ml fibronectin (Sigma-Aldrich; MO, USA).

siRNA transfection. Cells were transfected cells with 45nM small interfering RNA 1h after their plating using Lipofectamine RNAiMAX transfection reagent (Thermo Scientific, CA, USA) according to the manufacturer's instructions. The sequences of all siRNAs used in this study (from Sigma-Aldrich, MO, USA) are summarized in Table S1. For efficient depletion of FBL, we used a mixture of two previously reported siRNAs. Cells treated under the same conditions with transfection reagent without siRNA were used as controls (mock-depleted cells). Six hours after transfection, medium was replaced by fresh medium supplemented with 5% FBS and, the next day, by complete MEM medium. Phenotypes were analyzed and quantified 72h post-transfection.

Immunostaining. Fibroblasts were grown on sterilized glass coverslips coated with 50 µg/ml fibronectin (Sigma-Aldrich; MO, USA). Cells were fixed in freshly prepared 2% paraformaldehyde in PBS for 20 min or in 4% paraformaldehyde in PBS for 15 min in the case of 53BP1 and p21 markers. Following fixation, cells were rinsed in PBS, permeabilized with 0.3% Triton-X100 in PBS for 7 min and blocked in 0.05% Tween-20 in PBS (PBS-T) supplemented with 10% fetal bovine serum (FBS) for 1h at room temperature. Cells were then incubated overnight at 4ºC with primary antibodies diluted in PBS-T supplemented with 5% FBS as follows: rabbit anti-fibrillarin (ab5821, Abcam, Shanghai, China), 1:1000; mouse anti-fibrillarin (ab4566, Abcam, Shanghai, China), 1:3000; rabbit anti-p53 (sc-6243, Santa Cruz Biotechnology, CA, USA), 1:1000; rabbit anti-nucleolin (sc-13057, Santa Cruz Biotechnology, CA, USA) 1:500; rabbit anti-53BP1 (#4937, Cell Signaling Technology, MA, USA), 1:100; mouse anti-p21 (sc-6246, Santa Cruz Biotechnology, CA, USA), 1:1000; rabbit anti-Ki67 (ab15580, Abcam, Shanghai, China), 1:1500; mouse anti-NOL12 (sc-374257; Santa Cruz Biotechnology, CA), 1:800; mouse anti-UBF (sc-13125, Santa Cruz Biotechnology, CA, USA), 1:200; mouse anti-NPM (sc-271737, Santa Cruz Biotechnology, CA, USA), 1:100; rat anti-BrdU (ab6326, Abcam, Shanghai, China), 1:200; rabbit anti-

FoxM1 (Cell Signaling Technology, MA, USA), 1:200. Secondary antibodies AlexaFluor®-488, 568 and 647 were diluted 1:1500 in PBS-T supplemented with 5% FBS and coverslips were incubated for 45 min at room temperature. DAPI (Sigma-Aldrich, MO, USA) was used at 1 µg/ml for nuclei staining and coverslips were then mounted in slides with mounting solution (90% glycerol, 0.5% N propyl gallate and 20 nM Tris pH 8).

FUrd incorporation assay. To assess rDNA transcription in human primary fibroblasts, 5-Fluorouridine (FUrd; F5130, Sigma) was added to cell culture for 20 min at 2 mM. Coverslips were then rapidly washed in cold PBS, fixed in 1% PFA for 10 min, washed in cold PBS and permeabilized with 0.5% Triton-X100 in PBS for 10 min on ice. Coverslips were then immunostained as described above.

ClickiT assay. To visualize protein synthesis, we used Click-iT reagents (C10429, Invitrogen) according with the manufacture's protocol. Briefly, cells were incubated in methionine-free medium (21013-024, ThermoFisher) containing the methionine analog L-homopropargylglycine (HPG) for 30 min. After washing with PBS, cells were fixed with 4% paraformaldehyde for 15 min and rinsed twice in 10% FBS. Cells were permeabilized in 0.5% Triton X-100 for 20 min, and rinsed twice in 10% FBS followed by the click-iT reaction with Alexa 594 detection reagents, as described in manufacturer's protocol.

RNA quantification. Total RNA was isolated from cultured cells using the Quick RNA MicroPrep (R1050, Zymo Research) according to the manufacturer's instructions. After quantification in Nanodrop 1000 UV-Vis spectrophotometer, RNA was diluted at 100 ng/µL in RNAse free water and analyzed using the Experion RNA kit (Bio-Rad).

Drug treatments. For RNA Polymerase I inhibition, fibroblasts were incubated in medium containing 8 nM Actinomycin D (Sigma-Aldrich, MO, USA) for 4h before fixation. To enrich the cell culture mitotic index, the kinesin-5 inhibitor STLC (2799-07-7, Tocris) was used at 5µM for the last 16h, before cell harvesting by shake-off. To inhibit protein synthesis, cells were treated with cycloheximide (CHX) at 100 µg/mL for 30 min, before cell harvesting or fixation. For the puromycin assay, puromycin was added to cell culture at 10 µg/mL for 10 min at 37ºC, before cell harvesting.

Microscopy and image analysis. Slides were analyzed under HC PL APO CS 63x 1.30NA Glycerine objective in a Laser Scanning confocal microscope Leica TCS SP5 II (Leica Microsystems, Germany). Images were acquired at 1024x1024 pixel resolution and edited using Adobe Photoshop CS4 Extended Version 11.0. For ClickiT quantification, each cell was manually defined and integrated intensity was calculated in ImageJ. All the other image quantifications were based on fields acquired in IN Cell Analyzer 2000® (GE Healthcare, UK), equipped with a Photometrics CoolSNAP K4 camera. IN Cell Investigator software was used for measuring nucleolus and nucleus parameters (number and areas); nuclear area was measured in the DAPI channel whereas the total nucleolar area and the

number of nucleoli per nucleus were measured in the nucleolin channel. The mean pixel intensity of fibrillarin, nucleolin and FUrd (referred as “nuclear protein levels”) were measured based on mask defined by the DAPI channel.

Western blotting. Cell pellets were resuspended in lysis buffer (150 nM NaCl, 10 nM Tris-HCl pH=7.4, 1 nM EDTA, 1 nM EGTA, 0.5% IGEPAL) and instantaneously frozen in liquid nitrogen. Clarified lysates were quantified for protein content by the Lowry Method (DC™ Protein Assay; BioRad, CA, USA). 20 µg of total extract were loaded in SDS-PAGE gels and transferred into nitrocellulose membranes for western blot analysis. Membranes were blocked with 0.05% Tween-20 in TBS (TBS-T) containing 5% low fat milk, during 1h at RT. Primary antibodies were diluted in TBS-T containing 2% low fat milk as follows: rabbit anti-p53 (sc-6243), 1:1500; rabbit anti-fibrillarin (ab5821, Abcam, Shanghai, China), 1:1000; rabbit anti-nucleolin (sc-13057) 1:3000; mouse anti-fibrillarin (ab4566) 1:1000; mouse anti α tubulin (Sigma-Aldrich, CA, USA), 1:200000; mouse anti-GAPDH (Protein TechGroup, Inc., IL), 1:30000; mouse anti-FoxM1 (sc-271746, Santa Cruz Biotechnology, CA, USA) 1:500; rabbit anti-PARP1 (sc-7150, Santa Cruz Biotechnology, CA, USA), 1:500; mouse anti-puromycin (AB-2619605, DSHB), 1:1000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit antibodies (GENA9310 and GENA9340, GE Healthcare Life Sciences, USA, respectively) were used at a dilution of 1:1000 in TBS-T containing 2% low fat milk. Signal was detected using Clarity Western ECL Substrate reagent (BioRad) according to manufacturer’s instructions. A GS-800 calibrated densitometer (Bio-Rad Laboratories, CA, USA) was used for quantitative analysis of protein levels.

Statistical Analysis. P-values were obtained using GraphPad Prism version 7.00 (GraphPad, San Diego, CA, USA). Data were tested for parametric vs. non-parametric distribution using D'Agostino & Pearson omnibus normality test. Mann-Whitney, paired t-test, one-way ANOVA, Kruskal-Wallis or two-tailed $\chi 2$-square statistic tests were then applied accordingly to the experiment.

4. RESULTS

FoxM1 is required for nucleolar organization and regulates the expression of nucleolar proteins. FoxM1 repression was reported in naturally aged cells (Macedo et al., 2018) and aging has been increasingly associated with nucleolar alterations (Buchwalter and Hetzer, 2017; Pinho et al., 2019). We therefore asked whether FoxM1 could be required for proper nucleolar organization. To address this, we performed siRNA-mediated depletion of FoxM1 in human dermal fibroblasts (HDFs) to investigate changes in the immunolocalization patterns of known nucleolar protein markers. FoxM1 efficient depletion

(~85% knockdown) was validated in asynchronous populations of the MCF7 cancer cell line (Fig. S3.1A) and HDFs (Fig. S3.1B), as well as in interphasic and mitotic cell subpopulations of synchronous cultures of HDFs (Fig. S3.1C). Depletion efficiency was further confirmed by immunofluorescence analysis using a different antibody (Fig. S3.1D). We started by analyzing the levels of the fibrillarin (FBL) and nucleolin (NCL) nucleolar proteins in mock and FoxM1-depleted cells (Fig. 3.1A). FBL and NCL displayed increased immunofluorescence levels (Fig. 3.1B, C). Moreover, immunoblotting analysis confirmed increased FBL protein levels (Fig. 3.1D), but unchanged NCL protein levels (Fig. 3.1E), suggesting that FoxM1 repression leads to NCL relocalization rather than its upregulation. Furthermore, we found FBL upregulation as an upstream event essential for NCL nucleolar recruitment, as the double-depleted siFoxM1+siFBL fibroblasts showed restored NCL protein levels (Fig. 3.1B), whereas NCL depletion was not able to rescue FBL protein levels in FoxM1-depleted cells (Fig. 3.1C). We also found increased immunostaining levels of other nucleolar proteins such as NOL12, NPM and UBF (Fig. S3.2A-C).

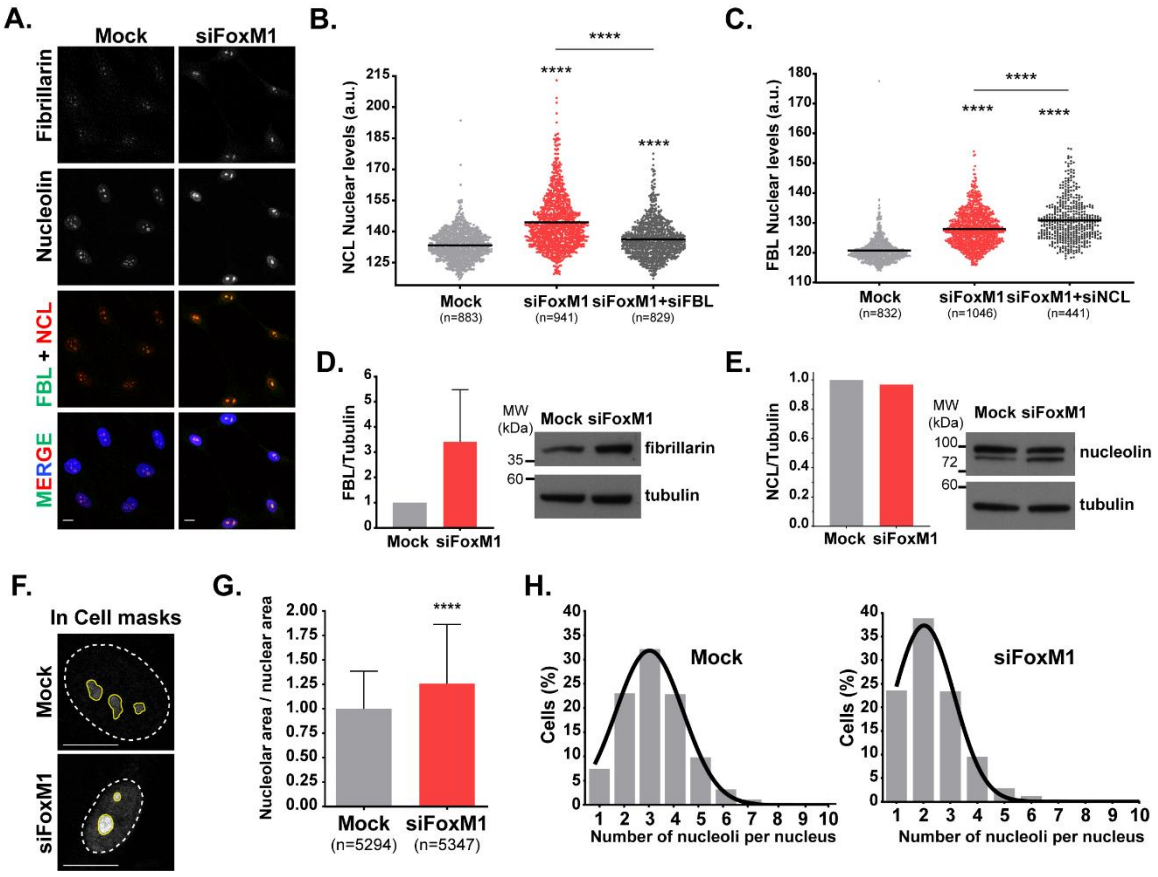

Figure 3.1. FoxM1 downregulation induces significant alterations in nucleolar morphology and impacts protein expression of nucleolar proteins. (A) Fibrillarin (FBL, grayscale/green) and Nucleolin (NCL, grayscale/red) immunostaining in control (mock-depleted) and FoxM1 siRNA-depleted (siFoxM1) cells. **(B)** Scatter plots of the mean pixel intensities of NCL nuclear levels in mock-, siFoxM1-, and siFoxM1+siFBL-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. a.u.,

arbitrary units. **(C)** Scatter plots of the mean pixel intensities of FBL nuclear levels in mock-, siFoxM1-, and siFoxM1+siNCL-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. a.u., arbitrary units. **(D)** Cell extracts from mock- and siFoxM1-treated neonatal fibroblasts were immunoblotted for fibrillarin. Tubulin levels were used for the loading control. In the graph, bars show mean values ± SD from five independent experiments, normalized to the values for the mock-treated control. MW, molecular weight. **(E)** Cell extracts from mock- and siFoxM1-treated neonatal fibroblasts were immunoblotted for nucleolin. Tubulin levels were used for the loading control. In the graph, bars show mean values from one independent experiment, normalized to the values for the mock-treated control. MW, molecular weight. **(F)** Nucleolin immunostaining (grayscale) in control (mock-depleted) and FoxM1 siRNA-depleted (siFoxM1) cells. The nuclear magnifications (x63), the white dashed and the yellow solid lines represent the masks used to define and measure nuclear and nucleolar areas, respectively. **(G)** Ratios of nucleolar and nuclear areas in mock- and siFoxM1-treated cells. Values are mean ± SD normalized to the mean value for the control. **(H)** Histograms and respective distribution curves for the percentages of mock- and siFoxM1-treated cells exhibiting total numbers of nucleoli as indicated. Scale bars represent 10μm in panels A and F. n = total number of cells analysed. ****, P≤0.0001, by Mann-Whitney and Kruskal-Wallis statistical tests in panels B, C, and G.

Finally, since dysregulation of several nucleolar proteins leads to significant changes in the nucleolar structure (Amin et al., 2008; Pinho et al., 2019; Ugrinova et al., 2007), we measured the nucleolar area and number of nucleoli in FoxM1-depleted cells, using a nucleolar mask based on NCL staining (Fig. 3.1F). We found an increased nucleolar size along with a decreased number of nucleoli per nucleus (Fig. 3.1G, H). Altogether, our data disclosed FoxM1 as a regulator of nucleolar organization, primarily repressing the expression and/or nucleolar recruitment of several proteins.

FoxM1 modulation of different nucleolar protein players impacts nucleolar organization parameters distinctly. We next investigated how the upregulation of NCL, FBL and NOL12 nucleolar proteins following FoxM1 repression contributes to the observed nucleolar phenotypes. To test this, we performed siRNA-double depletions of FoxM1 in combination with each one of the other players. Interestingly, FBL downregulation (Fig. S3.3A) in FoxM1-depleted cells did not alter the nucleolar area nor the number of nucleoli per nucleus (Fig. 3.2A, B). On the other hand, NCL downregulation (Fig. S3.3B) in FoxM1-depleted HDFs rescued the increased nucleolar area (Fig. 3.2C) while aggravating the number of nucleoli per nucleus (Fig. 3.2D). In a distinct manner, NOL12 downregulation did not restore the nucleolar size (Fig. 3.2E) but partially rescued the number of nucleoli per nucleus (Fig. 3.2F). Altogether, these results showed that NCL upregulation is a downstream event of FBL upregulation that is specifically causative of an increased nucleolar area observed in FoxM1-repressed fibroblasts. On the other hand, NOL12 upregulation seems to primarily contribute to the decreased number of nucleoli per nucleus

displayed by FoxM1-depleted HDFs. Further studies will be needed to ascertain whether NOL12 upregulation is a downstream event of FBL upregulation, similarly to NCL upregulation, or alternatively, directly regulated by FoxM1 repression.

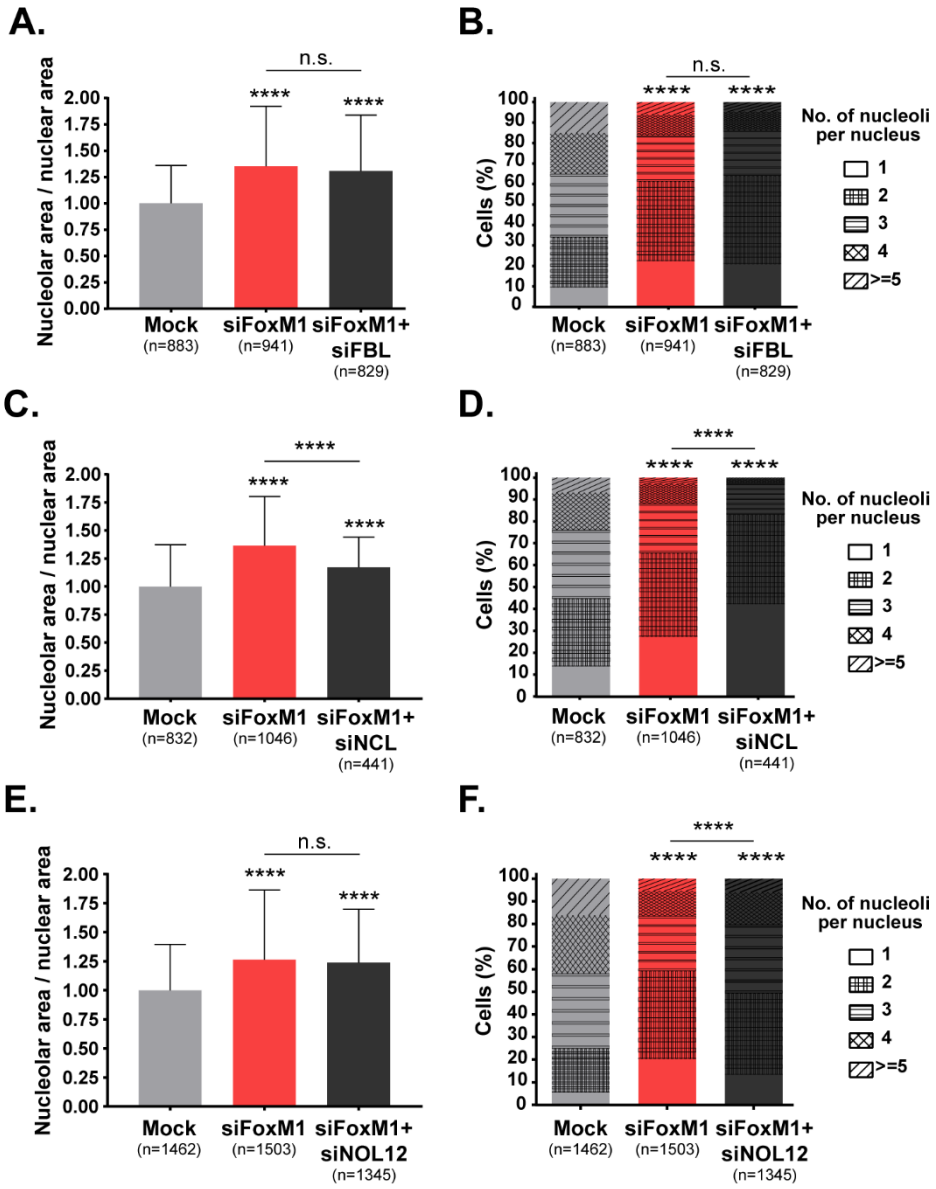

Figure 3.2. NCL and NOL12, but not FBL, mediate alterations of nucleolar morphology induced by FoxM1 depletion. (A) Ratios of nucleolar and nuclear areas in mock-, siFoxM1- and siFoxM1+siFBL-treated cells. Values are mean ± SD normalized to the mean value for the control. **(B)** Stacked bars showing the distribution of the total numbers of nucleoli per nucleus in mock-, siFoxM1-, and siFoxM1+siFBL-treated cells. **(C)** Ratios of nucleolar and nuclear areas in mock-, siFoxM1- and siFoxM1+siNCL-treated cells. Values are mean ± SD normalized to the mean value for the control. **(D)** Stacked bars showing the distribution of the total numbers of nucleoli per nucleus in mock-, siFoxM1-, and siFoxM1+siNCL-treated cells. **(E)** Ratios of nucleolar and nuclear areas in mock-, siFoxM1- and siFoxM1+siNOL12-treated cells. **(F)** Stacked bars showing the distribution of the total numbers of nucleoli per nucleus in mock-, siFoxM1-, and siFoxM1+siNOL12-treated cells. Values are mean ± SD normalized to the mean value for the control in the bar graphs A, C and E. Different patterns in bars

represent different numbers of nucleoli per nucleus in panels B, D and F. n = total number of cells analysed. ****, P≤0.0001, and n.s., not significant, by Kruskal-Wallis statistical tests.

FoxM1 repression increases rDNA transcription, imbalances rRNA processing and decreases protein synthesis. As we observed morphological changes in nucleoli from FoxM1-depleted cells along with deregulated expression of several nucleolar proteins, we hypothesized these events affect the nucleolar canonical function on ribosome biogenesis. To test that, we assessed different steps of ribosome biogenesis, starting by measuring rDNA transcription using FUrd incorporation into nascent rRNA transcripts (Kruhlak et al., 2007). To validate this procedure, we treated neonatal HDFs with a low dose of actinomycin D that inhibits RNA Pol I and, as expected, we found decreased levels of FUrd incorporation (Fig. 3.3A) (Perry and Kelley, 1970). Interestingly, upon FoxM1 depletion, we found significantly increased FUrd nuclear levels (Fig. 3.3B). Next, we asked whether increased rDNA transcription translated into increased production of 18S and 28S rRNAs. Indeed, 18S and 28S rRNAs were increased (Fig. 3.3C) suggesting that FoxM1 repression does not impair rRNA processing. However, the ratio 28S/18S was significantly higher (Fig. 3.3D), which reveals an imbalance between these two rRNA species. Nevertheless, as 18S and 28S rRNA species are assembled with other factors to generate functional small and large ribosome subunits, respectively, we next evaluated the rates of protein synthesis. To test this, we firstly measured the levels of incorporated L-homopropargylglycine (HPG) in newly synthesized proteins, by using a click reaction as previously described (Narita et al., 2011). To validate this procedure, we treated cells with cycloheximide (CHX), a protein synthesis inhibitor (Baliga et al., 1969), and as expected we found decreased incorporation of HPG (Fig. 3.3E). Interestingly, FoxM1 repression was found to significantly decrease HPG incorporation (Fig. 3.3F). In addition, we measured the incorporation of puromycin, an alternative method to read protein production. Similarly to the HPG incorporation assay, we found reduced puromycin incorporation in cells treated with CHX (Fig. 3.3G) as well as in FoxM1-depleted cells (Fig. 3.3H). Altogether, these data show that FoxM1 is required to maintain protein synthesis and balanced production of 28S and 18S rRNAs.

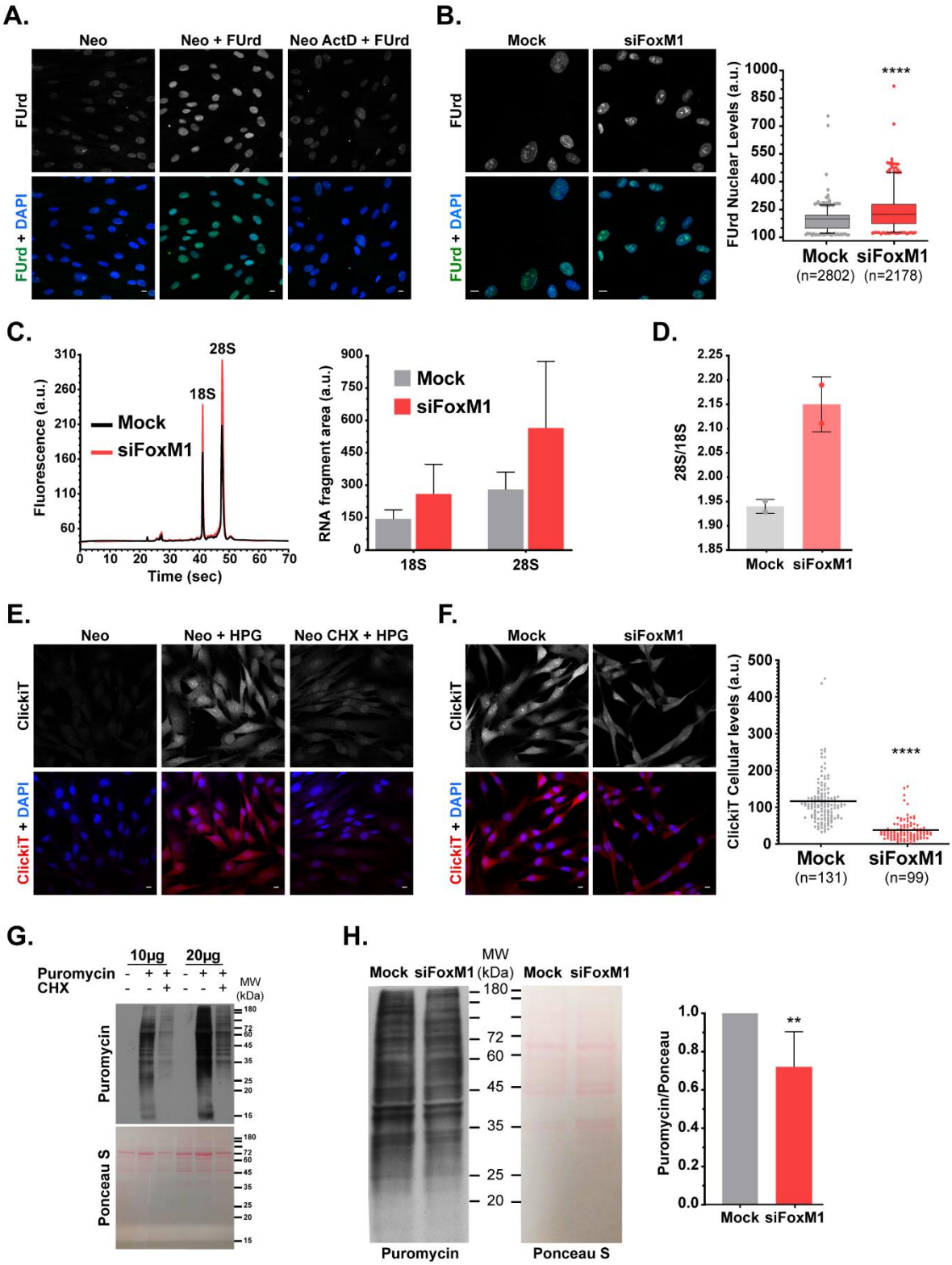

Figure 3.3. FoxM1 depletion increases rDNA transcription, impairs rRNA processing and decreases global protein synthesis. (A) Immunostaining of incorporated FUrd (grayscale/green) in control-, control+FUrd- and actinomycin D control+FUrd-treated human fibroblasts. DNA was stained with DAPI (blue). **(B)** Immunostaining of incorporated FUrd (grayscale/green) in mock- and siFoxM1-treated cells. Box-and-whiskers plot of median nuclear immunofluorescence levels of incorporated FUrd. Horizontal line within box represents the median fluorescence intensity; whiskers-representing boundaries of the box, 1st and 99th percentiles. **(C)** Representative profile of RNA fluorescence levels from mock- (black line) and siFoxM1- (red

line) treated cells. In the graph, bars show mean values ± SD of 18S and 28S rRNAs levels from two independent experiments. **(D)** Ratios of 28S/18S rRNAs in mock- and siFoxM1-treated human primary fibroblasts. Values are means ± SD from two independent experiments. **(E)** Immunostaining of incorporated L-HPG (grayscale/red) in control-, control+L-HPG- and CHX treated control+L-HPG-treated human fibroblasts. DNA was stained with DAPI (blue). **(F)** Immunostaining of incorporated L-HPG (grayscale/red) in mock- and siFoxM1-treated human fibroblasts. DNA was stained with DAPI (blue). Scatter plots of the mean pixel intensities of ClickiT cellular levels in mock- and siFoxM1-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. **(G)** Cell extracts (10µg or 20µg) from control-, control+puromycin- and CHX-treated control+puromycin-treated fibroblasts were immunoblotted for puromycin. Ponceau S levels were used for the loading control. MW, molecular weight. **(H)** Cell extracts from mock- and siFoxM1-treated cells were immunoblotted for puromycin. Ponceau S levels were used for the loading control. In bar graphs, values are means ± SD from six independent experiments, normalized to the values for the mock-treated control. MW, molecular weight. a.u., arbitrary units in panels B, C and F. Scale bars, 10µm in panels A, B, E and F. n = total number of cells analysed in panels B and F. **, P≤0.01 and ****, P≤0.0001 by Mann-Whitney statistical test.

FoxM1 downregulation activates P53 in an RPL11-dependent manner. Next, we asked whether FoxM1 repression and the associated nucleolar alterations could stabilize P53, as observed upon RNA Pol I inhibition (Choong et al., 2009) or NOL12 depletion-driven nucleolar stress (Pinho et al., 2019). Consistently, both immunofluorescence and immunoblotting data revealed an upregulation of P53 protein levels in response to FoxM1 siRNA-mediated depletion (Fig. 3.4A, B). We then ascertained if any of the FBL, NCL and NOL12 nucleolar proteins contributes for the P53 upregulation. Interestingly, increased P53 protein levels were blunted upon FBL repression (Fig. 3.4C) suggesting that FBL upregulation primarily accounts for P53 activation in FoxM1-depleted cells. Since nucleolar stress-induced P53 activation requires the 5S RNP complex, comprising RPL11, RPL5 and 5S rRNA, which binds MDM2 thereby preventing P53 proteasomal degradation (Sloan et al., 2013a), we additionally tested whether this mechanism contributes to P53 activation upon FoxM1 repression. Immunoblotting and immunofluorescence analysis of siFoxM1+siRPL11 double-depleted HDFs confirmed RPL11 to be required for P53 activation in response to FoxM1 repression (Fig. 3.4C, D). Overall, these data showed that FoxM1 repression activates P53 in an RPL11-dependent manner and revealed the nucleolar protein FBL as an important contributor for this P53 activation.

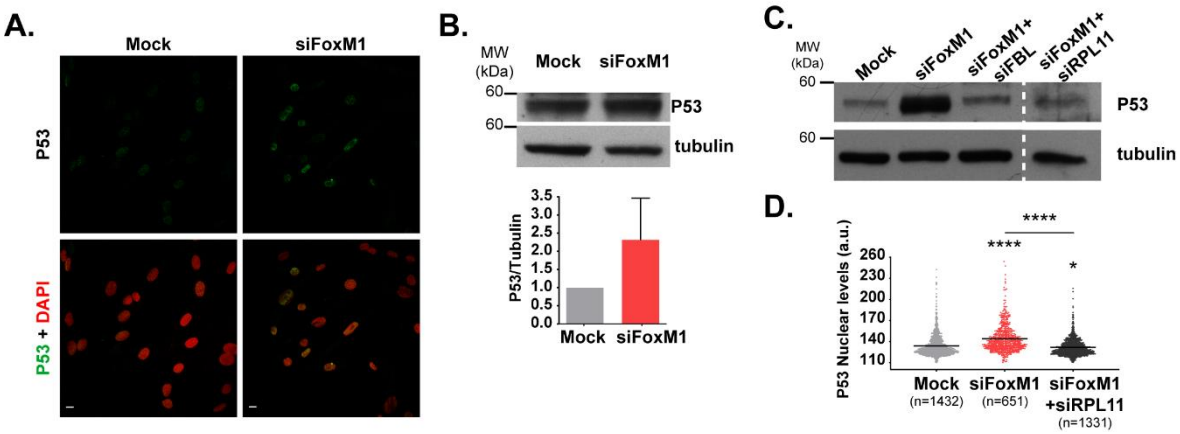

Figure 3.4. Both RPL11 and FBL mediate P53 activation elicited by FoxM1 depletion. (A) P53 immunostaining (green) of mock- and siFoxM1-treated human fibroblasts. DNA was stained with DAPI (red). Scale bars, 10µm. **(B)** Western blotting of P53 levels in cell extracts from mock- and siFoxM1-treated neonatal dermal fibroblasts. Tubulin protein levels were used as the loading control. In the graph, bars show mean values ± SD from three independent experiments, normalized to the values for the mock-treated control. **(C)** Cell extracts from mock-, siFoxM1-, siFoxM1+siFBL- and siFoxM1+siRPL11-treated neonatal fibroblasts were immunoblotted for P53. Tubulin protein levels were used as the loading control. **(D)** Scatter plots of the mean pixel intensities of P53 nuclear levels in mock-, siFoxM1- and siFoxM1+siRPL11-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. a.u., arbitrary units. n = total number of cells analysed. *, P≤0.05 and ****, P≤0.0001 by Kruskal-Wallis statistical tests.

FoxM1 repression decreases proliferation and increases senescence. Previous studies reported P53 activation as a major trigger of distinct cellular events, such as activation of DNA damage response, cell cycle arrest, apoptosis and senescence (Joerger and Fersht, 2016). In order to explore the cellular consequences of FoxM1 depletion-driven P53 activation, we immunostained mock- and FoxM1-depleted cell cultures for the proliferation marker Ki67. As illustrated in Fig. 3.5A, the percentage of cells staining negative for Ki67 markedly increased (26.23% ± 9.6% [mean ± standard deviation] vs 8.10% ± 1.8% in controls), showing that FoxM1 repression decreases the proliferative capacity. Furthermore, upon FoxM1 depletion we found an increased percentage of cells staining positive for double immunostaining of P21 cell cycle inhibitor and 53BP1 DNA damage markers, a readout of senescence (10.40% ± 1.1% vs 3.30% ± 1.3% in controls; Fig. 3.5B), which is in agreement with previous data (Macedo et al., 2018). Since RPL11 and FBL were found to mediate P53 activation induced by FoxM1 repression, we asked whether these two proteins contribute to the decreased proliferation capacity of FoxM1-depleted cell cultures. Surprisingly, none of the double-depleted fibroblast cultures siFoxM1+siRPL11 and siFoxM1+siFBL was able to rescue the reduced proliferative capacity caused by FoxM1 repression (Fig. 3.5C). This suggests that nucleolar stress activates P53-independent

mechanisms, as previously reported (James et al., 2014), which mainly contribute to decrease cell proliferation upon FoxM1 depletion.

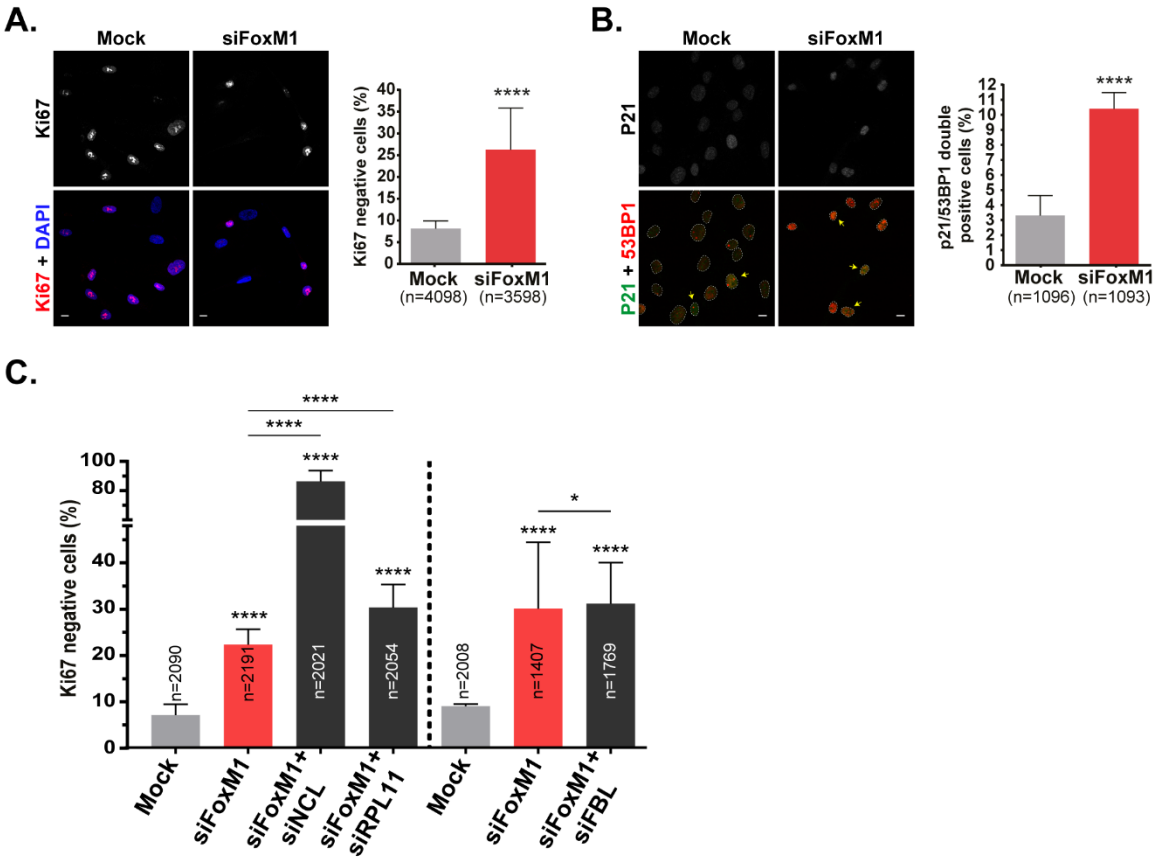

Figure 3.5. FoxM1 repression decreases cell proliferation and increases senescence. (A) Immunostaining of the proliferation marker Ki67 (grayscale/red) in mock- and siFoxM1-treated human fibroblasts. DNA was stained with DAPI (blue). Scale bars, 10µm. In bar graphs, percentages of cells staining negative for Ki67 are plotted. Values are means ± SD from four independent experiments. ****, P≤0.0001, by χ2 statistical test. **(B)** immunostaining of P21 (grayscale/green) and 53BP1 (red) in mock- and siFoxM1-treated human fibroblasts. Nuclear masking is shown in dashed white. Yellow arrows indicate P21/53BP1 double-positive cells. Scale bars, 10µm. In bar graphs, percentages of cells staining positive for P21/53BP1 are plotted. Values are means ± SD from two independent experiments. ****, P≤0.0001, by χ2 statistical test. **(C)** Percentages of cells staining negative for the proliferation marker Ki67 in mock-, siFoxM1-, siFoxM1+siNCL, siFoxM1+siRPL11- and siFoxM1+siFBL-treated cells. Values are means ± SD from two independent experiments. n = total number of cells analysed. *, P≤0.05 and ****, P≤0.0001 by χ2 statistical test.

PARP1 and PIM1 as putative FoxM1 transcriptional targets required for nucleolar homeostasis. Even though we found FBL and RPL11 to mediate P53 activation in FoxM1-depleted cells (Fig. 3.4C, D), we reasoned that transcriptional regulation of P53 by FoxM1 should not be disregarded. Indeed, increased P53 levels and transcriptional activity in response to FoxM1 depletion were previously described (Tan et al., 2007), as well as P53 feedback repression of FoxM1 expression (Barsotti and Prives, 2009). When

interrogating our previous RNA-sequencing datasets (Macedo et al., 2018) for P53 expression, we found that P53 transcripts are only slightly increased in FoxM1-depleted cells (Fig. 3.6A), thus excluding transcriptional regulation by FoxM1 as a major mechanism behind P53 upregulation. Interestingly, although naturally aged cells and FoxM1-depleted cells have low FoxM1 levels (Macedo et al., 2018), P53 transcripts are downregulated in elderly cells (Fig. 3.6B), and upregulated following expression of constitutively active FoxM1 (FoxM1dNdK) (Fig. 3.6C). Moreover, interrogation of the RNA-seq datasets for the nucleolar players NOL12, FBL, and NCL, for which we found increased protein levels upon FoxM1 repression, surprisingly revealed that these are unaltered or downregulated at the transcript level in siFoxM1-depleted cells and 87 year-old cells, and upregulated in FoxM1dNdK-overexpressing cells (Fig. 3.6A-C). This suggests for post-transcriptional increased stability of these players upon FoxM1 repression. To identify nucleolar FoxM1 transcriptional targets directly contributing to nucleolar disruption and senescence, we again interrogated the available RNA-seq datasets for a set of nucleolar genes (Huntley et al., 2015) to determine which are significantly responsive to FoxM1 levels. Intersection of i) the 5529 differentially expressed genes (DEGs) in mock vs siFoxM1 HDFs, ii) the 2818 DEGs in 87y vs 87y dNdK HDFs, iii) the 3188 DEGs in neo vs 87y HDFs, and iv) the 2364 nucleolar genes, came out with 42 genes as shown in Fig. 3.6D. Additionally, interrogation of a dataset of 1029 DEGs from targeted transcriptome analysis of asynchronous neonatal vs aged HDFs (Barroso-Vilares et al., 2020), also came out with 42 nucleolar genes of the Huntley et al. list (Fig. 3.6E). Intersection between the 42 genes found in each independent analysis revealed 9 overlapping genes (Fig. 3.6F). Three genes, SPECC1, GPRC5A and RASL11A, were excluded as candidate targets as their expression was not consistently responsive to FoxM1 levels (i.e., downregulation in both siFoxM1 and 87y, and upregulation in FoxM1dNdK) (Fig. 3.6G). This left us with 6 genes, 3 genes repressed by FoxM1 (HOXD9, YPEL2 and YPEL3), and 3 genes upregulated by FoxM1 (NFIB, PARP1 and PIM1). Interestingly, both HOXD9 (Fromental-Ramain et al., 1996) and YPEL2 (Wang et al., 2016) are mainly involved in development, while YPEL3 was shown to be transcriptionally regulated by P53 and an inducer of senescence (Kelley et al., 2010).

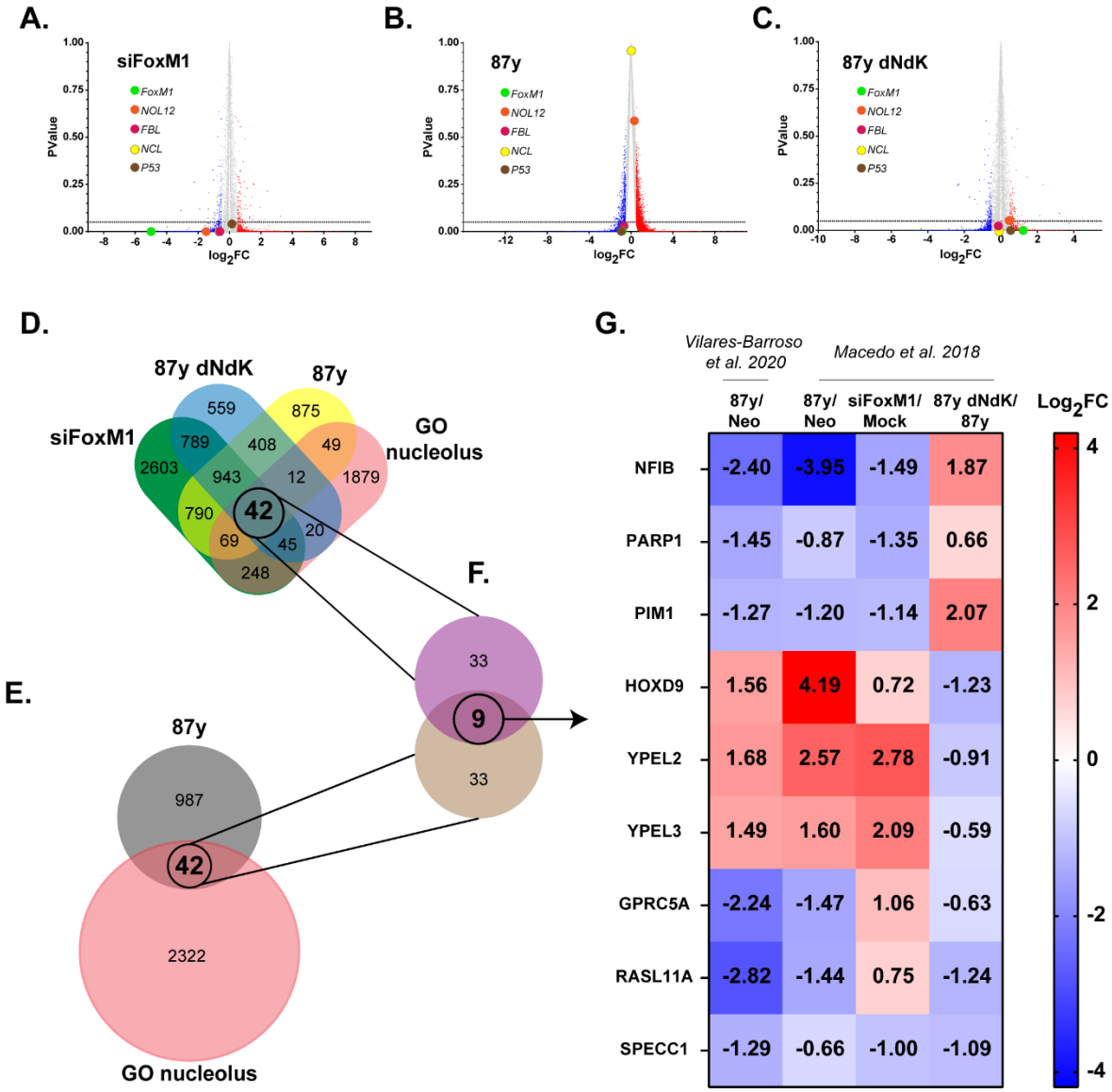

Figure 3.6. Six nucleolar proteins respond to FoxM1 modulation. (A), **(B)** and **(C)** Previously reported RNA sequencing data from different FoxM1 modulation conditions (Macedo et al., 2018) was plotted in volcano plots. Blue dots represent downregulated genes, red dots represent upregulated genes (|log2FC| ≥ 0.5) and gray dots represent unaltered expressed genes. Horizontal dashed line indicates a pvalue of 0.05. Specific genes are indicated. **(D)** Venn diagram showing the overlap of differentially expressed genes (DEGs) previously reported by (Macedo et al., 2018) and an RNA sequencing dataset of nucleolar genes. **(E)** Venn diagram showing the overlap of differentially expressed genes (DEGs) previously reported by (Vilares-Barroso al., 2020) and an RNA sequencing dataset of nucleolar genes. **(F)** Venn diagram showing the overlap of genes that result from the Venn diagrams in D and E panels. **(G)** Heatmap showing the log2FC values of the nine genes that result from the Venn diagram in F panel. Those values are indicated in the four conditions of FoxM1 modulation from (Macedo et al., 2018) and (Vilares-Barroso et al., 2020). Blue boxes indicate downregulated DEGs and red boxes indicate upregulated DEGs.

We next evaluated NFIB, PARP1 and PIM1 genes, which behave as putative direct transcriptional targets of FoxM1. We analysed available ChIP sequencing data (Mei et al., 2017; Zheng et al., 2019) to check whether and where FoxM1 binds in each of the three candidate gene sequences. As shown in Fig. 3.7A, there are no significant FoxM1-binding sites in the NFIB gene. In the PIM1 gene, there are at least two significant and strong FoxM1-binding sites, one in the promoter region and another in an intronic region, likely representing an enhancer cis-regulatory element since it displays the acetylated H3K27 mark (Fig. 3.7B). In addition, the PARP1 gene also displays a FoxM1-binding site in the promoter region displaying the acetylated H3K27 mark (Fig. 3.7C). Relevantly, our preliminary data confirmed that PARP1 is downregulated in FoxM1-depleted fibroblasts (Fig. 3.7D). Interestingly, while NFIB was previously shown to be essential for development of different organs (Steele-Perkins et al., 2005), PARP1 (Boamah et al., 2012; Kim et al., 2019) and PIM1 (Iadevaia et al., 2010) were implicated in ribosome biogenesis. Particularly, PARP1 seems to function as a structural support for the recruitment of rRNA processing factors, such as FBL (Boamah et al., 2012). Notably, PIM1 loss is associated with ribosomal stress and growth arrest (Iadevaia et al., 2010; Sagar et al., 2016). Altogether, these data strongly suggest PARP1 and PIM1 as putative functional links coupling nucleolar alterations, particularly impairment of nucleolar function, and decreased proliferation induced by FoxM1 repression.

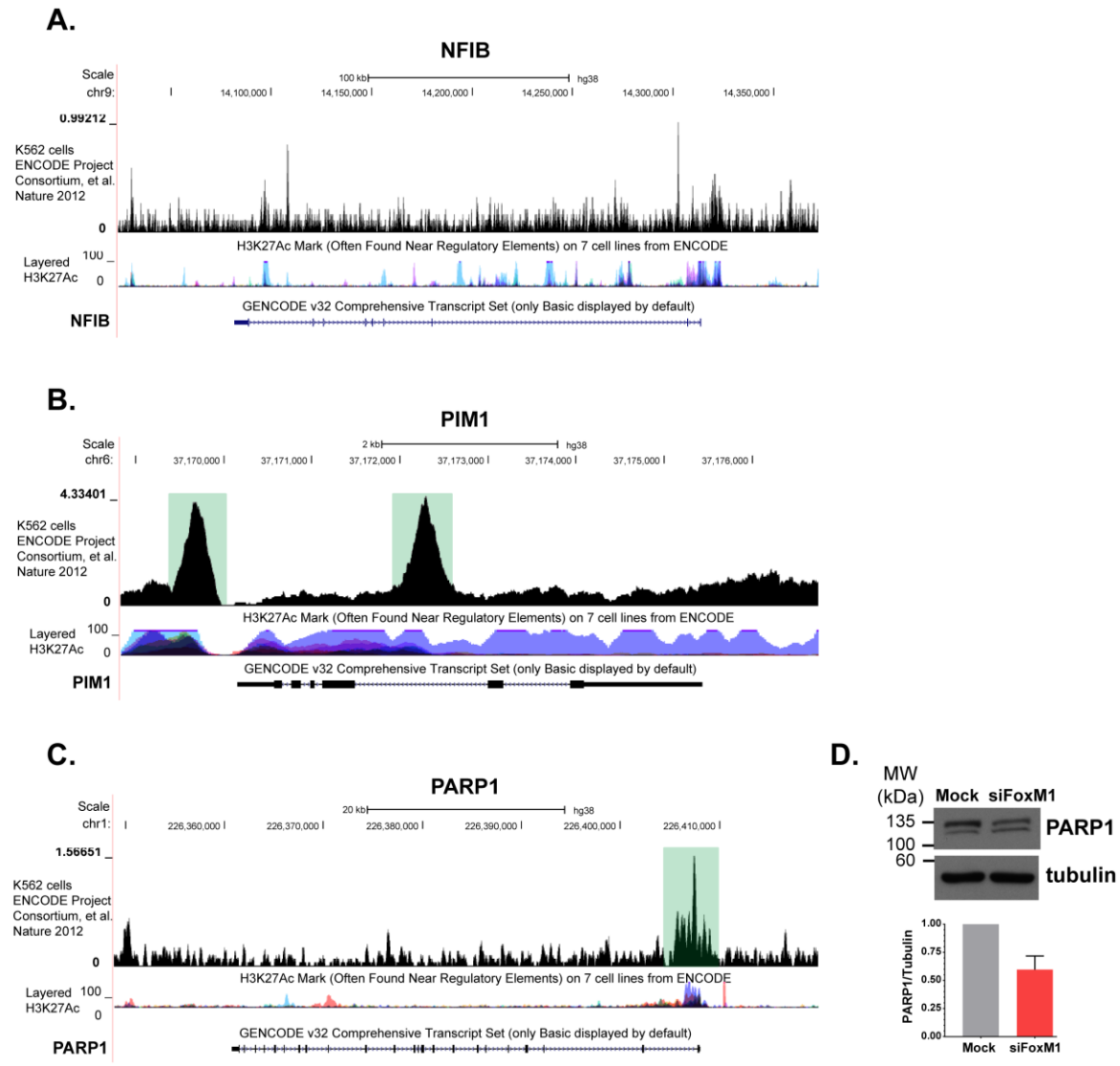

Figure 3.7. PIM1 and PARP1 are putative direct transcriptional nucleolar targets of FoxM1. (A) ChIP-sequencing data from K562 cell line aligned with sequences of NFIB gene and the H3K27 acetylation profile. Peaks correspond to interaction of the NFIB DNA sequence and FoxM1 protein. **(B)** ChIP-sequencing data from different K562 cell line aligned with sequences of PIM1 gene and the H3K27 acetylation profile. Peaks correspond to interaction of the PIM1 DNA sequence and FoxM1 protein. The strongest and most significant/relevant peaks are highlighted with a green box. **(C)** ChIP-sequencing data from K562 cell line aligned with sequences of PARP1 gene and the H3K27 acetylation profile. Peaks correspond to interaction of the PARP1 DNA sequence and FoxM1 protein. The strongest and most significant/relevant peaks are highlighted with a green box. **(D)** Western blotting of PARP1 levels in cell extracts from mock- and siFoxM1-treated neonatal dermal fibroblasts. Tubulin protein levels were used as the loading control. In the graph, bars show mean values ± SD from two independent experiments, normalized to the values for the mock-treated control.

5. DISCUSSION

Previously, we found FoxM1 to be a key transcriptional regulator in human normative aging, controlling cellular mitotic fitness and consequently aneuploidy, which is a main trigger of full-blown senescence (Macedo et al., 2018). Moreover, we found a correlation between nucleolar stress and aging, with aged cells displaying significant alterations in nucleolar organization and structure (Pinho et al., 2019). Based on these findings, we studied the role of FoxM1 transcriptional activity in nucleolar homeostasis.

By efficiently depleting FoxM1 in neonatal HDFs, we found a reduced number of nucleoli per nucleus. Importantly, our data disclosed NOL12 upregulation as key contributor for this phenotype, and thus further establishing NOL12's role in the regulation of nucleolar aggregation (Pinho et al., 2019). Furthermore, nucleoli from FoxM1-depleted fibroblasts exhibited an increased relative area when compared with controls. Interestingly, this was recently reported in HeLa cervical cancer cells upon treatment with latrunculin A, an inhibitor of the actin polymerization (Caragine et al., 2019). Furthermore, it was shown that this inhibitor is able to regulate the YAP/TEAD transcriptional activity (Ou et al., 2017), which was previously shown to target FoxM1 (Mizuno et al., 2012). Therefore, this seems to be a particular cell response to FoxM1 repression. Importantly, we found FBL upregulation as an upstream event relatively to NCL nucleolar recruitment although, surprisingly, increased FBL levels did not account for the altered nucleolar parameters tested upon FoxM1 repression. However, this is in agreement with a previous report showing FBL as playing a role in preserving nuclear morphology whereas none impact was observed in nucleoli from HeLa cells (Amin et al., 2007). Interestingly, our data revealed NCL nucleolar recruitment as a requirement for the increased nucleolar area of the FoxM1-depleted neonatal HDFs. Therefore, FBL upregulation, as an upstream event, might elicit several nucleolar modifications that counteract each other. This would explain that interference with a downstream event, such as NCL repression, induces a specific modification that is not revealed when FBL upregulation is blunted. Furthermore, the observed upregulation of other nucleolar proteins involved in ribosome biogenesis seems to support the effects of FoxM1 depletion in nucleolar function. Notably, we found an increased rDNA transcription, which is consistent with the upregulation of UBF since it is a nucleolar protein crucial for RNA Pol I-mediated transcription (Jantzen et al., 1992; Stefanovsky et al., 2001). Moreover, it was previously shown that UBF overexpression increases rDNA transcription in rat neonatal cardiomyocytes (Hannan et al., 1996). Importantly, while increase of 18S and 28S rRNA levels suggests that FoxM1-repressed cells were able to deal with the increased amount of rRNA transcripts, we found an increased 28S/18S level ratio, disclosing an imbalance between the two rRNA species. Therefore, these results suggest that the 18S

rRNA processing is not as efficient as the 28S rRNA processing. Altogether, these data reveal that FoxM1 depletion disrupts nucleolar organization and impairs the nucleolar function, driving cell into a state generally called nucleolar stress (Yang et al., 2018). In response to this stress, FoxM1-depleted fibroblasts activate P53, which is a common event often reported in response to nucleolar stress (James et al., 2014). Mechanistically, the 5S RNP complex, comprising RPL11, RPL5 and 5S rRNA, has been shown to mediate this activation by binding MDM2 hence preventing the P53 targeting to proteasomal degradation (Donati et al., 2013; Sloan et al., 2013a). Notably, our data strongly suggest this mechanism as a mediator of P53 activation in FoxM1-repressed neonatal HDFs. Interestingly, we found that FBL upregulation, but not NCL or NOL12 (data not shown), also strongly contributes for P53 activation. This could occur through RPL11, as previously reported for the nucleolar protein GRWD1 (Kayama et al., 2017). Since FBL protein levels are increased in siFoxM1+siRPL11 cells (data not shown), our data strongly suggest that P53 activation negatively regulates FBL protein levels. While the impact of FBL in regulating P53 has not been previously reported, some studies have revealed P53 as a negative regulator of FBL: in immortalized human mammary epithelial cells, P53 was shown to inhibit FBL transcription and protein levels (Marcel et al., 2013); in *C. elegans*, P53 was recently shown to indirectly reduce FBL protein levels (Chen et al., 2020). Nevertheless, although FBL depletion blunts P53 activation, it does not alter the nucleolar phenotypes of FoxM1-depleted HDFs. This suggests that in these cells, stabilized P53 does not regulate nucleolar morphology, which is in accordance with our previous results showing that nucleolar disruption induced by NOL12 depletion is not modified upon P53 depletion (Chapter 2).

Next, we inspected the cellular consequences of P53 activation upon FoxM1 repression. We observed decreased cell proliferation in primary neonatal fibroblasts upon FoxM1 depletion, along with an accumulation of senescent cells, as previously reported (Macedo et al., 2018). Interestingly, we formerly described a nucleolar stress-driven G2/M arrest that ultimately leads to decreased proliferation and increased senescence, in a P53-dependent manner (Pinho et al., 2019). Moreover, the decreased average of number of nucleoli per nucleus observed in FoxM1-depleted HDFs could be associated with an accumulation of cells arrested at G2/M. This is expected due to reduced transcription of the G2/M gene cluster regulated by FoxM1 (Wang et al., 2005). Thus, FoxM1 repression induces nucleolar stress-driven P53 activation and senescence even though our data suggest that P53 activation may not be essential for the reduction in proliferation. In line with decreased cell proliferation, we found reduced protein synthesis, a connection extensively reported. Notably, the cell cycle protein CDK1, which is a transcriptional target of FoxM1 (Macedo et al., 2018), was recently found to be required for global protein synthesis providing a support for proliferation in HeLa cells (Haneke et al., 2020). For

instance, a mouse model of increased lifespan exhibits decreased proliferation rate associated with a reduced protein synthesis (Thompson et al., 2016); fibroblasts from HGPS patients are hyperproliferative (Bridger and Kill, 2004) and display an increased protein synthesis rate (Buchwalter and Hetzer, 2017). Remarkably, using two independent assays we showed a significant decrease of newly synthesized proteins in FoxM1-depleted HDFs, suggesting impaired ribosome biogenesis due to uncoordinated 28S and 18S synthesis. Previously, FoxM1 repression was shown to downregulate several ribosomal proteins from both ribosome subunits (Chen et al., 2018). Hence, ribosomes might not be properly assembled, compromising their function. However, this might not be the case in our primary fibroblasts since we observed no differences in the protein levels of some ribosomal proteins (data not shown).

To get further insight into direct FoxM1 transcriptional targets with nucleolar function and accounting for impaired ribosome biogenesis, and eventually senescence, we took advantage from previous RNA-seq datasets as well as from ChIP-seq data. From this analysis, we found two interesting candidates, PARP1 and PIM1. PARP1 was shown to bind rDNA, creating a physical network and allowing the recruitment of nucleolar proteins involved in ribosome biogenesis, hence facilitating rRNA modifications and processing (Boamah et al., 2012). Accordingly, PARP inhibitors elicited a decrease of rDNA transcription, rRNA processing, modification and folding, and cell growth, in samples from human breast cancer patients (Kim et al., 2019). However, this contrasts with our data showing PARP1 downregulation concomitant with an increased rDNA transcription in FoxM1-depleted cells. One reconciling possibility is that PARP1 repression in FoxM1-depleted HDFs may disrupt the PARP1-TIP5-mediated epigenetic mechanism of rDNA silencing, resulting in an increased number of active rDNA copies (Guetg et al., 2012). Regarding PIM1, previous studies reported it as a constitutive active serine-threonine kinase responsible for decreasing the activity and increasing the degradation of the cell cycle inhibitor P27Kip1 (Morishita et al., 2008). Importantly, PIM1 downregulation was shown to induce P27Kip1 stabilization, leading to cell cycle arrest, independently of P53 status (Iadevaia et al., 2010). Although PIM1 is described as a sensor for ribosome deficiency (Iadevaia et al., 2010), its potential as a marker for the quality of ribosomes remains unexplored. Additionally, PIM1 was shown to bind RPS19 and both were found in association with both ribosome subunits and polysomes, suggesting that PIM1 plays a role during translation (Chiocchetti et al., 2005). Altogether, these data support PARP1 and PIM1 as attractive FoxM1 nucleolar targets, putatively mediating the impact of FoxM1 repression in protein synthesis. Promisingly, we found reduced PARP1 protein levels in FoxM1-depleted neonatal HDFs. Further analyses will be needed to disclose both PARP1

and PIM1 as nucleolar effectors of FoxM1, linking nucleolar homeostasis to protein synthesis and cellular fitness.

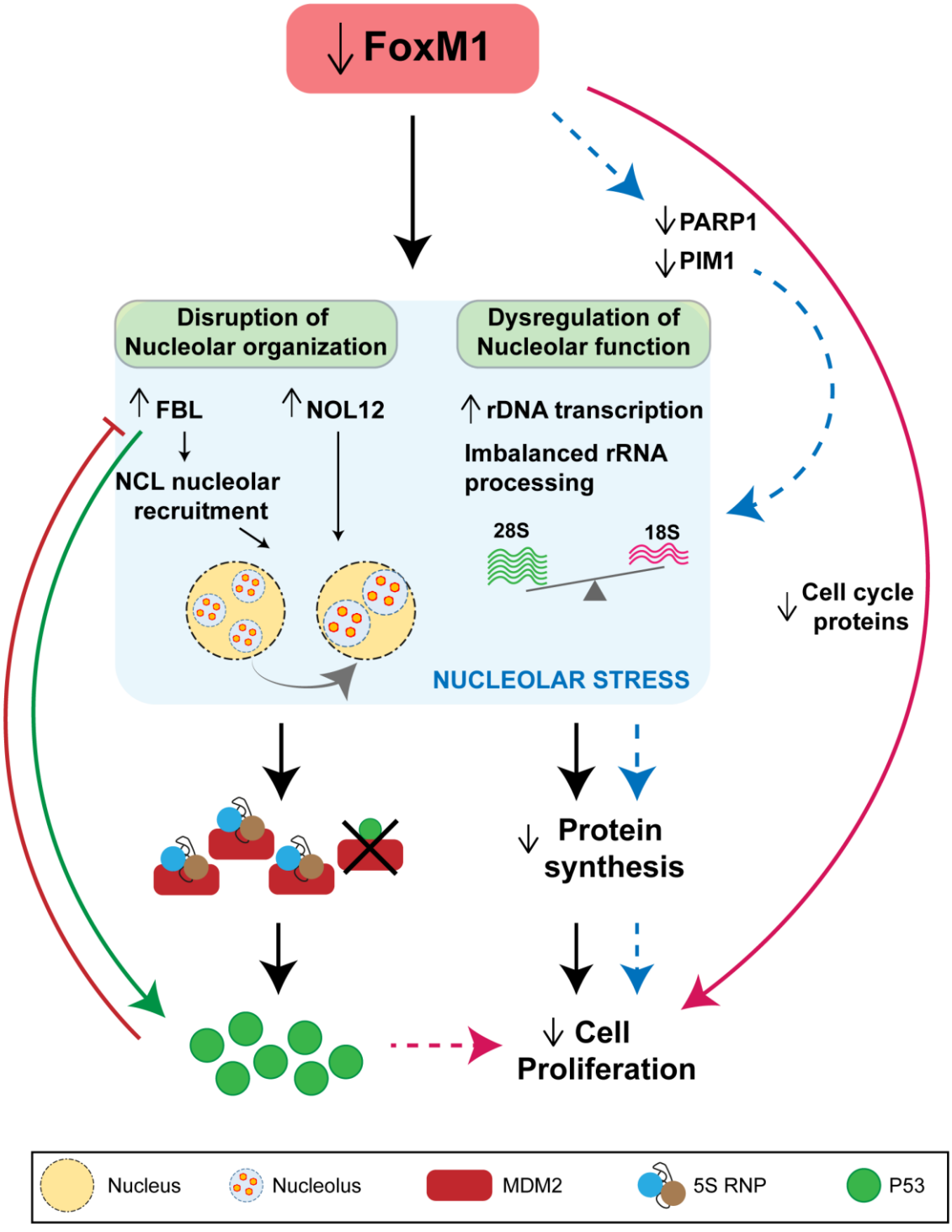

Figure 3.8. Model that summarizes and integrates the consequences of FoxM1 downregulation. FoxM1 downregulation in human primary fibroblasts leads to FBL upregulation, which is required for nucleolar recruitment of NCL, and NOL12 upregulation. This disrupts nucleolar morphology, increasing nucleolar size and decreasing the number of nucleoli per nucleus. Also, nucleolar function is perturbed by FoxM1 downregulation: both rDNA transcription and 28S and 18S rRNA levels increases. However, there is an imbalance between those rRNA species. Together, these nucleolar alterations configure a cell state called nucleolar stress which

activates P53. This activation is mediated by 5S RNP complex and FBL upregulation. In turn, FoxM1 depletion-induced P53 activation was shown to abrogate FBL protein levels. FoxM1-depleted cell cultures showed a reduced proliferative capacity which cannot be fully explained by P53 activation. Alternatively, both decreased global protein synthesis and downregulation of cell cycle genes that respond directly to FoxM1 downregulation are possible explanations. Furthermore, our preliminary data disclosed PARP1 and PIM1 as putative nucleolar proteins that respond directly to FoxM1. We hypothesize these downregulation events impact nucleolar homeostasis, leading to decreased protein synthesis thus being important contributors for decreased proliferative capacity of FoxM1-depleted fibroblasts. Solid arrows and lines exhibit observed and solid evidence, dashed arrows indicate work hypothesis.

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7. REFERENCES

Amin, M.A., Matsunaga, S., Ma, N., Takata, H., Yokoyama, M., Uchiyama, S., Fukui, K., 2007. Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells. Biochemical and biophysical research communications 360, 320-326.

Amin, M.A., Matsunaga, S., Uchiyama, S., Fukui, K., 2008. Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. The Biochemical journal 415, 345-351.

Baliga, B.S., Pronczuk, A.W., Munro, H.N., 1969. Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver. The Journal of biological chemistry 244, 4480-4489.

Barroso-Vilares, M., Macedo, J.C., Reis, M., Warren, J.D., Compton, D., Logarinho, E., 2020. Small-molecule inhibition of aging-associated chromosomal instability delays cellular senescence. EMBO reports 21, e49248.

Barsotti, A.M., Prives, C., 2009. Pro-proliferative FoxM1 is a target of p53-mediated repression. Oncogene 28, 4295-4305.

Boamah, E.K., Kotova, E., Garabedian, M., Jarnik, M., Tulin, A.V., 2012. Poly(ADP-Ribose) polymerase 1 (PARP-1) regulates ribosomal biogenesis in Drosophila nucleoli. PLoS genetics 8, e1002442.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J., Lamond, A.I., 2007. The multifunctional nucleolus. Nature reviews. Molecular cell biology 8, 574-585.

Bridger, J.M., Kill, I.R., 2004. Aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by hyperproliferation and increased apoptosis. Experimental gerontology 39, 717-724.

Buchwalter, A., Hetzer, M.W., 2017. Nucleolar expansion and elevated protein translation in premature aging. Nature communications 8, 328.

Caragine, C.M., Haley, S.C., Zidovska, A., 2019. Nucleolar dynamics and interactions with nucleoplasm in living cells. eLife 8.

Chen, J., Stark, L.A., 2019. Insights into the Relationship between Nucleolar Stress and the NF-kappaB Pathway. Trends in genetics : TIG 35, 768-780.

Chen, P.H., Chen, Y.T., Chu, T.Y., Ma, T.H., Wu, M.H., Lin, H.H., Chang, Y.S., Tan, B.C., Lo, S.J., 2020. Nucleolar control by a non-apoptotic p53-caspases-deubiquitinylase axis promotes resistance to bacterial infection. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 34, 1107-1121.

Chen, Z., Bu, N., Qiao, X., Zuo, Z., Shu, Y., Liu, Z., Qian, Z., Chen, J., Hou, Y., 2018. Forkhead Box M1 Transcriptionally Regulates the Expression of Long Noncoding RNAs Snhg8 and Gm26917 to Promote Proliferation and Survival of Muscle Satellite Cells. Stem cells 36, 1097-1108.

Chiocchetti, A., Gibello, L., Carando, A., Aspesi, A., Secco, P., Garelli, E., Loreni, F., Angelini, M., Biava, A., Dahl, N., Dianzani, U., Ramenghi, U., Santoro, C., Dianzani, I., 2005. Interactions between RPS19, mutated in Diamond-Blackfan anemia, and the PIM-1 oncoprotein. Haematologica 90, 1453-1462.

Choong, M.L., Yang, H., Lee, M.A., Lane, D.P., 2009. Specific activation of the p53 pathway by low dose actinomycin D: a new route to p53 based cyclotherapy. Cell cycle 8, 2810-2818.

Donati, G., Peddigari, S., Mercer, C.A., Thomas, G., 2013. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. Cell reports 4, 87-98.

Dutt, S., Narla, A., Lin, K., Mullally, A., Abayasekara, N., Megerdichian, C., Wilson, F.H., Currie, T., Khanna-Gupta, A., Berliner, N., Kutok, J.L., Ebert, B.L., 2011. Haploinsufficiency for ribosomal protein genes causes selective activation of p53 in human erythroid progenitor cells. Blood 117, 2567-2576.

Feric, M., Vaidya, N., Harmon, T.S., Mitrea, D.M., Zhu, L., Richardson, T.M., Kriwacki, R.W., Pappu, R.V., Brangwynne, C.P., 2016. Coexisting Liquid Phases Underlie Nucleolar Subcompartments. Cell 165, 1686-1697.

Fischer, M., Grossmann, P., Padi, M., DeCaprio, J.A., 2016. Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks. Nucleic acids research 44, 6070-6086.

Fromental-Ramain, C., Warot, X., Lakkaraju, S., Favier, B., Haack, H., Birling, C., Dierich, A., Doll e, P., Chambon, P., 1996. Specific and redundant functions of the paralogous Hoxa-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. Development 122, 461-472.

Fromont-Racine, M., Senger, B., Saveanu, C., Fasiolo, F., 2003. Ribosome assembly in eukaryotes. Gene 313, 17-42.

Goodfellow, S.J., Zomerdijk, J.C., 2013. Basic mechanisms in RNA polymerase I transcription of the ribosomal RNA genes. Sub-cellular biochemistry 61, 211-236.

Guetg, C., Scheifele, F., Rosenthal, F., Hottiger, M.O., Santoro, R., 2012. Inheritance of silent rDNA chromatin is mediated by PARP1 via noncoding RNA. Molecular cell 45, 790-800.

Haneke, K., Schott, J., Lindner, D., Hollensen, A.K., Damgaard, C.K., Mongis, C., Knop, M., Palm, W., Ruggieri, A., Stoecklin, G., 2020. CDK1 couples proliferation with protein synthesis. The Journal of cell biology 219.

Hannan, R.D., Stefanovsky, V., Taylor, L., Moss, T., Rothblum, L.I., 1996. Overexpression of the transcription factor UBF1 is sufficient to increase ribosomal DNA transcription in neonatal cardiomyocytes: implications for cardiac hypertrophy. Proceedings of the National Academy of Sciences of the United States of America 93, 8750-8755.

Huntley, R.P., Sawford, T., Mutowo-Meullenet, P., Shypitsyna, A., Bonilla, C., Martin, M.J., O'Donovan, C., 2015. The GOA database: gene Ontology annotation updates for 2015. Nucleic acids research 43, D1057-1063.

Iadevaia, V., Caldarola, S., Biondini, L., Gismondi, A., Karlsson, S., Dianzani, I., Loreni, F., 2010. PIM1 kinase is destabilized by ribosomal stress causing inhibition of cell cycle progression. Oncogene 29, 5490-5499.

James, A., Wang, Y., Raje, H., Rosby, R., DiMario, P., 2014. Nucleolar stress with and without p53. Nucleus 5, 402-426.

Jantzen, H.M., Chow, A.M., King, D.S., Tjian, R., 1992. Multiple domains of the RNA polymerase I activator hUBF interact with the TATA-binding protein complex hSL1 to mediate transcription. Genes & development 6, 1950-1963.

Joerger, A.C., Fersht, A.R., 2016. The p53 Pathway: Origins, Inactivation in Cancer, and Emerging Therapeutic Approaches. Annual review of biochemistry 85, 375-404.

Kayama, K., Watanabe, S., Takafuji, T., Tsuji, T., Hironaka, K., Matsumoto, M., Nakayama, K.I., Enari, M., Kohno, T., Shiraishi, K., Kiyono, T., Yoshida, K., Sugimoto, N., Fujita, M., 2017. GRWD1 negatively regulates p53 via the RPL11-MDM2 pathway and promotes tumorigenesis. EMBO reports 18, 123-137.

Kelley, K.D., Miller, K.R., Todd, A., Kelley, A.R., Tuttle, R., Berberich, S.J., 2010. YPEL3, a p53-regulated gene that induces cellular senescence. Cancer research 70, 3566-3575.

Kim, D.S., Camacho, C.V., Nagari, A., Malladi, V.S., Challa, S., Kraus, W.L., 2019. Activation of PARP-1 by snoRNAs Controls Ribosome Biogenesis and Cell Growth via the RNA Helicase DDX21. Molecular cell 75, 1270-1285 e1214.

Kruhlak, M., Crouch, E.E., Orlov, M., Montano, C., Gorski, S.A., Nussenzweig, A., Misteli, T., Phair, R.D., Casellas, R., 2007. The ATM repair pathway inhibits RNA polymerase I transcription in response to chromosome breaks. Nature 447, 730-734.

Leary, D.J., Huang, S., 2001. Regulation of ribosome biogenesis within the nucleolus. FEBS letters 509, 145-150.

Lee, J.J., Lee, H.J., Son, B.H., Kim, S.B., Ahn, J.H., Ahn, S.D., Cho, E.Y., Gong, G., 2016. Expression of FOXM1 and related proteins in breast cancer molecular subtypes. International journal of experimental pathology 97, 170-177.

Lindstrom, M.S., Latonen, L., 2013. The Nucleolus as a Stress Response Organelle. Proteins of the Nucleolus (11), 251-273.

Macedo, J.C., Vaz, S., Bakker, B., Ribeiro, R., Bakker, P.L., Escandell, J.M., Ferreira, M.G., Medema, R., Foijer, F., Logarinho, E., 2018. FoxM1 repression during human aging leads to mitotic decline and aneuploidy-driven full senescence. Nature communications 9, 2834.

Marcel, V., Ghayad, S.E., Belin, S., Therizols, G., Morel, A.P., Solano-Gonzalez, E., Vendrell, J.A., Hacot, S., Mertani, H.C., Albaret, M.A., Bourdon, J.C., Jordan, L., Thompson, A., Tafer, Y., Cong, R., Bouvet, P., Saurin, J.C., Catez, F., Prats, A.C., Puisieux, A., Diaz, J.J., 2013. p53 acts as a safeguard of translational control by regulating fibrillarin and rRNA methylation in cancer. Cancer cell 24, 318-330.

Mei, S., Qin, Q., Wu, Q., Sun, H., Zheng, R., Zang, C., Zhu, M., Wu, J., Shi, X., Taing, L., Liu, T., Brown, M., Meyer, C.A., Liu, X.S., 2017. Cistrome Data Browser: a data portal for ChIP-Seq and chromatin accessibility data in human and mouse. Nucleic acids research 45, D658-D662.

Mizuno, T., Murakami, H., Fujii, M., Ishiguro, F., Tanaka, I., Kondo, Y., Akatsuka, S., Toyokuni, S., Yokoi, K., Osada, H., Sekido, Y., 2012. YAP induces malignant mesothelioma cell proliferation by upregulating transcription of cell cycle-promoting genes. Oncogene 31, 5117-5122.

Morishita, D., Katayama, R., Sekimizu, K., Tsuruo, T., Fujita, N., 2008. Pim kinases promote cell cycle progression by phosphorylating and down-regulating p27Kip1 at the transcriptional and posttranscriptional levels. Cancer research 68, 5076-5085.

Myatt, S.S., Lam, E.W., 2007. The emerging roles of forkhead box (Fox) proteins in cancer. Nature reviews. Cancer 7, 847-859.

Narita, M., Young, A.R., Arakawa, S., Samarajiwa, S.A., Nakashima, T., Yoshida, S., Hong, S., Berry, L.S., Reichelt, S., Ferreira, M., Tavare, S., Inoki, K., Shimizu, S., Narita, M., 2011. Spatial coupling of mTOR and autophagy augments secretory phenotypes. Science 332, 966-970.

Nishimura, K., Kumazawa, T., Kuroda, T., Katagiri, N., Tsuchiya, M., Goto, N., Furumai, R., Murayama, A., Yanagisawa, J., Kimura, K., 2015. Perturbation of ribosome biogenesis drives cells into senescence through 5S RNP-mediated p53 activation. Cell reports 10, 1310-1323.

Ou, C., Sun, Z., Li, S., Li, G., Li, X., Ma, J., 2017. Dual roles of yes-associated protein (YAP) in colorectal cancer. Oncotarget 8, 75727-75741.

Perry, R.P., Kelley, D.E., 1970. Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. Journal of cellular physiology 76, 127-139.

Pinho, M., Macedo, J.C., Logarinho, E., Pereira, P.S., 2019. NOL12 Repression Induces Nucleolar Stress-Driven Cellular Senescence and Is Associated with Normative Aging. Molecular and cellular biology 39.

Sadasivam, S., Duan, S., DeCaprio, J.A., 2012. The MuvB complex sequentially recruits B-Myb and FoxM1 to promote mitotic gene expression. Genes & development 26, 474-489.

Sagar, V., Caldarola, S., Aria, V., Monteleone, V., Fuoco, C., Gargioli, C., Cannata, S., Loreni, F., 2016. PIM1 destabilization activates a p53-dependent response to ribosomal stress in cancer cells. Oncotarget 7, 23837-23849.

Scott, D.D., Trahan, C., Zindy, P.J., Aguilar, L.C., Delubac, M.Y., Van Nostrand, E.L., Adivarahan, S., Wei, K.E., Yeo, G.W., Zenklusen, D., Oeffinger, M., 2017. Nol12 is a

multifunctional RNA binding protein at the nexus of RNA and DNA metabolism. Nucleic acids research 45, 12509-12528.

Sloan, K.E., Bohnsack, M.T., Watkins, N.J., 2013a. The 5S RNP couples p53 homeostasis to ribosome biogenesis and nucleolar stress. Cell reports 5, 237-247.

Sloan, K.E., Mattijssen, S., Lebaron, S., Tollervey, D., Pruijn, G.J., Watkins, N.J., 2013b. Both endonucleolytic and exonucleolytic cleavage mediate ITS1 removal during human ribosomal RNA processing. The Journal of cell biology 200, 577-588.

Sloan, K.E., Warda, A.S., Sharma, S., Entian, K.D., Lafontaine, D.L.J., Bohnsack, M.T., 2017. Tuning the ribosome: The influence of rRNA modification on eukaryotic ribosome biogenesis and function. RNA biology 14, 1138-1152.

Steele-Perkins, G., Plachez, C., Butz, K.G., Yang, G., Bachurski, C.J., Kinsman, S.L., Litwack, E.D., Richards, L.J., Gronostajski, R.M., 2005. The transcription factor gene Nfib is essential for both lung maturation and brain development. Molecular and cellular biology 25, 685-698.

Stefanovsky, V.Y., Pelletier, G., Bazett-Jones, D.P., Crane-Robinson, C., Moss, T., 2001. DNA looping in the RNA polymerase I enhancesome is the result of non-cooperative in-phase bending by two UBF molecules. Nucleic acids research 29, 3241-3247.

Tan, Y., Raychaudhuri, P., Costa, R.H., 2007. Chk2 mediates stabilization of the FoxM1 transcription factor to stimulate expression of DNA repair genes. Molecular and cellular biology 27, 1007-1016.

Thompson, A.C., Bruss, M.D., Price, J.C., Khambatta, C.F., Holmes, W.E., Colangelo, M., Dalidd, M., Roberts, L.S., Astle, C.M., Harrison, D.E., Hellerstein, M.K., 2016. Reduced in vivo hepatic proteome replacement rates but not cell proliferation rates predict maximum lifespan extension in mice. Aging cell 15, 118-127.

Tiku, V., Antebi, A., 2018. Nucleolar Function in Lifespan Regulation. Trends in cell biology 28, 662-672.

Tiku, V., Jain, C., Raz, Y., Nakamura, S., Heestand, B., Liu, W., Spath, M., Suchiman, H.E.D., Muller, R.U., Slagboom, P.E., Partridge, L., Antebi, A., 2017. Small nucleoli are a cellular hallmark of longevity. Nature communications 8, 16083.

Tsai, R.Y., Pederson, T., 2014. Connecting the nucleolus to the cell cycle and human disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 28, 3290-3296.

Ugrinova, I., Monier, K., Ivaldi, C., Thiry, M., Storck, S., Mongelard, F., Bouvet, P., 2007. Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. BMC molecular biology 8, 66.

Wang, I.C., Chen, Y.J., Hughes, D., Petrovic, V., Major, M.L., Park, H.J., Tan, Y., Ackerson, T., Costa, R.H., 2005. Forkhead box M1 regulates the transcriptional network of

genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. Molecular and cellular biology 25, 10875-10894.

Wang, Y., Zhou, T., Wan, J., Yang, Y., Chen, X., Wang, J., Zhou, C., Liu, M., Ling, X., Zhang, J., 2016. Comparative transcriptome analysis reveals a regulatory network of microRNA-29b during mouse early embryonic development. Oncotarget 7, 53772-53782.

Yang, K., Yang, J., Yi, J., 2018. Nucleolar Stress: hallmarks, sensing mechanism and diseases. Cell stress 2, 125-140.

Zheng, R., Wan, C., Mei, S., Qin, Q., Wu, Q., Sun, H., Chen, C.H., Brown, M., Zhang, X., Meyer, C.A., Liu, X.S., 2019. Cistrome Data Browser: expanded datasets and new tools for gene regulatory analysis. Nucleic acids research 47, D729-D735.

8. SUPPLEMENTAL MATERIAL

Table S3.1. Sequences of siRNAs used in this study.

mRNA targets	***Sequence 5'-3'***
FoxM1	(SASI_Hs01_00243977) AACUCUUCUCCCUCAGAU[dT][dT] (sense) AUCUGAGGGAGAAGAGUU [dT][dT] (antisense)
FBL	siRNA1: UGGAGGACACUUUGUGAUU[dT][dT] (sense) AAUCACAAAGUGUCCUCCA[dT][dT] (antisense) siRNA2: GUCUUCAUUUGUCGAGGAA[dT][dT] (sense) UUCCUCGACAAAUGAAGAC[dT][dT] (antisense)
NCL	GGAAGAGCCUGUCAAAGAA[dT][dT] (sense) UUCUUUGACAGGCUCUUCC[dT][dT] (antisense)
NOL12	(SASI_Hs01_00047859) CAGAUGAGCUGGACCGGUU[dT][dT] (sense) AACCGGUCCAGCUCAUCUG[dT][dT] (antisense)
RPL11	CGCGAGCAGCCAAGGUGUUGGAGCA[dT][dT] (sense) UGCUCCAACACCUUGGCUGCUCGCG[dT][dT] (antisense)

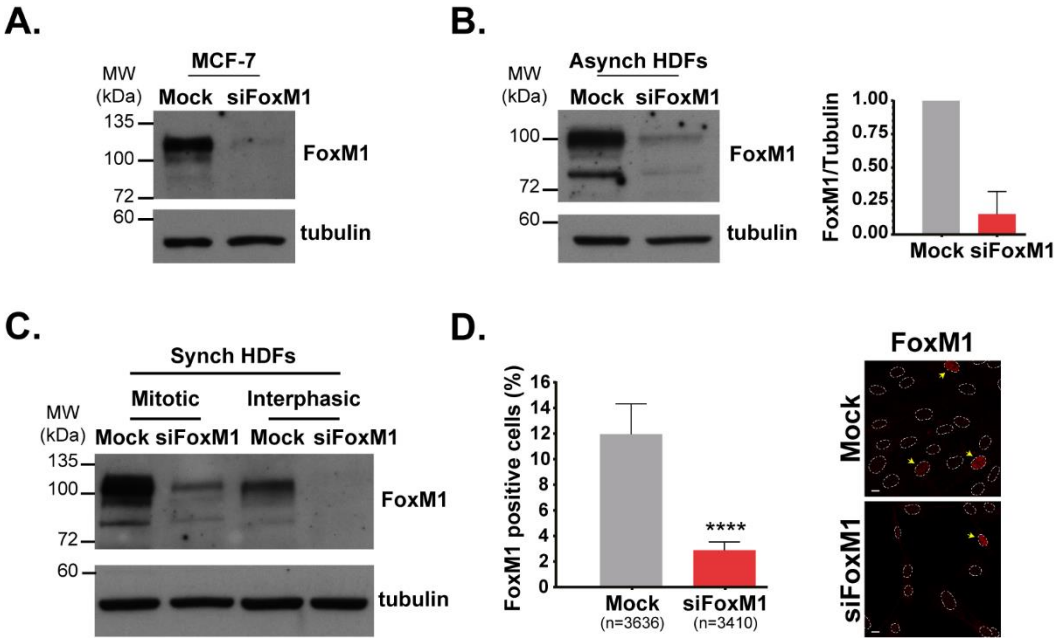

Figure S3.1. **Validation of FoxM1 downregulation in human primary fibroblasts. (A)** Western blotting of FoxM1 levels in cell extracts from mock- and siFoxM1-treated MCF7 cells, using an antibody anti-FoxM1 from Santa Cruz Biotechnology (sc-271746). Tubulin protein levels were used as the loading control. **(B)** Western blotting of FoxM1 levels in asynchronous cell extracts from mock- and siFoxM1-treated human dermal fibroblasts (HDFs), using an antibody anti-FoxM1 from Santa Cruz Biotechnology (sc-271746). Tubulin protein levels were used as the loading control. In the graph, bars show mean values ± SD from four independent experiments, normalized to the values for the mock-treated control. **(C)** Western blotting of FoxM1 levels in synchronous cell extracts from mock- and siFoxM1-treated human primary fibroblasts, using an antibody anti-FoxM1 from Santa Cruz Biotechnology (sc-271746). Both mitotic and interphasic cell extracts were run separately. Tubulin protein levels were used as the loading control. **(D)** Immunostaining of the FoxM1 (red) in mock- and siFoxM1-treated human fibroblasts using an antibody anti-FoxM1 from from Cell Signalling Technology (#5436). Nuclear masking is shown in dashed white. Yellow arrows indicate FoxM1 positive cells. Scale bars, 10μm. In bar graphs, percentages of cells staining positive for FoxM1 are plotted. Values are means ± SD from four independent experiments. ****, P≤0.0001, by $\chi 2$ statistical test.

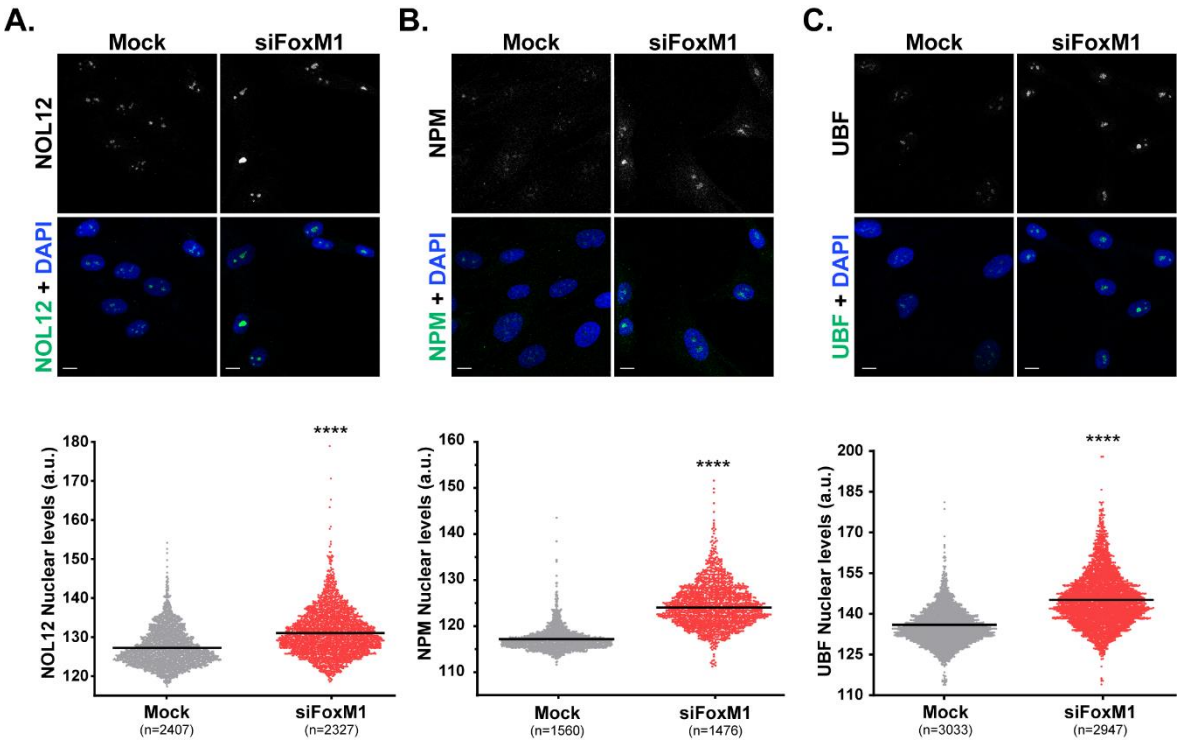

Figure S3.2. **FoxM1 downregulation leads to nucleolar upregulation of the NOL12, NPM and UBF proteins. (A)** Immunostaining of the nucleolar protein NOL12 (grayscale/green) in mock- and siFoxM1-treated human fibroblasts. DNA was stained with DAPI (blue). Scatter plots of the mean pixel intensities of NOL12 nuclear levels in mock- and siFoxM1-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. **(B)** Immunostaining of the nucleolar protein NPM (grayscale/green) in mock- and siFoxM1-treated human fibroblasts. DNA was stained with DAPI (blue). Scatter plots of the mean pixel intensities of NPM nuclear levels in mock- and siFoxM1-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. **(C)** Immunostaining of the nucleolar protein UBF (grayscale/green) in mock- and siFoxM1-treated human fibroblasts. DNA was stained with DAPI (blue). Scatter plots of the mean pixel intensities of UBF nuclear levels in mock- and siFoxM1-treated cells. Each dot represents the value for a single cell, and horizontal lines represent the mean values. Scale bars, 10μm. a.u., arbitrary units. n = total number of cells analysed. ****, P≤0.0001 by Mann-Whitney statistical test.

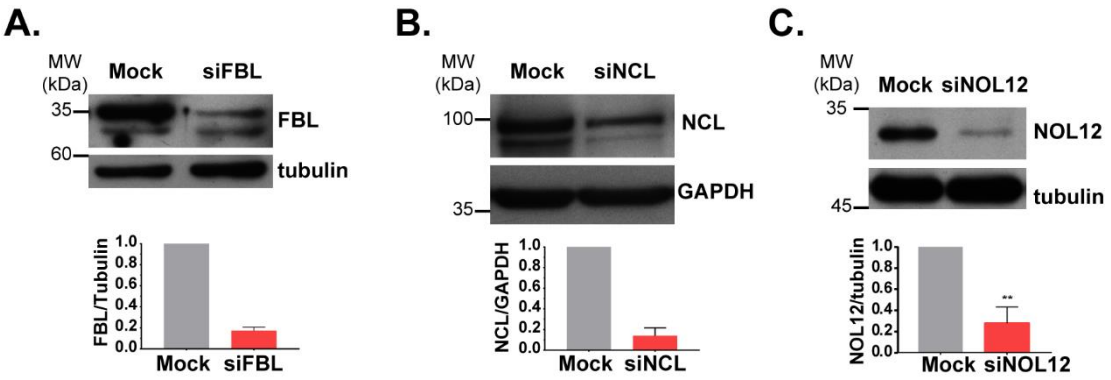

Figure S3.3. **Validation of FBL and NCL downregulation in human primary fibroblasts. (A)** Western blotting of FBL levels in cell extracts from mock- and siFBL-treated neonatal dermal fibroblasts. Tubulin protein levels were used as the loading control. In the graph, bars show mean values ± SD from two independent experiments, normalized to the values for the mock-treated control. **(B)** Western blotting of NCL levels in cell extracts from mock- and siNCL-treated neonatal dermal fibroblasts. GAPDH protein levels were used as the loading control. In the graph, bars show mean values ± SD from two independent experiments, normalized to the values for the mock-treated control. **(C)** Western blotting analysis of NOL12 protein levels in cell extracts from mock- and siNOL12-depleted fibroblasts. Tubulin levels were used as loading control. Values are mean ± SD from three independent experiments and normalized to mock controls. ** $p \leq 0.01$ by Mann-Whitney statistical test. MW, molecular weight.

CHAPTER 4

General Discussion

With this chapter, we discuss our findings in light of the current knowledge in the field, while pinpointing future research lines to pursue.

The nucleolus is a distinctive membraneless organelle that emerged in eukaryotic cells. Evolutionarily, it is thought that its tripartite organization arose with amniotes, being related with the increased rDNA intergenic region, whereas a bipartite organization characterizes anamniotes (Thiry and Lafontaine, 2005). Nevertheless, it was firstly described as the site where ribosomes, the molecular machines that ultimately lead to the production of all cellular proteins, are produced. This complex process of ribosome biogenesis is so important that cells dispense at least 60% of energy to ensure proper ribosome production (Zhou et al., 2015). Importantly, other functions were posteriorly attributed to the nucleolus, including cell cycle regulation, DNA repair, apoptosis and senescence (Boisvert et al., 2007). Recent studies have unveiled the nucleolus as an important player in several age-associated diseases, such as neurodegenerative, cardiovascular and cancer (Nunez Villacis et al., 2018). Therefore, understanding the regulation of nucleolar structure and function, as well as the response mechanisms that the nucleolus is able to activate, will ultimately disclose potential therapeutic targets.

1. NOL12 and FoxM1: how do they regulate nucleolar homeostasis?

Our findings described in Chapter 2 revealed NOL12 as a nucleolar protein in human neonatal fibroblasts, consistently to previous observations in yeast (Oeffinger et al., 2009), *Drosophila melanogaster* (Marinho et al., 2011) and mice (Suzuki et al., 2006). Furthermore, NOL12 repression was shown to elicit an increase of nucleolar relative area, a decreased number of nucleoli per nucleus and significant alterations in the nucleolar ultrastructure. Although repression of other nucleolar proteins, such as XRN2, did not mimic these nucleolar alterations (Chapter 2), the loss of FoxM1, a nuclear transcription factor mainly involved in cell cycle (Sadasivam et al., 2012), induced similar nucleolar alterations in neonatal HDFs (Chapter 3). This is an original observation since till now there are no reports showing FoxM1 as a regulator of nucleolar morphology. Unlike other nucleolar proteins, whose dysregulation did not show any impact in the expression of nucleolar proteins (Amin et al., 2007; Amin et al., 2008; Ma et al., 2007), NOL12 downregulation was able to upregulate the expression levels of other nucleolar proteins, such as FBL and NCL (Chapter 2). Also, upon FoxM1 repression, we found FBL upregulation, and as an upstream event required for nucleolar recruitment of NCL (Chapter 3). Interestingly, FBL upregulation in association with enlarged nucleoli was previously reported in *C. elegans* (Yi et al., 2015). Since recent studies have shown nucleoli as a liquid-like compartment, and prolonged stress was shown to induce a nucleolar shift from a liquid- into a solid-like state along with

nucleolar expansion (Frottin et al., 2019), our data suggests FBL as a determinant of nucleolar viscoelastic properties. Importantly, the liquid-solid transition of the nucleolus was recently found to impair rRNA processing (Zhu et al., 2019), and is also associated with the formation of nucleolar aggregates, characteristic of neurodegenerative diseases, such as Huntington's disease and spinocerebellar ataxias (Latonen, 2019).

Nucleolar function is nevertheless distinctly affected by FoxM1 and NOL12: while rDNA transcription is increased upon FoxM1 repression (Chapter 3), it decreases in NOL12-depleted cells (Chapter 2). This is interesting if we take into account that FoxM1 depletion increased NOL12 protein levels, possibly through protein stabilization (Chapter 3). Thus, our data strongly suggest NOL12 as a major positive regulator of rDNA transcription. Since NOL12 was shown to be required for proper rRNA processing in yeast (Oeffinger et al., 2009) and HeLa cells (Sloan et al., 2013b), the decreased rDNA transcription might be a feedback response to the accumulation of unprocessed rRNA transcripts, in NOL12-depleted cells. On the other hand, FoxM1-depleted cells seem to handle with the higher amount of synthesized 47S pre-rRNA by increasing both 18S and 28S rRNAs (Chapter 3), although we found an imbalance towards 28S rRNA. Interestingly, mRNA levels of NOB1, an endonuclease involved specifically in the 18S processing, and WDR46, a nuclear protein required for structuring the 18S rRNA processing machinery (Hirai et al., 2013), were both found to be decreased in FoxM1-depleted cells (Macedo et al., 2018), which could explain the smaller efficiency of 18S rRNA processing. Alternatively, one could speculate that 28S rRNA processing is more efficient and faster. The increased levels of XRN2 upon FoxM1 depletion (data not shown) might explain this, due to its involvement in rRNA processing and its ability to clean the unprocessed transcripts (Wang and Pestov, 2011).

Altogether, our data demonstrated that NOL12 and FoxM1 repressions induce similar alterations in the nucleolar morphology, but distinct responses in nucleolar function.

2. P53 activation in response to nucleolar stress

Nucleolar stress occurs when nucleolar homeostasis is disrupted, thus it is characterized by both structural and functional defects in the nucleolus (Yang et al., 2018). Frequently, in response to this stress, P53 accumulates in the cell (Holmberg Olausson et al., 2012), activating its signalling pathway. Notably, both NOL12 and FoxM1 repression showed to induce P53 activation (Chapters 2 and 3). Importantly, 5S RNP, comprising RPL11, RPL5 and 5S rRNA, has been shown to be the main mediator of P53 activation in response to nucleolar stress by binding MDM2 preventing its repressor activity over P53 (Bursac et al., 2012; Donati et al., 2013; Sloan et al., 2013a). We found P53 activation upon

NOL12 and FoxM1 depletions to be mediated by RPL11, strongly supporting the involvement of the 5S RNP. Moreover, FBL upregulation was shown to also mediate P53 activation upon FoxM1 repression (Chapter 3), while its contribution in the context of NOL12 depletion was not addressed. Interestingly, when P53 activation is abrogated in double-depleted siFoxM1+siRPL11 cells, FBL protein levels increased (data no shown). Together, these results suggest that under FoxM1 downregulation, P53 represses FBL, which is in agreement with the transcriptional repressing activity of P53 over FBL previously reported in human cancer cells (Marcel et al., 2013). Interestingly, the depletion of P53 in non-senescent cells elicited an increase of FBL protein levels (Chapter 2). Although interestingly, we cannot actually compare directly these two findings since ablation of P53 activation (by doing siFoxM1+siRPL11) might not elicit the same effects as reduction of *P53* mRNA levels (by doing siP53). In fact, *P53* mRNA could activate mechanisms that result into the upregulation of FBL. Therefore, the measurement of FBL protein levels in double-depleted siFoxM1+siP53 is a key experiment missed here. Additionally, FBL emerged as an unforeseen positive regulator of P53, in siFoxM1 cells, whereas its depletion increased P53 protein levels in normal fibroblasts (data not shown). This is a very interesting finding which could reveal a specific impairment of FBL/P53 crosstalk in senescent cells.

Altogether, these data revealed P53 activation as a common event in response to nucleolar stress in primary human cells.

3. Nucleolar stress induces cellular senescence

The activation of P53 in NOL12- and FoxM1-depleted fibroblasts was shown to induce upregulation of the P53 transcriptional target *CDKN1A* (Chapter 2) (Macedo et al., 2018). As P53 is involved in major cellular events, we hypothesized its activation would impact cell homeostasis. We did not find any impact of P53 in nucleolar morphology in neonatal primary fibroblasts. However, NOL12 depletion led to the accumulation of G2 arrested cells in a P53-dependent manner. Interestingly, NCL repression in HeLa cells (Ugrinova et al., 2007) and FoxM1 depletion in MCF-7 cells (Barsotti and Prives, 2009) were previously reported to elicit a G2 arrest. So, although P53 did not account for maintenance of nucleolar morphology, it had a major role in downstream events, in NOL12-depleted cells. Accordingly, our data revealed loss of proliferative capacity of upon NOL12 or FoxM1 repression, which ultimately led to the accumulation of cells stained positive for different senescence biomarkers (Chapters 2 and 3). Therefore, our data disclosed senescence as an outcome of nucleolar stress.

4. Nucleolar stress-driven loss of proliferative capacity is likely due to defective protein synthesis

Although we observed loss of proliferative capacity in both NOL12- and FoxM1-depleted fibroblasts, P53 was found to partially mediate this loss in NOL12 repression (Chapter 2), but not in FoxM1 repression (Chapter 3). Interestingly, increased protein synthesis has been associated with enhanced proliferative capacity (Pardee, 1989; Polymenis and Aramayo, 2015). So, we hypothesized that protein synthesis and cell proliferation are coupled in neonatal fibroblasts under nucleolar stress. Accordingly, we found decreased protein synthesis in NOL12-depleted cells, which indicates that a decreased ribosome biogenesis (suggested by decreased rDNA transcription) compromises protein synthesis (Chapter 2). Similarly, FoxM1-depleted fibroblasts displayed a decreased protein synthesis rate, even though under increased rDNA transcription and 18S and 28S rRNAs levels, which suggests that ribosome production is elevated (Chapter 3). This might result from a compensatory mechanism through which decreased protein synthesis may signal cells to produce more ribosomes. Nevertheless, our aim was to find nucleolar factors able to causally link decreased protein synthesis to reduced proliferative capacity. From our analysis, PIM1 emerged as a promising candidate (Chapter 3), since it is required for proper ribosome production (Iadevaia et al., 2010). Downregulation of PIM1 in FoxM1-depleted cells could reconcile the increased amount of 18S and 28S rRNAs with defective ribosome production and consequent decrease of protein synthesis. Additionally, PARP1 arose as an auspicious candidate, as it was described to be required for recruitment of factors such as FBL, hence controlling rRNA processing and pre-ribosome assembly (Boamah et al., 2012). Indeed, we found downregulation of PARP1 in FoxM1-depleted fibroblasts (Chapter 3).

Noteworthy, although NOL12 and FoxM1 repressions impact ribosome biogenesis distinctly, they both inhibit protein synthesis and cell proliferation. Interestingly, as human aged cells displayed NOL12 (Pinho et al., 2019) and FoxM1 downregulation (Macedo et al., 2018), our data contrasts with recent data showing an increased protein synthesis in premature aged cells (Buchwalter and Hetzer, 2017).

5. Nucleolar stress during aging: a new research avenue

In agreement with the increased number of senescent cells upon NOL12 and FoxM1 repressions, naturally aged cells displayed reduced levels of both proteins (Macedo et al., 2018; Pinho et al., 2019). This led us to explore the role of nucleolar stress during human aging. Remarkably, fibroblasts from elderly donors exhibited a similar nucleolar phenotype

as NOL12-depleted neonatal fibroblasts (Chapter 2). Taking into account recent studies showing nucleolar stress as a potential hallmark of aging (Buchwalter and Hetzer, 2017; Tiku et al., 2017), we asked whether nucleolar organization, as well as proliferation and senescence, could be rescued upon re-establishment of NOL12 levels in elderly cells. Except for FBL expression, we did not find any improvement in the general structure of the nucleolus. Moreover, even though we found proliferation rate slightly rescued, the accumulation of senescent cells remained unchanged (Chapter 2). This was in contrast with previous data showing that the expression of constitutively active FoxM1 in aged cell rescues senescence (Macedo et al., 2018). There are possible explanations. Firstly, NOL12 is a nucleolar protein, with a limited spectrum of action in comparison to FoxM1 wide transcriptional activity that impacts the expression of many genes. Moreover, senescence is a complex multifactorial process unlikely rescued by simply restored nucleolar structure and NOL12 expression. However, we cannot discard the hypothesis that NOL12 overexpression protocol may technically require further optimization, regarding time of infection, doxycycline induction and end point analysis (Chapter 2). Also, it would be very interesting to determine if FoxM1 induction in elderly cells is able to rescue nucleolar stress. Second, FoxM1 appears to regulate NOL12 levels (Chapter 3), with FoxM1 induction leading to increased *NOL12* transcripts (Macedo et al., 2018). Thus, it would be important to also measure NOL12 protein levels to disclose a putative synergistic effect between NOL12 and FoxM1 in the regulation of nucleolar homeostasis.

In another perspective, our data showed FBL upregulation as a common event in response to nucleolar stress, shared by NOL12-depleted, FoxM1-depleted and elderly fibroblasts (Chapters 2 and 3). Although FBL dysregulation and nucleolar morphology have been correlated with lifespan (Tiku and Antebi, 2018), the mechanism behind FBL upregulation remains unknown. Also, the mechanism through which NOL12 is downregulated in elderly aged cells awaits further studies.

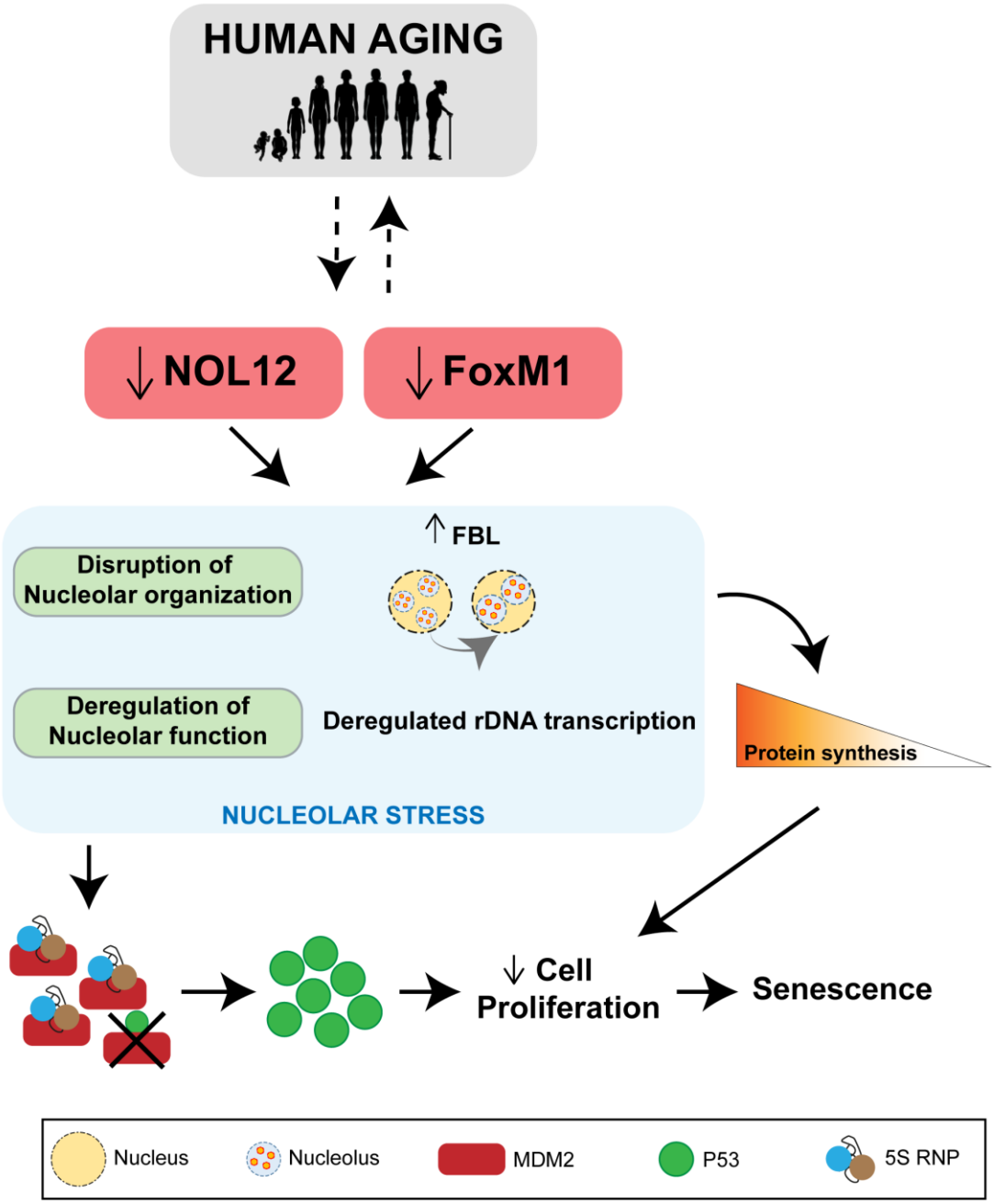

Figure 4.1. Proposed model for the impact of NOL12 and FoxM1 repressions in nucleolar homeostasis, senescence and aging. Both NOL12 or FoxM1 repressions induce nucleolar stress, particularly by increasing nucleolar size and decreasing the number of nucleoli per nucleus, along with FBL upregulation and deregulation of nucleolar function. Nucleolar stress elicts P53 activation likely mediated by the accumulation of 5S RNP bound to MDM2. Activation of P53 decreases cell proliferation ultimately leading to senescence. Additionally, both NOL12- and FoxM1-depleted cells exhibit decreased global protein synthesis which could support the reduced proliferative capacity of these cells. Two independent studies reported NOL12 and FoxM1 downregulation in elderly cells. How do they crosstalk to disorganize nucleolar morphology and function remains elusive.

CHAPTER 5

Future Perspectives

In this chapter, we propose future directions, taking our data as a starting point. Importantly, we expect that these research lines will lead to deepened understanding of basic cell biology and physiology processes, which might be explored as therapeutic targets in the context of human diseases, particularly in cancer.

As typically in science, from our interesting findings, many other questions arose as described below.

Explore the impact of FBL upregulation in the RPs pool and protein translation

A missing piece in our study was the evaluation of the composition of the ribosomal proteins (RPs) pool in the different nucleolar stress conditions (NOL12 and FoxM1 repression cellular models and fibroblasts from different age donors). This represents a relevant research topic as ribosomes are now recognized as heterogeneous entities, meaning that ribosomes have distinct RP compositions specialized on translating specific mRNA populations (Shi et al., 2017; Xue and Barna, 2012). Examples include the tissue-specific RP expression (Xue and Barna, 2012), the sensitivity of a certain mRNA population to RPL40 depletion (Lee et al., 2013) and the biased translation of mRNAs associated to stress pathways upon RPS26 depletion (Ferretti et al., 2017). Therefore, in line with this, it would be interesting to find which mRNAs are more or less abundant in response to different nucleolar stress stimuli. On the other hand, our data revealed FBL upregulation as a common event to distinct nucleolar stress conditions. Noteworthy, modulation of FBL expression was shown to bias mRNA translation towards IRES-dependent vs. CAP-dependent translation (Erales et al., 2017; Marcel et al., 2013). Therefore, future studies will be needed to investigate the contribution of FBL upregulation and changes in the RP pool for a hypothetic biased translation under different nucleolar stress conditions. This would be relevant in the context of neurodegenerative diseases, since they have been associated with nucleolar stress (Parlato and Kreiner, 2013), as well as in cancer, as cancer cells often exhibit structurally altered ribosomes driven by RP mutations (Penzo et al., 2019).

Disclose a NOL12 role in cancer development

The involvement of the nucleolus in neoplasia processes has been increasingly addressed (Stepinski, 2018). Our data unveiled NOL12 as a nucleolar protein required for nucleolar homeostasis, regulating both nucleolar structure and function. Also, previous data showed that NOL12 is distinctly dysregulated in different types of cancer (Marinho, 2011), which is in agreement with the observation that NOL12 functions either as a good or a bad prognostic marker (Uhlen et al., 2017) (Human Protein Atlas available from http://www.proteinatlas.org). On the one hand, reports have been consensual to show that cancer cells exhibit increased ribosome biogenesis to cope with increased proliferation (Penzo et al., 2019). Therefore, given its role in rRNA processing, NOL12 upregulation would be expected (Scott et al., 2017; Sloan et al., 2013b). On the other hand, it is more difficult to reconcile NOL12 downregulation and cancer development. Firstly, NOL12 repression could elicit downregulation of specific RPs triggering cell transformation and

giving support for cancer development, as previously described (Wang et al., 2010). Second, our data showed NOL12 downregulation as a pro-senescence event (Chapter 2), a feature with great importance in the cancer context (Chan and Narita, 2019). Strikingly, senescent cells are able to produce SASP, whose capacity to non-autonomously influence microenvironment contributes for chronic inflammation (Lecot et al., 2016), tumour invasion (Kim et al., 2017) and vascularization (Coppe et al., 2006), among other features that generally characterize cancers (Chan and Narita, 2019). Additionally, our data also disclosed that the roles played by NOL12 in nucleolar homeostasis, and proliferation and senescence are independent or dependent on P53, respectively. Therefore, the P53 status of a certain cancer cell could likely influence the NOL12 impact for cancer development. This is particularly relevant if we take into account that nearly half of the human cancers were reported to exhibit mutations on the *TP53* gene (Perri et al., 2016).

Altogether, these data highlight NOL12 as an interesting protein to study in the cancer context. Different cancer lines, with distinct NOL12 levels and P53 status should be investigated, to further understand its role in ribosome biogenesis and senescence, and disclose it as a potential therapeutic target.

Functional analysis of NOL12 during mitosis

The nucleolar dynamics (Caragine et al., 2019) and the association of some nucleolar proteins to the perichromosomal region during mitosis (Booth et al., 2014) indicate a nucleolar role for efficient chromosome segregation. Accordingly, nucleolar proteins as dyskerin (Alawi and Lin, 2013) and nucleolin (Ma et al., 2007; Ugrinova et al., 2007) were shown to be required for mitotic fidelity. Therefore, it would be interesting to understand the role of NOL12 during mitosis, particularly whether its repression affects chromosome segregation, being important to distinguish between acrocentric and submetacentric/metacentric chromosomes, as acrocentric chromosomes contain rDNA repeats (McStay, 2016). Noteworthy, naturally aged fibroblasts, which we found to be NOL12 downregulated (Chapter 2), exhibit higher rate of chromosome mis-segregation (Macedo et al., 2018). Additionally, the impact of NOL12 repression in the dynamics of other nucleolar proteins, such as FBL, would provide important clues about the way nucleolar structure is post-mitotically established under nucleolar stress conditions.

Search for a putative crosstalk between NF-κB signalling pathway and NOL12

As mentioned in Chapter 1, the activation of the NF-κB signalling pathway was shown to increase senescence and contribute for aging, thus representing an interesting target in the context of rejuvenation (Osorio et al., 2016). In addition, NF-κB activation was shown to enlarge the nucleolus (Chen et al., 2018), in particular upon heat stress-induced

changes in the conformation of the nucleolar NF-κB repressing factor, leading to rRNA processing impairment, due to the XRN2 mislocalization (Coccia et al., 2017). Therefore, it seems that the NF-κB signalling pathway plays a role during nucleolar stress, hence it would be interesting to explore whether and how NOL12 crosstalks with this signalling pathway. For instance, the analysis of the impact of NOL12 and FoxM1 modulations in NF-kB activation, and the use of available tools targeting NF-κB signaling pathway (Cvek and Dvorak, 2007; Garg and Aggarwal, 2002; Sethi et al., 2008), would allow to mechanistically uncover the role of NOL12 during aging and eventually also disclose a crosstalk between NF-κB signaling pathway and FoxM1.

REFERENCES

Alawi, F., Lin, P., 2013. Dyskerin localizes to the mitotic apparatus and is required for orderly mitosis in human cells. PloS one 8, e80805.

Amin, M.A., Matsunaga, S., Ma, N., Takata, H., Yokoyama, M., Uchiyama, S., Fukui, K., 2007. Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells. Biochemical and biophysical research communications 360, 320-326.

Amin, M.A., Matsunaga, S., Uchiyama, S., Fukui, K., 2008. Depletion of nucleophosmin leads to distortion of nucleolar and nuclear structures in HeLa cells. The Biochemical journal 415, 345-351.

Barsotti, A.M., Prives, C., 2009. Pro-proliferative FoxM1 is a target of p53-mediated repression. Oncogene 28, 4295-4305.

Boamah, E.K., Kotova, E., Garabedian, M., Jarnik, M., Tulin, A.V., 2012. Poly(ADP-Ribose) polymerase 1 (PARP-1) regulates ribosomal biogenesis in Drosophila nucleoli. PLoS genetics 8, e1002442.

Boisvert, F.M., van Koningsbruggen, S., Navascues, J., Lamond, A.I., 2007. The multifunctional nucleolus. Nature reviews. Molecular cell biology 8, 574-585.

Booth, D.G., Takagi, M., Sanchez-Pulido, L., Petfalski, E., Vargiu, G., Samejima, K., Imamoto, N., Ponting, C.P., Tollervey, D., Earnshaw, W.C., Vagnarelli, P., 2014. Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. eLife 3, e01641.

Buchwalter, A., Hetzer, M.W., 2017. Nucleolar expansion and elevated protein translation in premature aging. Nature communications 8, 328.

Bursac, S., Brdovcak, M.C., Pfannkuchen, M., Orsolic, I., Golomb, L., Zhu, Y., Katz, C., Daftuar, L., Grabusic, K., Vukelic, I., Filic, V., Oren, M., Prives, C., Volarevic, S., 2012. Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. Proceedings of the National Academy of Sciences of the United States of America 109, 20467-20472.

Caragine, C.M., Haley, S.C., Zidovska, A., 2019. Nucleolar dynamics and interactions with nucleoplasm in living cells. eLife 8.

Chan, A.S.L., Narita, M., 2019. Short-term gain, long-term pain: the senescence life cycle and cancer. Genes & development 33, 127-143.

Chen, J., Lobb, I.T., Morin, P., Novo, S.M., Simpson, J., Kennerknecht, K., von Kriegsheim, A., Batchelor, E.E., Oakley, F., Stark, L.A., 2018. Identification of a novel TIF-IA-NF-kappaB nucleolar stress response pathway. Nucleic acids research 46, 6188-6205.

Coccia, M., Rossi, A., Riccio, A., Trotta, E., Santoro, M.G., 2017. Human NF-kappaB repressing factor acts as a stress-regulated switch for ribosomal RNA processing and

nucleolar homeostasis surveillance. Proceedings of the National Academy of Sciences of the United States of America 114, 1045-1050.

Coppe, J.P., Kauser, K., Campisi, J., Beausejour, C.M., 2006. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. The Journal of biological chemistry 281, 29568-29574.

Cvek, B., Dvorak, Z., 2007. Targeting of nuclear factor-kappaB and proteasome by dithiocarbamate complexes with metals. Current pharmaceutical design 13, 3155-3167.

Donati, G., Peddigari, S., Mercer, C.A., Thomas, G., 2013. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. Cell reports 4, 87-98.

Erales, J., Marchand, V., Panthu, B., Gillot, S., Belin, S., Ghayad, S.E., Garcia, M., Laforets, F., Marcel, V., Baudin-Baillieu, A., Bertin, P., Coute, Y., Adrait, A., Meyer, M., Therizols, G., Yusupov, M., Namy, O., Ohlmann, T., Motorin, Y., Catez, F., Diaz, J.J., 2017. Evidence for rRNA 2'-O-methylation plasticity: Control of intrinsic translational capabilities of human ribosomes. Proceedings of the National Academy of Sciences of the United States of America 114, 12934-12939.

Ferretti, M.B., Ghalei, H., Ward, E.A., Potts, E.L., Karbstein, K., 2017. Rps26 directs mRNA-specific translation by recognition of Kozak sequence elements. Nature structural & molecular biology 24, 700-707.

Frottin, F., Schueder, F., Tiwary, S., Gupta, R., Korner, R., Schlichthaerle, T., Cox, J., Jungmann, R., Hartl, F.U., Hipp, M.S., 2019. The nucleolus functions as a phase-separated protein quality control compartment. Science 365, 342-347.

Garg, A., Aggarwal, B.B., 2002. Nuclear transcription factor-kappaB as a target for cancer drug development. Leukemia 16, 1053-1068.

Hirai, Y., Louvet, E., Oda, T., Kumeta, M., Watanabe, Y., Horigome, T., Takeyasu, K., 2013. Nucleolar scaffold protein, WDR46, determines the granular compartmental localization of nucleolin and DDX21. Genes to cells : devoted to molecular & cellular mechanisms 18, 780-797.

Holmberg Olausson, K., Nister, M., Lindstrom, M.S., 2012. p53 -Dependent and -Independent Nucleolar Stress Responses. Cells 1, 774-798.

Iadevaia, V., Caldarola, S., Biondini, L., Gismondi, A., Karlsson, S., Dianzani, I., Loreni, F., 2010. PIM1 kinase is destabilized by ribosomal stress causing inhibition of cell cycle progression. Oncogene 29, 5490-5499.

Kim, Y.H., Choi, Y.W., Lee, J., Soh, E.Y., Kim, J.H., Park, T.J., 2017. Senescent tumor cells lead the collective invasion in thyroid cancer. Nature communications 8, 15208.

Latonen, L., 2019. Phase-to-Phase With Nucleoli - Stress Responses, Protein Aggregation and Novel Roles of RNA. Frontiers in cellular neuroscience 13, 151.

Lecot, P., Alimirah, F., Desprez, P.Y., Campisi, J., Wiley, C., 2016. Context-dependent effects of cellular senescence in cancer development. British journal of cancer 114, 1180-1184.

Lee, A.S., Burdeinick-Kerr, R., Whelan, S.P., 2013. A ribosome-specialized translation initiation pathway is required for cap-dependent translation of vesicular stomatitis virus mRNAs. Proceedings of the National Academy of Sciences of the United States of America 110, 324-329.

Ma, N., Matsunaga, S., Takata, H., Ono-Maniwa, R., Uchiyama, S., Fukui, K., 2007. Nucleolin functions in nucleolus formation and chromosome congression. Journal of cell science 120, 2091-2105.

Macedo, J.C., Vaz, S., Bakker, B., Ribeiro, R., Bakker, P.L., Escandell, J.M., Ferreira, M.G., Medema, R., Foijer, F., Logarinho, E., 2018. FoxM1 repression during human aging leads to mitotic decline and aneuploidy-driven full senescence. Nature communications 9, 2834.

Marcel, V., Ghayad, S.E., Belin, S., Therizols, G., Morel, A.P., Solano-Gonzalez, E., Vendrell, J.A., Hacot, S., Mertani, H.C., Albaret, M.A., Bourdon, J.C., Jordan, L., Thompson, A., Tafer, Y., Cong, R., Bouvet, P., Saurin, J.C., Catez, F., Prats, A.C., Puisieux, A., Diaz, J.J., 2013. p53 acts as a safeguard of translational control by regulating fibrillarin and rRNA methylation in cancer. Cancer cell 24, 318-330.

Marinho, J., 2011. Molecular and cellular analysis of the function of viriato gene during drosophila development. PhD Thesis (Coimbra) 4, 128-129.

Marinho, J., Casares, F., Pereira, P.S., 2011. The Drosophila Nol12 homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth. Development 138, 349-357.

McStay, B., 2016. Nucleolar organizer regions: genomic 'dark matter' requiring illumination. Genes & development 30, 1598-1610.

Nunez Villacis, L., Wong, M.S., Ferguson, L.L., Hein, N., George, A.J., Hannan, K.M., 2018. New Roles for the Nucleolus in Health and Disease. BioEssays : news and reviews in molecular, cellular and developmental biology 40, e1700233.

Oeffinger, M., Zenklusen, D., Ferguson, A., Wei, K.E., El Hage, A., Tollervey, D., Chait, B.T., Singer, R.H., Rout, M.P., 2009. Rrp17p is a eukaryotic exonuclease required for 5' end processing of Pre-60S ribosomal RNA. Molecular cell 36, 768-781.

Osorio, F.G., Soria-Valles, C., Santiago-Fernandez, O., Freije, J.M., Lopez-Otin, C., 2016. NF-kappaB signaling as a driver of ageing. International review of cell and molecular biology 326, 133-174.

Pardee, A.B., 1989. G1 events and regulation of cell proliferation. Science 246, 603-608.

Parlato, R., Kreiner, G., 2013. Nucleolar activity in neurodegenerative diseases: a missing piece of the puzzle? Journal of molecular medicine 91, 541-547.

Penzo, M., Montanaro, L., Trere, D., Derenzini, M., 2019. The Ribosome Biogenesis-Cancer Connection. Cells 8.

Perri, F., Pisconti, S., Della Vittoria Scarpati, G., 2016. P53 mutations and cancer: a tight linkage. Annals of translational medicine 4, 522.

Pinho, M., Macedo, J.C., Logarinho, E., Pereira, P.S., 2019. NOL12 Repression Induces Nucleolar Stress-Driven Cellular Senescence and Is Associated with Normative Aging. Molecular and cellular biology 39.

Polymenis, M., Aramayo, R., 2015. Translate to divide: control of the cell cycle by protein synthesis. Microbial cell 2, 94-104.

Sadasivam, S., Duan, S., DeCaprio, J.A., 2012. The MuvB complex sequentially recruits B-Myb and FoxM1 to promote mitotic gene expression. Genes & development 26, 474-489.

Scott, D.D., Trahan, C., Zindy, P.J., Aguilar, L.C., Delubac, M.Y., Van Nostrand, E.L., Adivarahan, S., Wei, K.E., Yeo, G.W., Zenklusen, D., Oeffinger, M., 2017. Nol12 is a multifunctional RNA binding protein at the nexus of RNA and DNA metabolism. Nucleic acids research 45, 12509-12528.

Sethi, G., Sung, B., Aggarwal, B.B., 2008. Nuclear factor-kappaB activation: from bench to bedside. Experimental biology and medicine 233, 21-31.

Shi, Z., Fujii, K., Kovary, K.M., Genuth, N.R., Rost, H.L., Teruel, M.N., Barna, M., 2017. Heterogeneous Ribosomes Preferentially Translate Distinct Subpools of mRNAs Genome-wide. Molecular cell 67, 71-83 e77.

Sloan, K.E., Bohnsack, M.T., Watkins, N.J., 2013a. The 5S RNP couples p53 homeostasis to ribosome biogenesis and nucleolar stress. Cell reports 5, 237-247.

Sloan, K.E., Mattijssen, S., Lebaron, S., Tollervey, D., Pruijn, G.J., Watkins, N.J., 2013b. Both endonucleolytic and exonucleolytic cleavage mediate ITS1 removal during human ribosomal RNA processing. The Journal of cell biology 200, 577-588.

Stepinski, D., 2018. The nucleolus, an ally, and an enemy of cancer cells. Histochemistry and cell biology 150, 607-629.

Suzuki, S., Kanno, M., Fujiwara, T., Sugiyama, H., Yokoyama, A., Takahashi, H., Tanaka, J., 2006. Molecular cloning and characterization of Nop25, a novel nucleolar RNA binding protein, highly conserved in vertebrate species. Experimental cell research 312, 1031-1041.

Thiry, M., Lafontaine, D.L., 2005. Birth of a nucleolus: the evolution of nucleolar compartments. Trends in cell biology 15, 194-199.

Tiku, V., Antebi, A., 2018. Nucleolar Function in Lifespan Regulation. Trends in cell biology 28, 662-672.

Tiku, V., Jain, C., Raz, Y., Nakamura, S., Heestand, B., Liu, W., Spath, M., Suchiman, H.E.D., Muller, R.U., Slagboom, P.E., Partridge, L., Antebi, A., 2017. Small nucleoli are a cellular hallmark of longevity. Nature communications 8, 16083.

Ugrinova, I., Monier, K., Ivaldi, C., Thiry, M., Storck, S., Mongelard, F., Bouvet, P., 2007. Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. BMC molecular biology 8, 66.

Uhlen, M., Zhang, C., Lee, S., Sjostedt, E., Fagerberg, L., Bidkhori, G., Benfeitas, R., Arif, M., Liu, Z., Edfors, F., Sanli, K., von Feilitzen, K., Oksvold, P., Lundberg, E., Hober, S., Nilsson, P., Mattsson, J., Schwenk, J.M., Brunnstrom, H., Glimelius, B., Sjoblom, T., Edqvist, P.H., Djureinovic, D., Micke, P., Lindskog, C., Mardinoglu, A., Ponten, F., 2017. A pathology atlas of the human cancer transcriptome. Science 357.

Wang, M., Pestov, D.G., 2011. 5'-end surveillance by Xrn2 acts as a shared mechanism for mammalian pre-rRNA maturation and decay. Nucleic acids research 39, 1811-1822.

Wang, S., Huang, J., He, J., Wang, A., Xu, S., Huang, S.F., Xiao, S., 2010. RPL41, a small ribosomal peptide deregulated in tumors, is essential for mitosis and centrosome integrity. Neoplasia 12, 284-293.

Xue, S., Barna, M., 2012. Specialized ribosomes: a new frontier in gene regulation and organismal biology. Nature reviews. Molecular cell biology 13, 355-369.

Yang, K., Yang, J., Yi, J., 2018. Nucleolar Stress: hallmarks, sensing mechanism and diseases. Cell stress 2, 125-140.

Yi, Y.H., Ma, T.H., Lee, L.W., Chiou, P.T., Chen, P.H., Lee, C.M., Chu, Y.D., Yu, H., Hsiung, K.C., Tsai, Y.T., Lee, C.C., Chang, Y.S., Chan, S.P., Tan, B.C., Lo, S.J., 2015. A Genetic Cascade of let-7-ncl-1-fib-1 Modulates Nucleolar Size and rRNA Pool in Caenorhabditis elegans. PLoS genetics 11, e1005580.

Zhou, X., Liao, W.J., Liao, J.M., Liao, P., Lu, H., 2015. Ribosomal proteins: functions beyond the ribosome. Journal of molecular cell biology 7, 92-104.

Zhu, L., Richardson, T.M., Wacheul, L., Wei, M.T., Feric, M., Whitney, G., Lafontaine, D.L.J., Brangwynne, C.P., 2019. Controlling the material properties and rRNA processing function of the nucleolus using light. Proceedings of the National Academy of Sciences of the United States of America 116, 17330-17335.