

The Nucleolus and Ribosomal Genes in Aging and Senescence

Nadine Hein^{1,*}, Elaine Sanij^{1,2,*},
Jaclyn Quin^{1,3}, Katherine M. Hannan¹,

Austen Ganley^{4,#} and Ross D. Hannan^{1,3,5,6,#}

¹*Division of Cancer Research, Peter MacCallum Cancer Centre, Melbourne,*

²*Department of Pathology, University of Melbourne, Melbourne,*

³*Department of Biochemistry and Molecular Biology,
University of Melbourne, Melbourne,*

⁴*Institute of Natural Sciences, Massey University, Auckland,*

⁵*Department of Biochemistry and Molecular Biology,
Monash University, Melbourne,*

⁶*School of Biomedical Sciences, The University of Queensland, Melbourne,
^{1,2,3,5,6}Australia*

⁴*New Zealand*

1. Introduction

The nucleolus forms around the tandem repeats of the ribosomal RNA (rRNA) genes (rDNA) that are transcribed by RNA polymerase I (Pol I), giving rise to the production of rRNAs. These represent the nucleic acid backbone of the functional ribosomes in the cytoplasm, and as such rDNA transcription dictates the cells' protein translational capacity. More recently it has become apparent that the epigenetic status of these rDNA repeats and the integrity of the nucleolus can modulate cellular homeostasis beyond ribosome biogenesis. Such roles include mediating the titration of tumor suppressors and oncogenes, modulating the heterochromatic state of many RNA Polymerase II (Pol II) transcribed genes, and importantly, regulating the process of aging and senescence. This chapter will focus on the molecular and cellular evidence that the nucleolus and the rDNA repeats play critical roles in the control of aging and cellular senescence in yeast and mammals.

2. Introduction to rDNA transcription and the nucleolus

This section will provide a brief overview of the regulation of rDNA transcription, however, for more details refer to (Tschochner & Hurt, 2003; McStay & Grummt, 2008).

* These authors contributed equally to this work.

Corresponding Authors

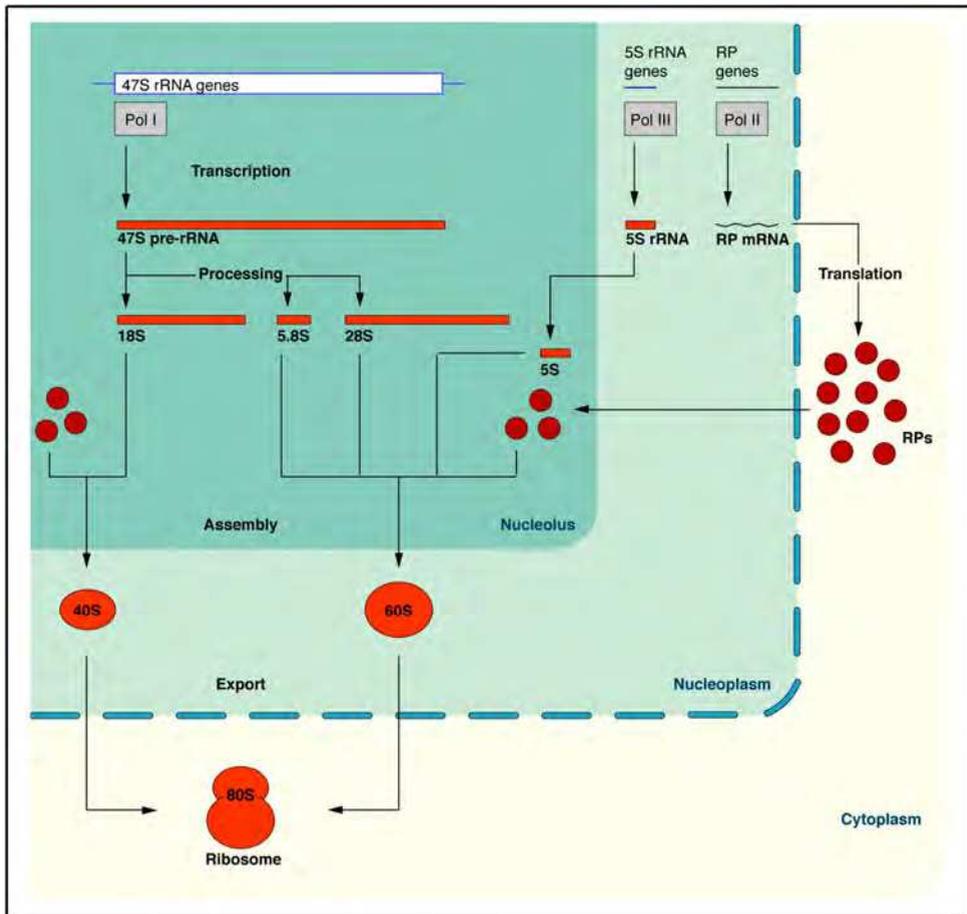


Fig. 1. Overview of ribosome biogenesis in mammalian cells.

2.1 Ribosome biogenesis

Ribosome biogenesis dictates the capacity of a cell to grow and proliferate, and is one of the most energy consuming processes in eukaryotic cells (Grummt & Pikaard, 2003). The synthesis of a ribosome is a highly complex, yet exquisitely coordinated process, which utilizes all three DNA-dependent RNA polymerases (Pol I, Pol II and Pol III) to produce approximately equimolar amounts of numerous ribosomal proteins (RP) and four rRNA (Fig. 1). Transcription of the Pol I-transcribed rRNA genes (the rDNA) has traditionally been considered the major rate-limiting step in ribosome biogenesis. Consistent with this dogma, any perturbations in the cellular environment, such as nutrient withdrawal, altered growth factor signaling, cell cycle cues and stress, are directly accompanied by modulation of the rate of rDNA transcription. However, in addition to the two key components of the ribosome (rRNAs and RPs) a multitude of non-ribosomal proteins and non-coding RNAs have been identified as essential for various steps in the generation of new ribosomes. These

steps not only include Pol I transcription of the pre-rRNA precursor but its subsequent processing and modifications, the nuclear import of RP's, and the final assembly of the large and small ribosomal subunits followed by their export to the cytoplasm (Grummt & Pikaard, 2003; Moss et al., 2007). The process of ribosome biogenesis is fundamental to cellular life and consequently is highly conserved, and this is illustrated by the numerous similarities between yeast and mammals.

In budding yeast (*Saccharomyces cerevisiae*) and mammals the rRNA gene is transcribed exclusively by Pol I in the nucleolus. While in yeast this generates a 35S rRNA precursor which is processed into the mature 18S, 5.8S and 26S rRNAs (Fig. 2a), in mammals a 47S rRNA precursor is generated and processed to give 18S, 5.8S and 28S rRNAs (Fig. 2b). There are also numerous similarities in the other component of the ribosome, the RPs, which in both cases are transcribed by Pol II in the nucleoplasm. In growing yeast it has been established that ~40 nascent ribosomes leave the nucleolus every second, 80% of the total RNA is rRNA, and ~50% of total protein consists of RPs (Tschochner & Hurt, 2003). Overall the process of ribosome biogenesis consumes between 60-80% of the cells total energy both in yeast and mammals (Moss & Stefanovsky, 2002). Thus for both systems, even minor perturbations to ribosome biogenesis are likely to have major repercussions for the cell.

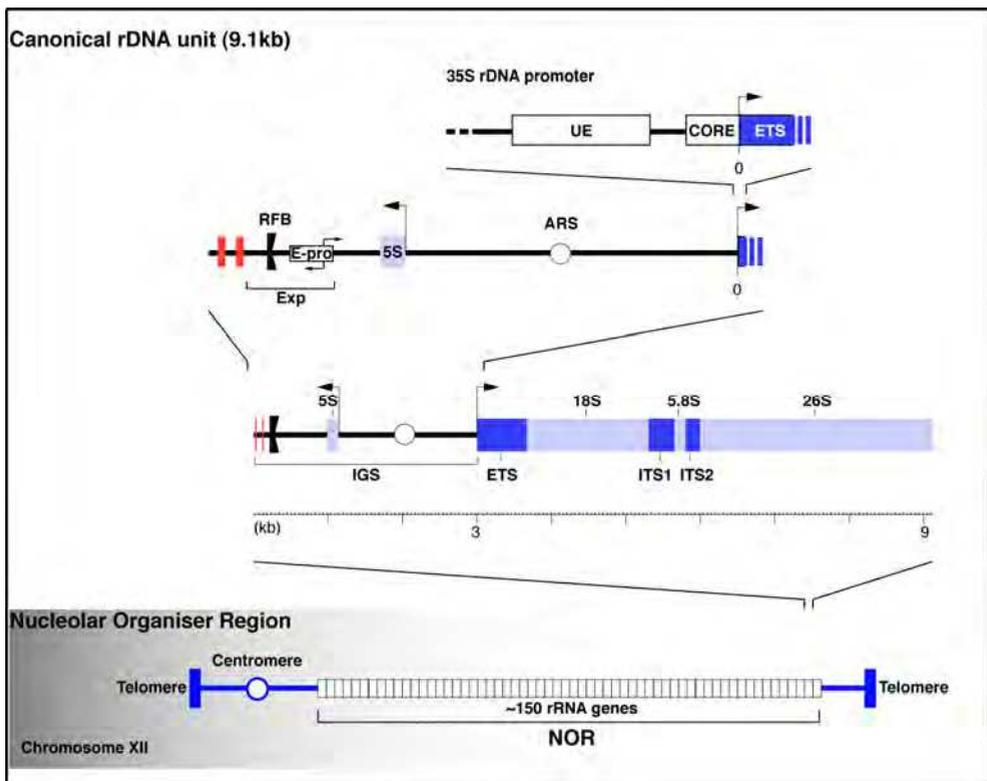


Fig. 2a. Organization of ribosomal RNA genes in yeast.

2.2 Organization of eukaryotic ribosomal RNA genes

The number of rDNA units per cell varies among eukaryotes, from 40 to ~19,000 in animals and from 150 to 26,000 in plants and correlates positively with genome size (Richard et al., 2008). In human cells there are up to 200 hundred copies of the ribosomal genes per haploid genome which are arranged in a head to tail orientation in clusters of tandem repeats. In yeast a single cluster consisting of ~150 copies of rRNA genes termed the nucleolar organizer region (NOR) is located on chromosome XII (Fig. 2a) and comprises over 10% of the whole genome (Kobayashi, 2011). During interphase a single nucleolus forms around this cluster. Two transcriptional regulatory DNA elements have been identified (Kulkens et al., 1991; Musters et al., 1989): the upstream element (UE), which is the binding site for the upstream activating factor (UAF); and the core promoter (CORE), which recruits the core factor (CF) (Elion & Warner, 1984). In addition to these regulatory DNA elements several non-coding regions have been identified within the gene and the IGS, including an origin of replication, called the ribosomal autonomous replicating sequence (ARS), and an expansion sequence containing a replication fork barrier (RFB) plus a bidirectional promoter (E-pro) that is required for rDNA amplification (Fig. 2a) (Brewer et al., 1992; Kobayashi et al., 1992).

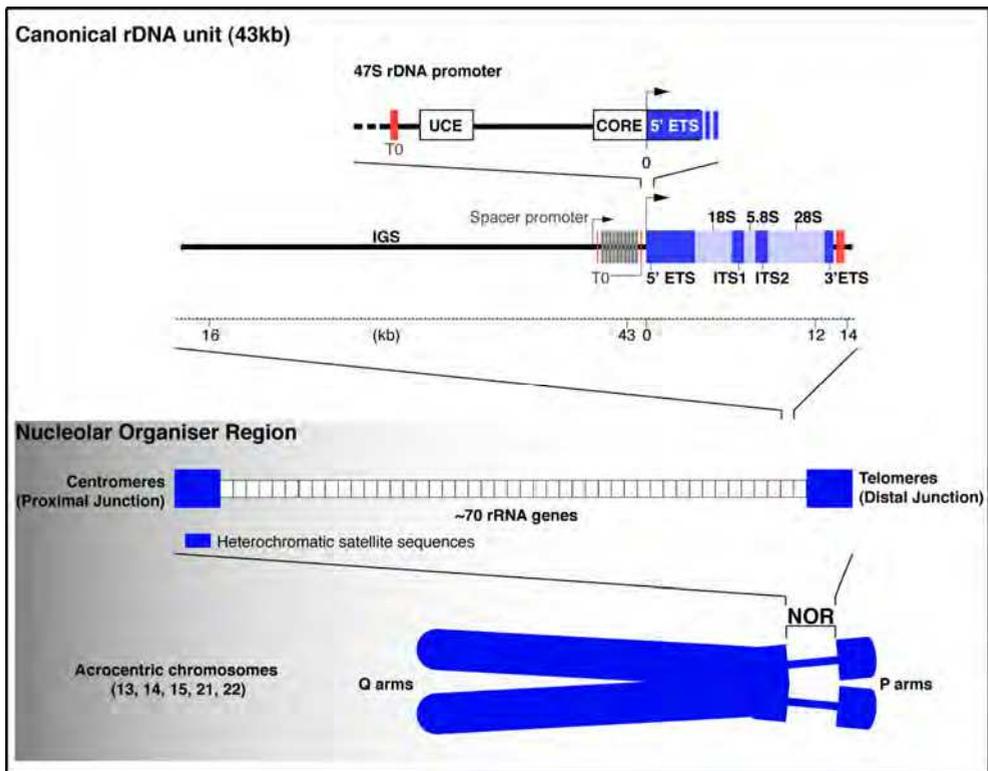


Fig. 2b. Organization of ribosomal RNA genes in mammalian cells.

In contrast to yeast, the rDNA repeats of higher eukaryotes are located in multiple NORs (Fig. 2b). For example, humans have five NORs located on the short arms of the acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) that contain ~70 copies of the rRNA genes (Sakai et al., 1995). During interphase two or more NORs coalesce to form multiple nucleoli in exponentially growing cells (McStay & Grummt, 2008).

A single rDNA unit, containing a transcribed region followed by intergenic spacer (IGS) is referred to as a canonical rDNA unit. However, non-canonical units, which form a palindromic structure are present and arranged as a mosaic with the canonical units in human NORs (Caburet et al., 2005). These non-canonical units are likely to be non-functional and it is believed that they are silenced since their transcription would result in antisense transcripts that could use the RNAi machinery to degrade the 47S precursor rRNA. However, this has not been formally tested.

The rDNA repeats were not sequenced during the human genome project due to their repetitive nature and high copy number, thus the full sequence of only two repeats have been reported (Kuo et al., 1996). A canonical human rDNA repeat (43 kb) consists of a 13 kb coding region that encodes the 47S pre-rRNA, which is rapidly processed at the external transcribed spacer (ETS) and internal transcribed spacer (ITS) regions and ~30 kb of IGS (Fig. 2b). The IGS harbors several DNA regulatory elements, including the 47S rDNA promoter. The rDNA promoter has a bipartite architecture composed of a CORE and an upstream control element (UCE) located 100 bp upstream (McStay & Grummt, 2008). One or more terminator elements are located at the 5' and 3' ends of each mammalian rDNA repeat. Intriguingly, the IGS also contains one or more regions that are almost identical to the 47S rDNA promoter, and these are termed spacer promoters. Recent studies demonstrated that non-coding IGS transcripts play a role in the epigenetic control of rRNA gene silencing through modulation of the activity of the nucleolar remodeling complex (NoRC), the rDNA silencing complex (Mayer et al., 2006) (see below for more detail).

2.3 Epigenetic regulation of Pol I transcription

It is well established that even in exponentially growing cells only a subset of rRNA genes are active. In yeast, active and inactive rRNA genes are randomly distributed in the single NOR (French et al., 2003). However in higher eukaryotes it is thought that active and silent rRNA genes are clustered, thus generating active and silent NORs. Silent NORs are condensed and do not contribute to the formation of nucleoli during interphase. It is likely that active NORs may contain a mosaic of active and inactive units however this remains a matter for discussion (McStay & Grummt, 2008).

2.3.1 Epigenetic silencing of rDNA

While the precise mechanism controlling the silencing of rRNA genes is yet to be fully elucidated, there appears to be a marked difference between yeast and higher organisms in this process. For example, *S. cerevisiae* lacks the two major repressive methylation marks (CpG dinucleotide and H3K9 methylation) that are associated with the silencing of higher eukaryotic genes. Indeed, little is known about what regulates the number of active/inactive repeat units in yeast, although the histone deacetylase, Rpd3, is thought to play a role (Sandmeier et al, 2003), and TOR signaling regulates Pol I transcription and alters nucleolar

compaction (Tsang et al, 2007). Instead, the majority of studies looking at rDNA silencing in yeast have looked at the silencing of Pol II genes in the rDNA, and it is this form of silencing that is most strongly linked to senescence and aging, particularly through the Pol II-dependent E-pro promoter in the rDNA (Kobayashi & Ganley, 2005).

Silencing of E-pro is regulated by Sir2p, a member of the Sirtuin family of NAD⁺ dependent protein deacetylases. Sir2p also regulates the silencing of the telomeres and the mating loci (Guarente, 1999). The Sir2p analog in mammals SIRT1 is a key component of the energy-dependent nucleolar silencing complex (eNoSC), which has been reported to repress rRNA gene transcription in response to altered intracellular energy status (Murayama et al., 2008). Sir2p together with Net1 and Cdc14 are part of a well-known epigenetic regulator of the yeast rDNA locus, the regulator of nucleolar silencing and telophase exit (RENT) complex (Huang & Moazed, 2003). In addition, Sir2p in a complex with accessory proteins such as condensin or cohesin can repress recombination events within the rDNA repeats (Huang et al., 2006; Machin et al., 2004). More recently it has become clear that the stability of the rDNA repeats and their accurate replication depends on the proportion of the epigenetically silenced rRNA genes (Kobayashi, 2011).

In higher eukaryotes methylation of CpG dinucleotides is a common modification associated with establishing stable transcriptional repression. This covalent modification is catalyzed by the DNA methyltransferases DNMT1, DNMT3a and DNMT3b, and is maintained during cell division (Klose & Bird, 2006). Stanchev et al (1979) demonstrated that CpG dinucleotide methylation marks are predominantly present in the promoter and enhancer regions of inactive rRNA genes (Fig. 3). In murine cells, methylation of a single CpG dinucleotide within the UCE (position -133 relative to the start of transcription) impairs the association of the Pol I transcription factor, upstream binding factor (UBF), to the rDNA and thus inhibits the assembly of the preinitiation complex (PIC) at the promoter (McStay & Grummt, 2008). In contrast, the human rDNA promoter contains ~25 CpG islands none of which are completely methylated or non-methylated. This suggests that the overall level of methylation rather than a binary on/off switch, dictates the transcriptional status of the rDNA. NoRC is the major complex involved in CpG methylation silencing of rDNA repeats (Santoro et al., 2002) (Fig. 3). The evolutionary logic underlying the additional complexity of rDNA silencing in higher eukaryotes compared to yeast is not clear but it potentially relates to regulation of cell differentiation and multicellular development.

2.3.2 Active rDNA repeats and activation of Pol I transcription

Regulation of rDNA transcription can occur at multiple levels, through regulatory elements defined by the primary DNA sequence as described above and also via the structure of the chromatin, which determines the accessibility of the DNA. Similar to regulation of Pol II and Pol III transcription, post-translational modification of the histones, such as acetylation, methylation, phosphorylation and ubiquitination, represent a key mechanism for the regulation of transcription by Pol I of active rDNA (Fig. 3).

In yeast it has been shown that the chromatin of actively transcribed rRNA genes is largely devoid of histone molecules, and instead is associated with the high-mobility group protein Hmo1, which interacts with the Pol I subunit Rpa49, binds across the entire 35S rDNA sequence and stabilize open rRNA gene chromatin (Hall et al., 2006; Merz et al., 2008).

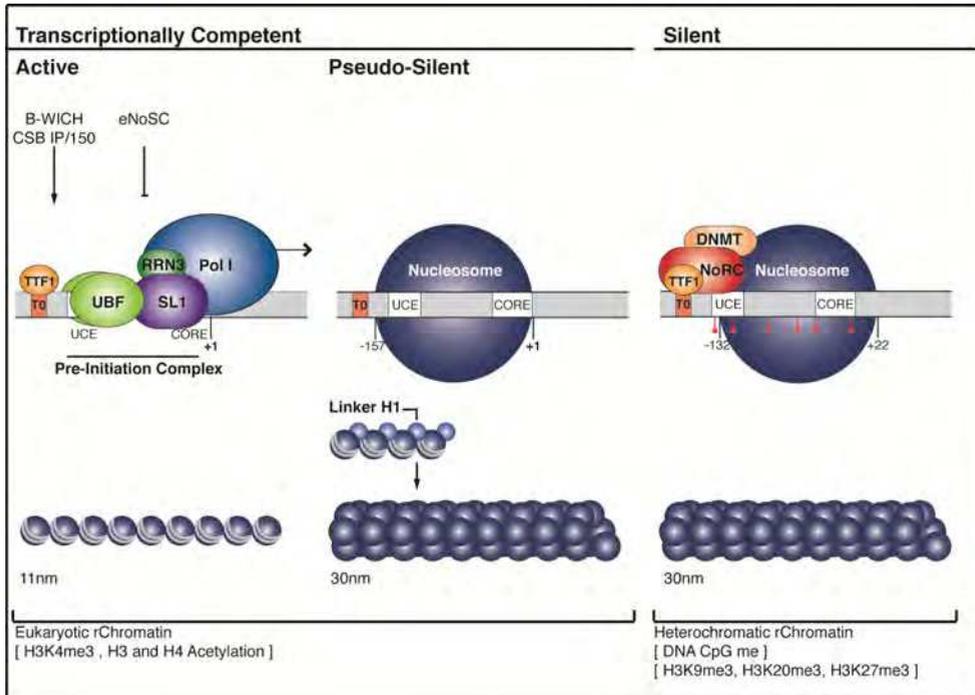


Fig. 3. Regulation of Pol I transcription in mammalian cells.

In mammals transcriptionally active rRNA genes lack repressive histone modifications such as H3K9, H3K20 and H3K27 methylation and CpG DNA methylation (Conconi et al., 1989). Furthermore, they are associated with markers for active genes including H3K4 methylation and acetylation of histone H3 and H4 (Fig. 3). Importantly, transcriptionally active mammalian rDNA are characterized by the presence of UBF, which is enriched at the promoter and the transcribed regions of the repeat, and to a lesser extent at the IGS (Fig. 3) (Sanij et al., 2008; Wright et al., 2006). UBF seems to play multiple roles at the rDNA including transcriptional initiation, promoter escape and elongation control (Stefanovsky et al., 2006). Most likely these functions relate to the essential role UBF plays in maintaining active genes in an open, uncondensed configuration, which is achieved, in part, through the ability of UBF to outcompete histone linker H1, thus preventing the formation of higher order chromatin (Sanij & Hannan, 2009; Sanij et al., 2008). Of note, active rRNA genes are around tenfold less condensed than adjacent DNA and remain uncondensed during mitosis (Heliot et al., 1997). This is undoubtedly due to the continual association of UBF and a subset of the Pol I transcription machinery with the rDNA repeats, which maintains them in an under-condensed configuration to allow the rapid resumption of rDNA transcription as cells re-enter the cell cycle (Prieto & McStay, 2007; Roussel et al., 1996).

One complex that has been described to promote the formation of an active chromatin environment for Pol I transcription is the chromatin remodeling complex B-WICH, which is

composed of the William syndrome transcription factor (WSTF), SNF2h and nuclear myosin (NM1) has been described to promote the formation of an active chromatin environment for Pol I transcription (Vintermist et al., 2011) (Fig. 3). The remodeling activity of the B-WICH complex is restricted to a specific 200 bp region around the promoter, which includes the UCE, CORE and transcriptional start site (Vintermist et al., 2011). An ATP-dependent chromatin remodeling complex (CSB IP/150) also promotes transcription of active rRNA genes. CSB IP/150 consists of the Crockayne syndrome protein B (CSB), TFIIF and TIF1B (Bradsher et al., 2002) (Fig. 3).

A key similarity between yeast and mammals is that the rate of rRNA transcription is regulated in response to stress signals and the availability of nutrients as sensed by the TOR pathway. In mammals the Pol I transcription factors, UBF and Pol I-specific transcription initiation factor 1A (TIF-1A)/RRN3 (Hannan et al., 2003; Mayer et al., 2004) have been reported to be activated by TOR kinase. Similar findings have been made in yeast for Hmo1 and Rrn3p. Specifically binding of Hmo1 to the rDNA is TORC1 dependent, and nutrient starvation or rapamycin (inhibitor of mTOR) treatment prevents this association (Berger et al., 2007). In the absence of Hmo1 the histone H4 deacetylase, Rpd3, can associate with the rDNA, resulting in rDNA condensation and a reduction of nucleolar size (Tsang et al., 2003).

The basal Pol I transcription machinery in yeast involves two multiprotein complexes, the UAF consisting of Rrn5, Rrn9, and Rrn10 and UAF30 (Keys et al., 1996; Siddiqi et al., 2001) and the CF, consisting of Rrn7p, Rrn11p, and Rrn6p (Steffan et al., 1996). Both complexes interact with the TATA-box-binding protein TBP. Binding of UAF to the promoter is essential for the recruitment of CF, once the UAF-CF is established active Pol I is recruited to initiate transcription. Initiation in yeast and mammals requires the essential Pol I-associated factor Rrn3p. Rrn3p is only found associated with a small fraction of Pol I, and in yeast this association requires Pol I phosphorylation. Upon initiation of transcription Pol I enters into the elongation phase of transcription and Rrn3p is released.

As with yeast, in mammals the initiation of transcription of active rRNA genes requires the assembly of a PIC at the promoter, although some of the components are species specific (Grummt, 2003; Moss et al., 2007). In the mammalian system the PIC (Fig. 3) contains the selectivity factor I (SL1), a complex itself of 4 or more TATA association factors (TAFs) unique to Pol I transcription plus the TBP that is utilized by all three Pol's (Learned et al., 1985; Zomerdijk et al., 1994). Our current understanding is that SL1 is recruited to the promoter by UBF, and consequently stabilizes UBF interaction with the rDNA promoter. Upon formation of a stable UBF/SL1 complex, active Pol I (defined by its association with RRN3 (Hempel et al., 1996; Yuan et al., 2002) is then recruited to complete the PIC. Following Pol I transcription mediated initiation and promoter clearance, RRN3 is thought to be released to be recycled for another round of transcription. Various steps in transcription including initiation or elongation, are also regulated in response to extracellular signals such as nutrients, amino acids, ATP, stress, which is mediated by signaling pathways, including the PI3K/AKT/mTOR, RAS/RAF/ERK and JNK pathways (Chan et al., 2011; Hannan et al., 2003; Mayer et al., 2005; Stefanovsky et al., 2001). More recently the transcription termination factor (TTF1), which was originally identified for its role in the termination of Pol I transcription, has been implicated in modulating DNA looping at the rDNA repeat thus facilitating a

specific interaction between the promoter and terminator elements of actively transcribed rDNA repeats (Sander & Grummt, 1997). Thus by creating an rDNA loop, TTF1 is thought to promote efficient re-initiation of the Pol I complex at the rDNA promoter (Grummt et al., 1985; Henderson & Sollner-Webb, 1986).

2.3.3 Pseudo-silenced rDNA repeats in higher eukaryotes

Interestingly, in mammals not all the transcriptionally active rRNA genes of interphasic nucleoli are transcribed at any one time (Sanij & Hannan, 2009). Transcriptionally competent genes can be subdivided into two categories; active genes and pseudo-silent genes (Fig. 3). Active genes are undermethylated, bound by the cytoarchitectural chromatin remodeling factor UBF and are highly transcribed, whereas pseudo-silent rRNA genes are undermethylated and bound by linker-histone H1, but not by UBF, and thus are not transcriptionally active. This pseudo-silenced conformation of rDNA repeats, when induced by RNA interference mediated knock down of UBF, is stably propagated throughout the cell cycle of many generations in the absence of changes in CpG methylation and can be reversed by restoration of UBF to wild-type level (Sanij et al., 2008). Importantly, pseudo-silencing seems to be a physiologically relevant phenomenon. For example, terminal differentiation of various cell types is associated with decreased UBF expression and a concomitant increase in the number of pseudo-silent rRNA genes (Poortinga et al., 2004; Poortinga et al., 2011; Sanij & Hannan, 2009). Moreover, the transition from a pre-malignant to malignant state is also associated with a decrease in the proportion of pseudo-silenced rRNA genes (Hannan RD and Bywater M, unpublished observation).

3. rDNA stability and aging in yeast

S. cerevisiae and its unique genetic and biochemical attributes have proven to be an outstanding model organism to analyze many aspects of eukaryotic ribosome biogenesis. This is evident as much of our current understanding of the link between the nucleolus/rDNA transcription with aging and senescence comes from studies utilizing the experimental advantages of budding yeast.

3.1 Maintenance of rDNA copy number in yeast

The highly repetitive nature of eukaryotic rDNA makes it one of the most fragile and dynamic regions of the genome, as recombination events within these repeats can cause either loss or gain of rDNA copies. Typically, such recombination events are highly regulated and are essential for maintenance of the rDNA copy number and the evolutionary stability of the rDNA repeats (Hawley & Marcus, 1989). Two mechanisms have been shown to be utilized for the repair of DNA double strand breaks (DSB) in the rDNA: homologous recombination (HR) and the single strand annealing (SSA) (Fishman-Lobell et al., 1992). Both these repair pathways can cause loss of rDNA copies, however the HR pathway can also result in a gain of copies. Despite the fluctuation resulting from the loss or gain of rDNA copies, an rDNA maintenance system provides a mechanism for the cell to keep copy number at a uniform level and ensure genomic stability (Fig. 4) (Kobayashi, 2006). Part of this maintenance system utilizes amplification of the rDNA, which rectifies the loss of rDNA copies (Kobayashi et al., 2004). Interestingly two of the

key players in the regulation of rDNA copy number are also known as “aging associated genes”, Fob1p and Sir2p (Fig. 4).

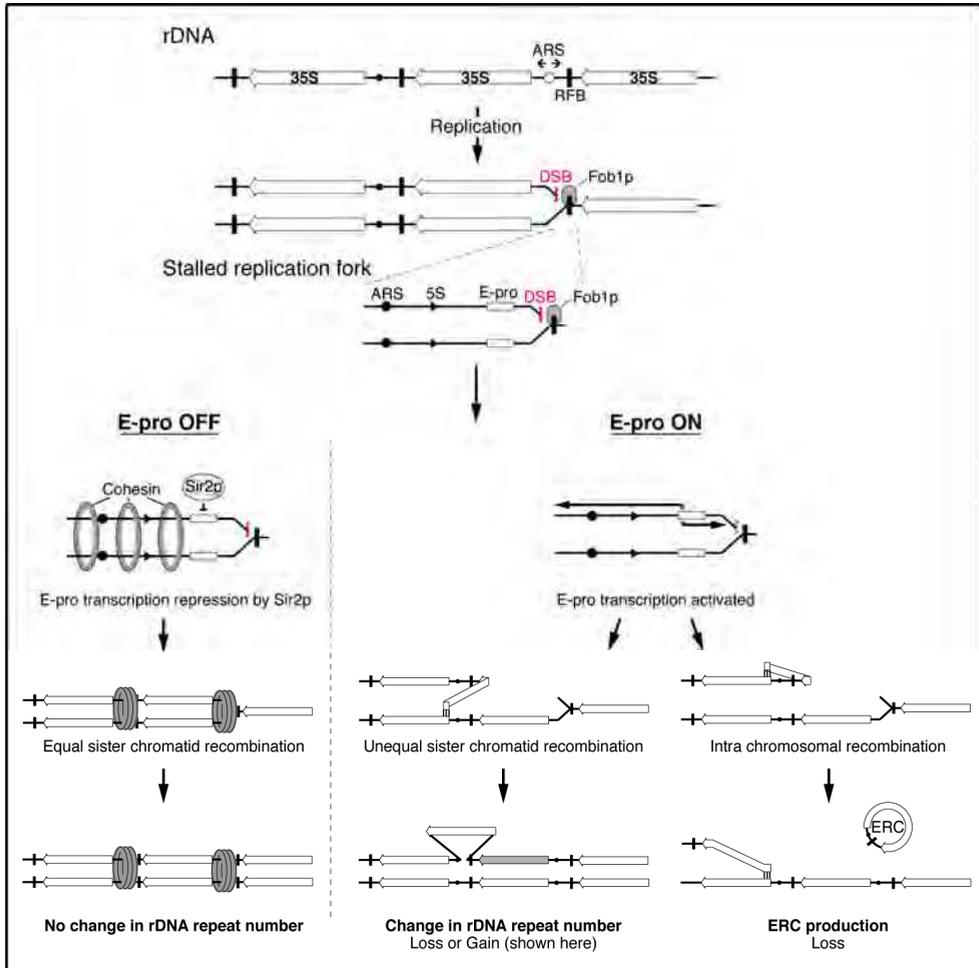


Fig. 4. Maintenance of rDNA copy number in yeast. Following initiation of replication from an ARS, Fob1p binding at the RFB inhibits replication fork progression. The outcome of the repair of these DSBs is dependent on the copy number of the rDNA. “E-pro OFF” illustrates cells containing wild-type copy number with no change in rDNA copy number. “E-pro ON” illustrates cells where rDNA copy number is altered, resulting in the production of an ERC.

During S-phase, initiation of DNA replication occurs at the origins of a subset of rDNA repeats (Pasero et al., 2002). The protein Fob1p binds in a sequence-specific manner to the rDNA at the RFB site, stalling the replication fork in one direction (Kobayashi, 2003). DNA

DSB can occur at these paused replication forks, and they are repaired using HR. The outcome of this repair is dependent on the copy number of the rDNA (Kobayashi & Ganley, 2005). In cells containing wild-type copy number, Sir2p represses noncoding Pol II-dependent transcription at the bidirectional promoter located in the IGS (Fig. 4: E-pro OFF), enabling the association of cohesin with the IGS. Cohesin is a chromosome-associated multisubunit complex that connects sister chromatids and plays an essential role in the correct segregation of chromosomes during cell division and post-replicative DNA repair (Merkenschlager, 2010). Cohesin complex association with the IGS prevents the broken end from using a non-cognate repeat as the template for HR, thereby ensuring repair through equal sister chromatid recombination, with no rDNA copy number change. If the number of rDNA copies is reduced, however, a transcription-dependent rDNA amplification mechanism is activated whereby Sir2p repression is lifted, thus activating bidirectional E-pro transcription (Fig. 4: E-pro ON). This non-coding transcription promotes the dissociation of cohesin from the IGS, allowing the broken end to use an unequal repeat as the repair template, resulting in a change of copy number. Copy number can either increase or decrease depending on whether the repeat used as the template for repair is upstream or downstream of the broken repeat (Ganley et al., 2005; Kobayashi & Ganley, 2005; Santangelo et al., 1988). If the template for repair is the same sister chromatid, a circular pop-out molecule, called an extra-chromosomal ribosomal circle (ERC) is formed. When rDNA copy number reaches wild-type levels E-pro transcription is silenced by Sir2p again, and rDNA amplification is inhibited. Sir2p mutant yeast cells can accumulate up to 300 copies due to non-restricted rDNA amplification (Kobayashi et al., 2004). Fob1p also mediates recombination events that are important for sequence homogenization of rDNA repeats and thus maintenance of the rRNA genes with identical or similar sequences (Ganley & Kobayashi, 2007). Whilst the mechanism by which cells monitor their rDNA copy number remains to be determined, it is clear that the maintenance of numerous copies of the rDNA is very important for genomic stability.

3.2 Silenced rDNA copies and DNA damage

A recent landmark study (Ide et al., 2010) demonstrated that the number of silenced rDNA copies determines the cells sensitivity to DNA damage inducing agents such as ultraviolet (UV) radiation and methyl methanesulfonate (MMS) (Fig. 5). By using low-rDNA copy number strains (20 copies), the ratio of actively transcribed rRNA genes increased, and these strains were deficient in their ability to repair DNA damage during S-phase. Low copy number strains mutated in the Pol I subunit Rpa135p or Rrn3p were not impaired in their DNA repair capacity and consequently do not exhibit a higher sensitivity to DNA damaging agents. These findings suggest that rDNA transcription determines the sensitivity to DNA damage by inhibiting DNA repair (Ide et al., 2010). The authors also reported that this transcription-dependent sensitivity resulted from the inability of the multi subunit complex condensin, that is important for establishing and maintaining chromosome condensation, to associate with actively-transcribing rDNA units. This results in premature sister-chromatid separation, which impairs accurate sister-chromatid recombination required for DNA repair. Strikingly the major site of condensin complex occupation in the genome is the NOR. Consistent with this, the binding of mitotic condensin to the rDNA was shown to be reduced when Pol I transcription was elevated, and this impaired proper DNA repair and chromatid cohesion, thus resulting in increased rDNA instability (Wang et al., 2006).

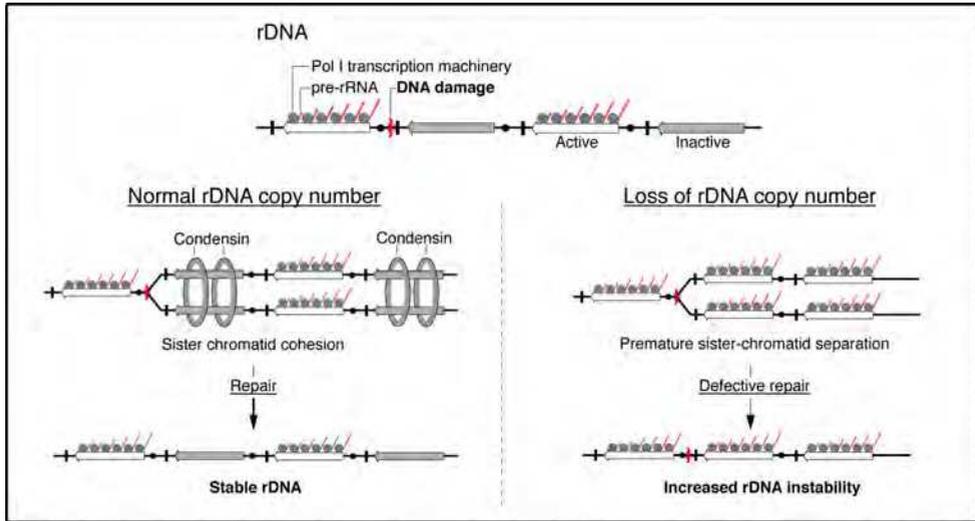


Fig. 5. Silenced rDNA copies and DNA damage in yeast. In cells with normal rDNA copy number, a proportion of genes are transcriptionally silent. Following DNA damage, condensin binds to untranscribed rDNA copies and facilitates sister chromatid cohesion, allowing repair of the rDNA by HR between sister chromatids at the stalled replication fork. Loss of rDNA copy number results in increased rates of transcription from the remaining rDNA repeats. Pol I transcription hinders the association of condensin with the rDNA, resulting in premature sister chromatid separation and preventing repair of the rDNA.

3.3 Aging in budding yeast

Aging can generically be defined as a progressive functional decline, or a gradual deterioration of physiological function and loss of viability (Partridge & Mangel, 1999). In *S. cerevisiae* an aged phenotype becomes apparent when the mother cell gets beyond ~10 asymmetric cell divisions, and this includes enlargement of the cell and vacuole, extension of the cell division cycle, and sterility. Furthermore aged wild-type cells display an extremely enlarged and often fragmented nucleolus (Shore, 1998). The average lifespan of wild-type yeast is ~ 20 buddings, after which time the mother cell dies (Jazwinski, 2001). In contrast, daughter cells are born with a full budding capacity independent of the age of the mother cell. Studies in the late 90's implicated a role for the nucleolus in yeast aging (reviewed in Guarente, 1997). As mentioned above Sirtuins are a protein family of NAD⁺ dependent protein deacetylases, and they are linked to the process of yeast aging by acting as silencing factors at a site termed the *AGE* locus, prolonging life span. It has been shown that Sir3p relocates to the nucleolus with age and that deletion of Sir2, 3 and 4 can abbreviate yeast life span (Kennedy et al., 1997). Later studies revealed that Sir2p could repress recombination in the rDNA locus, maintain rDNA stability and promote yeast longevity (Kaerberlein et al., 1999), suggesting a role for rDNA stability in the regulation of life span. Intriguingly, extra copies of Sir2 orthologs are capable of extending the lifespan of both worms and flies (Bauer et al., 2009; Tissenbaum & Guarente, 2001), suggesting an evolutionary role for Sir2 in regulation of longevity. As discussed earlier in section 2, the

mammalian Sir2p orthologue, SIRT1 is part of the eNoSC complex, which mediates epigenetic silencing of rDNA in response to varying intracellular energy status (Murayama et al., 2008). It has been proposed that the SIRT1-eNoSC complex and epigenetic regulation of rDNA may provide a novel regulatory pathway for mammalian aging, which is associated with lower metabolic rates (Salminen & Kaarniranta, 2009).

There is a clear prediction for aging factors in *S. cerevisiae*, as cell division is asymmetrical and the daughter cell receives a full lifespan, thus any aging factor must be preferentially sequestered in the mother cell and not passed on to the daughter. Indeed, Sinclair & Guarente (1997) demonstrated that aging wild-type yeast accumulated ERC, and these accumulated exclusively in mother cells. ERC accumulated even more rapidly in mutants (*sgs1*) that exhibit premature aging. In addition, accumulation of other extra-chromosomal genetic elements (i.e. plasmids) in the mother were shown to induce senescence. It was proposed that the accumulation of extra-chromosomal elements, including ERC and episomes, in the mother titrates genomic factors important for the maintenance of a young phenotype. A recent study suggested that it is not the ERC themselves that are the aging factor, but instead the rDNA recombination process that produces the ERC (Ganley et al., 2009). This study used strains with altered rDNA replication efficiencies. ERC exist in the cell effectively as plasmids because they harbour a replication origin. In the absence of selection, plasmid stability correlates with replication origin strength. Thus by altering rDNA replication strength, ERC production could be separated from their maintenance, and a strain with very little ERC accumulation was shown to age quickly when rDNA recombination was high. This study also reported that other episomes can induce genomic instability (Ganley et al., 2009), reconciling their results with those of Guarente and colleagues. ERCs have also been identified in *Drosophila* and humans (Gagnon-Kugler et al., 2009; Peng & Karpen, 2007) however its origin and role in aging is yet to be determined.

Other aging theories propose that senescence is caused by an accumulation of DNA damage or cytoplasmic senescence factors that remain within the mother cell due to asymmetrical segregation. These theories are supported by the observation showing that oxidized (damaged) proteins predominantly accumulate in mother rather than daughter cells (Erjavec et al., 2007). Nucleolar rDNA is proposed to be particularly sensitive to the presence of elevated levels of oxidized proteins, as this leads to an impaired protein turnover and defects in DNA repair. Furthermore, the asymmetrical segregation of oxidized proteins is Sir2p dependent (Erjavec et al., 2007), leads to rDNA instability and the accumulation of ERC in the mother cell, which then promotes cellular senescence (Kobayashi, 2008).

3.4 A specific role for rDNA in aging

The role of rDNA in aging is most clearly demonstrated in yeast with rDNA instability and cellular aging strongly correlating with rDNA copy number (Burkhalter & Sogo, 2004; Kobayashi et al., 2004). A recent review proposed the “rDNA theory” for aging. Specifically that dysfunction of DNA repair and the replication proteins, predominantly within the nucleolus of the mother cell, is a cause of increased rDNA instability. Because the nucleolus is the most sensitive cellular component to age-related DNA damage, the stability of the rDNA will in turn dictate the stability of the whole genome (Kobayashi,

2008). In both yeast and humans mutations within DNA repair genes result in a reduced lifespan (Park et al., 1999). In humans mutations associated with a premature aging phenotype (Werner and Bloom syndrome) are prominently found in RecQ homolog helicases, which are involved in rDNA repair (Ellis et al., 1995; Yu et al., 1996). In yeast, mutations in genes involved in rDNA transcription and elongation have been identified as modulators of rDNA stability and longevity (Heo et al., 1999; Hoopes et al., 2002; Merker & Klein, 2002). In conclusion whilst the findings in yeast clearly link the stability of rDNA, to aging and cellular senescence, relatively few studies in mammals investigating this link have been reported, predominantly due to the difficulties in studying this complicated part of the genome.

The idea that the rDNA has other, extra-coding functions has received increasing attention over the last few years (Kobayashi, 2011). In yeast, due to the fact that the rDNA cluster comprises ~10% of the genome, rDNA copy number and stability can influence the effective concentration of proteins and protein complexes located within the nucleus through titration. For example, studies investigating Sirtuins revealed that Sir2p is released from the nucleolus upon loss of rDNA copies. Sir2p, together with Sir3p, Sir4p and RAP1 can mediate silencing of telomeres and the mating-type loci. Intriguingly, depletion of ~50% of the rDNA repeats caused an increase in telomeric and mating-type gene silencing, suggesting that the effective concentration of Sir2p at different genomic loci plays a critical role in epigenetic regulation. The fact that mammalian rDNA comprises only ~0.3% of the genome raises the question of whether the association of genomic factors with the rDNA is sufficient to titrate silencing complexes to a similar extent to that reported in yeast. However, the mammalian nucleolus has been reported to influence various cellular functions via sequestering or releasing factors important for various cellular processes that regulate senescence and aging and this is described in more detail in section 4.

4. The nucleolus and senescence in mammals

Senescence is considered a manifestation of organismal aging at a cellular level, although this remains mechanistically unproven (Guarente, 1997; McCormick & Campisi, 1991). However, a number of studies have shown that senescent cells accumulate within mammalian tissues with increasing chronological age (Dimri et al., 1995; Y. Li et al., 1997; Pawelec et al., 1999). As discussed above, in yeast the rDNA locus, and hence the nucleolus, has been implicated in the regulation of longevity and senescence (Kobayashi, 2008). Consistent with this, a nontraditional role for the mammalian nucleolus is also now emerging that involves sequestration and release of tumor suppressors or oncogenes, cell cycle regulators and factors involved in modulating telomerase function (Olson et al., 2002; Olson, 2004; Olson & Dunder, 2005). Thus, similar to yeast, the mammalian nucleolus has been proposed to play an active role in senescence (Mehta et al., 2007; Olson et al., 2000).

Changes in nucleolar morphology are detected in aging cells (Mehta et al., 2007). While pre-senescent cells show a higher number of smaller nucleoli (Bemiller & Lee, 1978), senescent cells have a single prominent nucleolus. Cellular senescence however, does not always correlate with a concomitant decrease in rRNA gene transcription (Halle et al., 1997; Machwe et al., 2000). Even so, studies from many laboratories indicate that

inhibition of rRNA synthesis and ribosome biogenesis and subsequent changes in nucleolar structure and function induce cell cycle arrest, implicating the nucleolus in regulation of cell survival and proliferation (Boisvert et al., 2007; Boulon et al., 2010). Indeed, a number of proteins regulated by their localization to the nucleolus, including the tumor suppressor protein ARF (alternative reading frame; p19ARF in mouse, p14ARF in human) and nucleophosmin (NPM), are involved in cellular senescence by mediating p53 stability (Colombo et al., 2002; Daniely et al., 2002). Therefore, it is likely that the nucleolus plays an active role in establishing and maintaining the senescent phenotype.

Senescent cells are growth arrested in the transition from G1 to S-phase of the cell cycle (Sherwood et al., 1988). The role of the G1-S and G2-M cell cycle checkpoints is to ensure that the cell accurately duplicates its genome and successfully divide into the two daughter cells. The tumor suppressor factors p53 and retinoblastoma protein (RB) regulate these cell cycle checkpoints, which upon activation induce cell cycle arrest, senescence or apoptosis. Correct cell division requires increased protein synthesis that, in turn, is achieved by upregulation of ribosome biogenesis, thus these processes are tightly linked and potentially regulated by common mechanisms (Montanaro, 2008). For example, rDNA transcription and subsequent assembly of the nucleoli during G1 have been shown to be prerequisites for G1-S progression (Pardee, 1989; Sirri et al., 2002). Conversely, decreases in rates of rDNA transcription and disassembly of the nucleoli are observed during mitosis (Grummt, 1999; Pyronnet et al., 2001; Sirri et al., 2002). In fact, the nucleolus can sense and respond to cellular stress by modifying its size and content throughout interphase (Nalabothula et al., 2010). Consequently, it is not surprising that the nucleolus has been recognized as a central regulatory link between ribosome biogenesis and cell cycle progression (Carmo-Fonseca et al., 2000). Certainly, an ever increasing number of nucleolar proteins have been reported to play multiple roles in regulating ribosome biogenesis and cell cycle progression (Boisvert et al., 2007). Although, the exact molecular mechanisms responsible for mediating this crosstalk remain largely unknown, the p53 pathway is prevailing as an important link between ribosome biogenesis and the cell cycle (Pestov et al., 2001).

Early evidence for p53 as a key mediator of the crosstalk between ribosome biogenesis and cell cycle progression came from studies showing that inhibition of Bop1 (block of proliferation), a factor involved in rRNA synthesis and assembly, led to a p53-dependent G1 checkpoint arrest (Pestov et al., 2001). This is consistent with the notion that ongoing ribosome synthesis acts as a checkpoint at the G1-S boundary. In support of this inhibition of rRNA synthesis by microinjecting antibodies to UBF, disruption of the *TIF-IA* gene by Cre-dependent HR, or low doses of actinomycin D (Act D), leads to perturbations in nucleolar structure and function, p53-dependent G1-S cell cycle arrest and apoptosis (Montanaro et al., 2007; Rubbi & Milner, 2003; Yuan et al., 2005).

We have previously shown that inhibition of Pol I transcription by CX-5461, a small molecule inhibitor of initiation of rRNA synthesis, induces senescence in solid tumor cell lines (Drygin et al., 2011). Moreover, CX-5461 treatment or low doses of Act D induces premature senescence in TERT immortalized primary human fibroblasts (BJ-TERT) (Fig. 6) (Hahn et al., 1999). Within 24 hours of treatment with Act D or CX-5461, the protein levels of p53 and its transcriptional target, p21, are upregulated and sustained for a further 24 hours (Fig. 6a). Inhibition of rRNA synthesis correlates with the appearance of

single nucleoli, as visualized by fluorescence *in situ* hybridization (FISH) of rDNA (Fig. 6b), while in interphasic control cells the rDNA repeats are present as multiple nucleoli. The induction of p53 and nucleolar disorganization correlates with the subsequent appearance of γ H2A.X foci indicative of DNA damage, which is associated with senescence (Gire et al., 2004). After 96 hours of Pol I transcription inhibition, BJ-TERT cells appear bigger in size with a flat cell morphology and display acidic β -galactosidase activity, characteristic phenotypic markers of senescence (Fig. 6c) (Dimri et al., 1995). UBF depletion in BJ-TERT cells is also associated with nucleolar disorganization and premature senescence, consistent with its role in establishing and maintaining nucleolar structure. Intriguingly, UBF depletion does not lead to decreased rates of rRNA synthesis, suggesting that the premature senescence may be a consequence of nucleolar disruption (Sanj E and Hannan RD, manuscript in preparation).

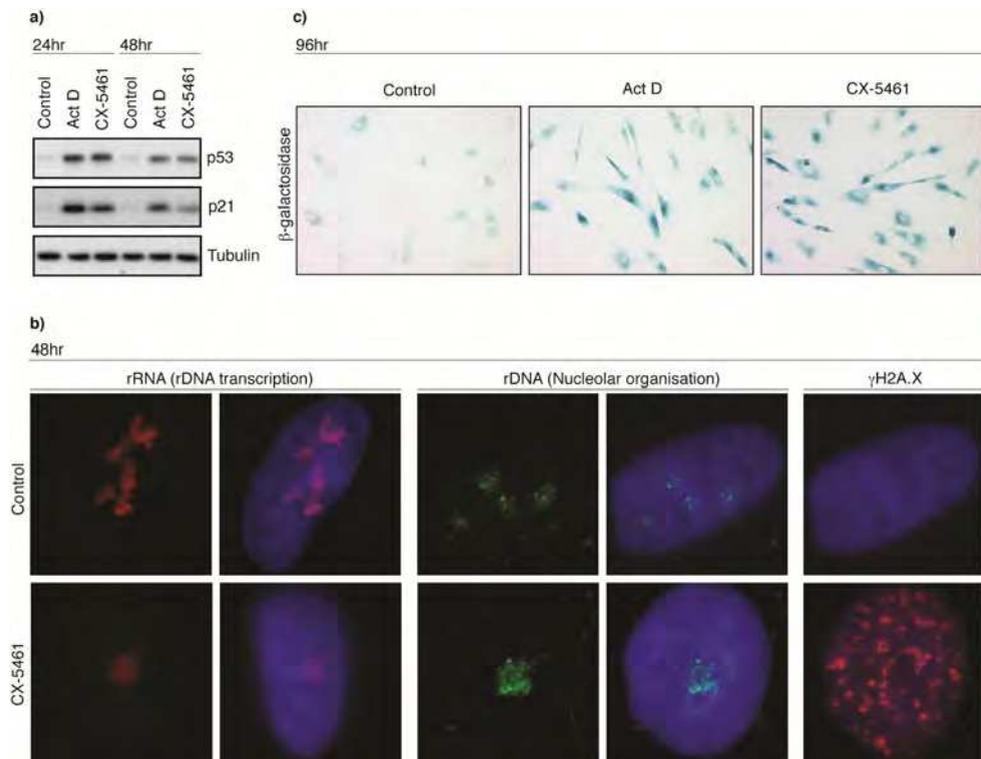


Fig. 6. Inhibition of Pol I transcription induces senescence. Following inhibition of Pol I transcription by treatment with either 5nM Act D or 1 μ M CX-5461, BJ-TERT fibroblasts display hallmarks of senescence. a) Western blot analysis of p53 and p21 after treatment with either Act D or CX-5461. b) Following 48hr CX-5461 treatment, from left to right: RNA FISH to the 5'ETS region of the 47S pre-rRNA (red); DNA FISH to the rDNA (green) combined with immunofluorescent analysis for γ H2A.X (red). gDNA is visualized by DAPI (blue) c) β -galactosidase staining of BJ-TERT cells after 96hr treatment with Act D or CX-5461.

Recent studies exploring the nucleolar proteome have revealed the involvement of the nucleolus in multiple biological processes including senescence, regulation of telomerase function, cell cycle regulation, and stress signaling (Boisvert et al., 2010; Boisvert et al., 2007). Furthermore, nucleolar structure and function are intimately linked with the regulation of p53 stabilization and activation of the p53 pathway, which firmly places the integrity of rDNA transcription and ribosome biogenesis at the centre of control of the cell cycle progression. In addition, over the last few years, significant advances have been made in understanding the higher order organization of nuclear structures, including the nucleolus, and their importance for the regulation of nuclear functions (Nemeth & Langst, 2011). Here, we address the impact of these recent results and discuss the molecular mechanisms underlying nucleolar function in the regulation of cell cycle progression and senescence.

4.1 The nucleolus in control of cell cycle regulation and senescence

An increasing number of nucleolar proteins have been reported to play multiple roles in regulating ribosome biogenesis and cell cycle progression. For instance, components of the DNA replication initiation machinery, origin of replication complex (ORC) and minichromosome maintenance (MCM) proteins, have been purified from human nucleoli (Boisvert et al., 2007; Couté et al., 2006) and were shown, in yeast, to associate with several 60S ribosomal synthesis factors that are required for pre-rRNA processing and are also essential for initiation of DNA replication by mediating the association of the ORC and MCM proteins at replication origins (Zhang et al., 2002). However, the most direct role for nucleoli in regulation of the cell cycle is via the sequestration or release of proteins directly involved in cell cycle progression.

The tumor suppressor protein RB is an important regulator of senescence (Campisi & di Fagagna, 2007). RB is generally active during senescence, indeed its enforced expression has been shown to induce senescence (Narita et al., 2003). RB was initially reported to accumulate in the nucleolus and to have a repressive role in Pol I transcription (Cavanaugh et al., 1995; Hannan et al., 2000). Subsequently, Nucleolin (NCL), a multifunctional nucleolar protein essential for rRNA processing (Mongelard & Bouvet, 2007) was reported to associate with hypophosphorylated (active) RB (pRB) during the G₁ phase of the cell cycle (Grinstein et al., 2006). pRB mediates a cell cycle checkpoint between G₁ and S phase (Bartek et al., 1996) by targeting members of the E2F family of transcriptional activators (Chellappan et al., 1991) that are essential for cellular proliferation. While pRB has been reported to reside in the nucleoli in a cell type dependent manner (Angus et al., 2003) hyperphosphorylated RB (ppRB) is eliminated from the nucleolus until late S or G₂ phase. Import of ppRB into the nucleolus in late S or G₂ phase is mediated by its interaction with nucleolar NPM (Takemura et al., 2002). Although the functional significance of nucleolar retention and release of RB is currently unresolved, it has been proposed that its retention could represent a negative regulatory mechanism to sequester RB to prevent checkpoint activation during the cell cycle (Angus et al., 2003).

By far the most convincing studies linking the dynamic release of nucleolar proteins and cell cycle progression were performed in yeast. Cdc14p is a protein phosphatase that is crucial

for promoting exit from mitosis into the G1 phase (Jin et al., 2008; Visintin et al., 1998; Zachariae et al., 1998). Cdc14p activation is also a prerequisite for successful chromosome segregation as it is necessary for condensin enrichment at the rDNA, which triggers rDNA segregation and ensures the completion of chromosome segregation (D'Amours et al., 2004). In G1 or during S phase, Cdc14p is sequestered to the nucleolus by its inhibitor Net1/Cfi1p, a component of the multifunctional protein complex RENT, where it remains inactive until the onset of anaphase, thereby preventing the premature onset of mitotic exit (Shou et al., 1999). The release of Cdc14p from the nucleolus is mediated through the sequential action of two regulatory networks: FEAR (CDC Fourteen Early Anaphase Release) and MEN (Mitotic Exit Network) that lead to Net1/Cfi1p phosphorylation reducing its affinity for Cdc14p and thus disassociation of the complex (Shou et al., 2002; Yoshida & Toh-e, 2002). The roles of the two human orthologs of Cdc14p (CDC14A and CDC14B) are not yet established. CDC14B has been shown to translocate from the nucleolus to the nucleoplasm following genotoxic stress in G2, leading to the activation of the APC/CDH1 complex and the establishment of a DNA damage induced G2 checkpoint (Bassermann et al., 2008). Nevertheless, a wider role in promoting mammalian cell cycle progression has been proposed for CDC14B including the governing of cell cycle re-entry after G2 block (De Wulf & Visintin, 2008).

Another example of nucleolar sequestration-mediated regulation of specific cellular activity during the cell cycle is the regulation of telomerase, the enzyme that adds telomeric repeats sequences to the ends of chromosomes (Fig. 7). Telomeres are composed of TTAGGG repeats that form a 3' overhang of 100-400 nucleotides forming a T-loop structure that is stabilized by telomeric proteins (Griffith et al., 1999). Telomeres maintain chromosome integrity by protecting against end shortening and end-to-end fusions (de Lange, 2005; Sahin & Depinho, 2010). If telomere length is not maintained, the telomeres will reach a critically short length, triggering the cell to undergo replicative senescence (Harley et al., 1990; Stewart et al., 2003). Although the telomeres and the nucleolus are separate subnuclear domains, multiple telomeric components have been detected in the nucleolus suggesting an underlying regulatory connection between the nucleolus and telomeres (Tsai, 2009).

The ribonucleoprotein (RNP) telomerase is composed of the telomerase RNA component (TERC) and the telomerase reverse transcriptase (TERT), which catalyzes *de novo* repeat addition by utilizing TERC as a template (Greider & Blackburn, 1989). Outside of S phase, TERC and TERT are localised within distinct nucleoplasmic foci separate from telomeres. In early S-phase, TERT moves to nucleoli while Cajal bodies containing TERC accumulate at the periphery of nucleoli (Fig. 7). Nucleolar transportation of TERC and TERT has been proposed as a prerequisite step in the process of telomerase RNP biogenesis (Etheridge et al., 2002; Narayanan et al., 1999; Yang et al., 2002) and/or the transport of active telomerase, which occurs during mid-S phase (Tomlinson et al., 2006). Nucleolar localization of telomerase has also been reported to be mediated by NCL, which interacts with the active telomerase complex and is involved either in the assembly or maturation of telomerase. Nucleolar NCL-telomerase complexes are exported and maintained in the nucleoplasm and delivered to the telomeres (Khurts et al., 2004). Cell-cycle dependent nucleolar localization of telomerase is lost in transformed cells or following DNA damage (Wong et al., 2002).

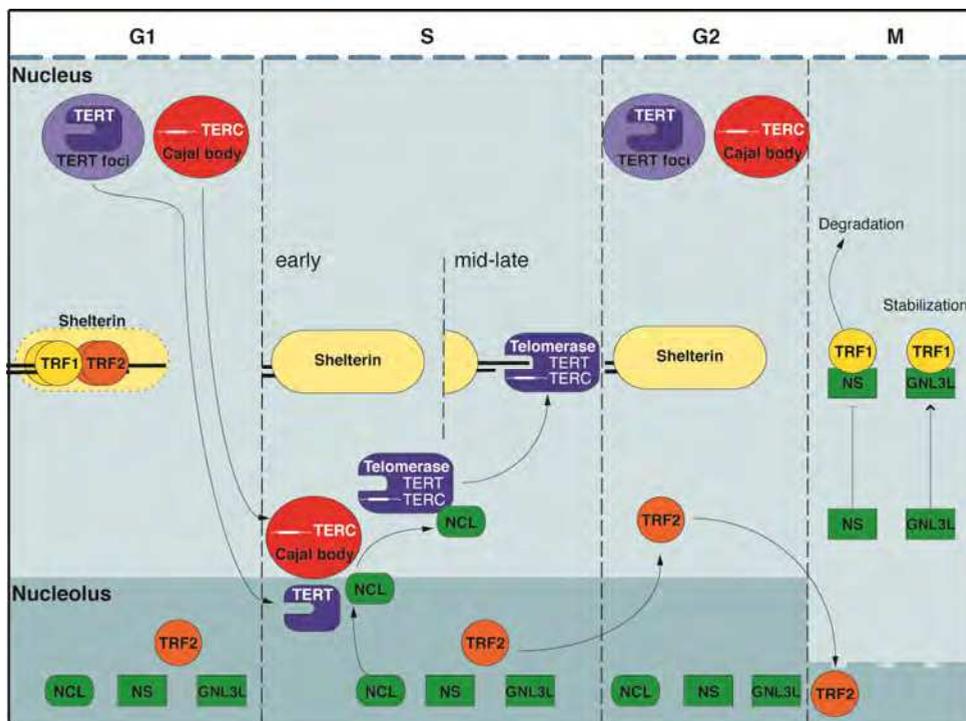


Fig. 7. Nucleolar regulation of telomere stability. Telomerase and the shelterin complex are regulated by the nucleolus through sequestration and release. In early S-phase, TERT moves to the nucleoli and Cajal bodies containing TERC accumulate at the periphery of the nucleoli. NCL-telomerase complexes are then exported to the telomeres during mid-S phase. TRF1 is regulated by NS and GNL3L in opposing manners. TRF2 is sequestered in the nucleolus during G1 and S phase, released to the nucleoplasm in G2, and returned to the nucleoli at cytokinesis.

In addition to telomerase, components of the shelterin telomere binding complex, including telomeric repeat binding factors 1 (TRF1) and TRF2 have been reported as regulated in part by localization to the nucleolus (Fig. 7) (Lin et al., 2008; Tsai, 2009; Wong et al., 2002; Zhang et al., 2004). TRF1, which is required for establishing 'closed' structures at the telomeres that are inaccessible to telomerase, has been shown to be regulated by a number of nucleolar proteins, including nucleostemin (NS) and guanine nucleotide binding protein-like 3-like (GNL3L). NS binding to TRF1 enhances its degradation, while GNL3L binding stabilizes TRF1 (Zhu et al., 2009). Since the majority of TRF1 resides in the nucleoplasm, nucleolar retention of NS and GNL3L renders them inactive in modulating TRF1 activity. However, their nucleoplasmic localization during mitosis or in response to nucleolar stress may allow modulation of TRF1 in regulating telomere capping (Tsai, 2009).

In addition, TRF2, the component of shelterin considered responsible for the formation of the protective telomeric T-loop structure required for protecting the telomeres, localizes to the nucleolus at G0 and S but diffuses into the nucleoplasm in G2 and returns to the nucleolus at cytokinesis (Fig. 7). Low dose of Act D, which specifically inhibits Pol I transcription, causes a delay in TRF2 release from nucleoli in G2 and mitotic cells displaying end-to-end chromosomal fusions, suggesting that the timely nucleolar retention/release of TRF2 regulates its nucleoplasmic function (Zhang et al., 2004).

Telomere attrition is recognized as a hallmark of aging cells (Harley et al., 1990). The p53 and p16INK4a-RB pathways are critical for establishing senescence in human cells (Campisi & di Fagagna, 2007). p53 is presumed to sense dysfunctional telomeres as damaged DNA, upon which it elicits the senescence response in part by increasing expression of the p21 CDKI, which in turn prevents the phosphorylation and inactivation of RB (Sherr & Roberts, 1999). In several mouse models, inappropriate p53 activity, either through deregulated expression of p53 or in response to constitutive stress like DNA damage, leads to premature aging (Maier et al., 2004; Tyner et al., 2002). As discussed later in this chapter, the nucleolus has been proposed as a central hub for sensing major cellular stress and transmitting signals for regulation of p53 levels and activity (Olson, 2004). It is therefore tempting to suggest that nucleoli may sense the DNA damage signal induced by damaged telomeres and activate a p53 response to implement senescence. Taken together, the nucleolus has emerged as a highly complex and multifunctional regulatory compartment involved in diverse biological processes including the regulation of proliferation and the execution of anti-proliferative responses such as cell cycle arrest and senescence.

4.2 The nucleolus as a sensor of cellular stress

One of the most intriguing roles proposed for the nucleolus is as a sensor of cellular stress and a means to couple cellular stress to the p53 pathway (Rubbi & Milner, 2003), a key regulator of senescence and longevity (Fig. 8) (Vigneron & Vousden, 2010). In this paradigm, under normal conditions, the nucleolus contributes to the maintenance of low p53 levels, while in response to cellular stress p53 levels and activity are dramatically elevated through the actions of select nucleolar proteins. Key to the nucleolar control of p53 is the oncogene MDM2 (mouse double minute 2; or HDM2 in humans). In proliferating cells, p53 activity is kept under surveillance by MDM2, via two complimentary mechanisms: (i) MDM2 acts as an E3 ubiquitin ligase directly transferring ubiquitin onto p53 thereby targeting it for 26S proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997); and (ii) the direct binding of MDM2 to the N-terminal domain of p53 inhibits its transcriptional activity by abrogating its interaction with the basal Pol II transcription machinery (Momand et al., 1992; Oliner et al., 1993). The two best characterized mechanisms by which cellular stress modulates MDM2/p53 pathway in a nucleolar specific manner are in response to oncogenes (oncogenic stress) and to perturbations that alter ribosome biogenesis (nucleolar stress).

4.2.1 ARF and oncogenic / replicative stress

The *Ink4/Arf* locus encodes two tumor-suppressor proteins, p16INK4a and p19ARF, that govern the antiproliferative functions of RB and p53 proteins, respectively (Fig. 8). ARF

binds to MDM2 and sequesters it into the nucleolus, thereby preventing negative-feedback regulation of p53 by MDM2, leading to the activation of p53 in the nucleoplasm (Honda & Yasuda, 1999; Palmero et al., 1998; Zindy et al., 1998). Under normal conditions, ARF is expressed at very low levels and is sequestered into the nucleolus, due to its association with the nucleolar protein NPM (Gjerset & Bandyopadhyay, 2006; Korgaonkar et al., 2005). This prevents its interaction with MDM2. In contrast during replicative senescence of MEFs or stress induced by activation of oncogenes such as c-MYC and H-RAS, ARF rapidly accumulates to sufficient quantities and is able to bind and sequester MDM2 leading to p53 activation (Palmero et al., 1998; Sharpless et al., 2001; Weber et al., 1999). In addition ARF directly suppresses rRNA synthesis and processing to modulate ribosome biogenesis (Ayrault et al., 2006; Lessard et al., 2010; Sugimoto et al., 2003). Although, the importance of the later for modulation of p53 is unclear, a ribosome biogenesis-dependent-ARF pathway may complement ARF's function in modulating the p53 pathway.

4.2.2 Nucleolar stress

The protein content of the nucleolus has been shown to change dramatically under various stress conditions (Boulon et al., 2010). It is now clear that, the nucleolar proteome undergoes distinct spatial and temporal alterations in response to different stress insults, suggesting that the nucleolus responds to different stress stimuli in a unique and specific manner (Moore et al., 2011). The landmark study by Rubbi and Milner (2003) proposed that disruption of nucleolar structure and function and subsequent release of nucleolar components into the nucleoplasm as a common denominator in most or possibly all p53-inducing stresses (Reviewed in Olson, 2004). Consistent with this, it is now recognized that inactivation of rDNA transcription, RP synthesis, rRNA processing, and the assembly and nucleolar export of the 40S and 60S ribosomal subunits (Zhang & Lu, 2009) are established mechanisms for causing nucleolar disruption and activation of the p53 pathway. From these observations a model of nucleolar surveillance of ribosome biogenesis (also termed nucleolar stress) has been proposed to integrate a diverse array of metabolic irregularities and oncogenic stimuli whereby the rate or efficiency of ribosome production serves as a signal by which cells could regulate cell-cycle progression via controlling p53 levels (Fig. 8) (Boulon et al., 2010; Deisenroth & Zhang, 2010; Ruggero & Pandolfi, 2003; Shcherbik & Pestov, 2010). In addition, due to the repetitive nature of the rRNA genes as well as the high rate of transcription by Pol I complexes, the rDNA is considered unstable and has been proposed to act as a potential sensor for DNA damage (Boisvert & Lamond, 2010). Signals associated with stalled polymerases and/or reduced rRNA transcription could activate p53 and possibly other DNA damage response pathways (Boisvert & Lamond, 2010; Kobayashi, 2008) leading to cell cycle arrest or programmed cell death (Drygin et al., 2009). Under conditions of "nucleolar stress", p53 stabilization can be achieved via different mechanisms including posttranslational modifications, protein-protein interactions and increases in the translation rate of p53 mRNA. Of these mechanisms perhaps the best documented is the role of RPs which are able to interact directly with MDM2 leading to p53 stabilization in response to ribosomal stress. Interestingly, while the RPs are required for p53 response to ribosomal stress, ARF in this context is not required, suggesting that different cellular conditions, oncogenic stress or ribosomal stress modulate the binding of either ARF or RPs to MDM2 and subsequent activation of p53 (Pan et al., 2011).

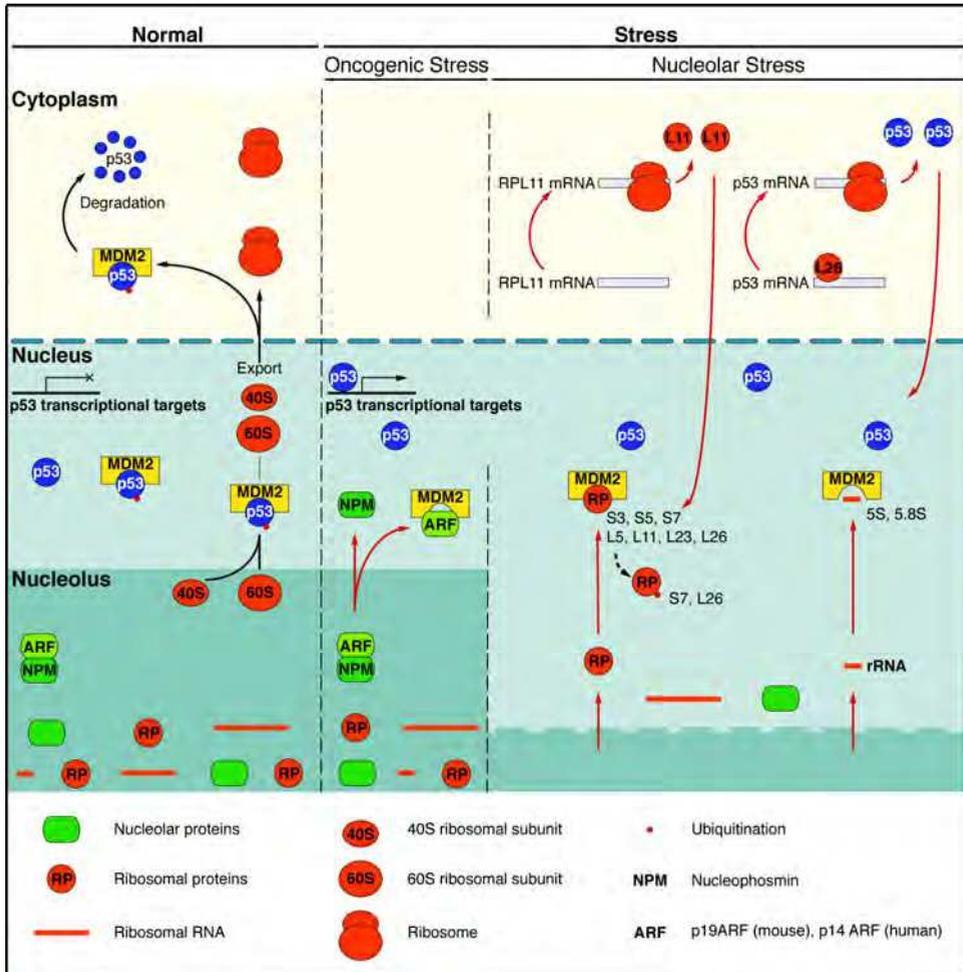


Fig. 8. The nucleolus as a sensor of cellular stress. Under normal conditions, p53 activity is maintained at low levels by MDM2, via two mechanisms: First, MDM2 ubiquitinates p53 thereby promoting its degradation; second, the binding of MDM2 to p53 abrogates its interaction with Pol II transcription machinery. Following oncogenic stress, ARF binds MDM2 and sequesters it in the nucleolus. Under nucleolar stress, p53 can be activated by the following mechanisms: (i). The co-transport of p53 and/or MDM2 with the ribosomal subunits to the cytoplasm is impaired; (ii). RPs interact directly with MDM2; (iii). 5.8S and 5S rRNA interact directly with MDM2; (iv). RPL26 binds p53mRNA and enhances its translation; (v). Increased RPL11 mRNA translation results in enhanced interaction between RPL11 and MDM2.

An increasing number of RPs (RPS3, RPS5, RPS7, RPL5, RPL11, RPL23, RPL26) as well as the 5.8S and 5S rRNAs are capable of interacting with MDM2 leading to p53 stabilization (Deisenroth & Zhang, 2010; Fontoura et al., 1992; Fumagalli et al., 2009; Ofir-Rosenfeld et al., 2008; Riley & Maher, 2007; Zhang & Lu, 2009). A number of different models have been

proposed for how these various interactions might regulate p53 (Fig. 8). Under normal growth conditions, RPs are synthesized in equimolar amounts with the rRNAs and assembled with large and small ribosomal subunits in the nucleolus and transported to the cytoplasm to form functional ribosomes. In one model, so called "riding the ribosome", the interaction of p53 and/or MDM2 with the ribosomal subunits may facilitate p53/MDM2 transport from the nucleolus to the cytoplasm thus preventing p53 from interacting with its target genes in the nucleoplasm and/or promoting its ubiquitin-mediated degradation in the cytoplasm (Boulon et al., 2010). Conversely, stress signaling that impairs production and export of ribosome subunits, would be predicted to decrease p53/MDM2 transport to the cytoplasm, thus allowing p53 to activate transcription of its target genes in the nucleoplasm (Boulon et al., 2010). In a second and perhaps better described model, conditions that inhibit rRNA transcription or stall ribosome synthesis and assembly in the nucleolus are postulated to create a pool of free RPs (such as RPL5, RPL11 and RPL23) that are directly interact and sequester MDM2 resulting in suppression of p53 ubiquitination (Daniely et al., 2002; Deisenroth & Zhang, 2010; Lindstrom & Nister, 2010; Pestov et al., 2001; Warner & McIntosh, 2009; Zhang & Lu, 2009). However, Horn and Vousden (2008) observed a synergistic suppression of MDM2 activity through cooperation of RPL11 and RPL5, suggesting they have distinct roles in inhibiting MDM2 function. In addition, binding sites for ARF, RPL5, and RPL11 on MDM2 do not appear to overlap (Lindstrom et al., 2007; Zhang et al., 2003) suggesting that ARF and RPL5/L11 may respond to different stimuli and converge at the point of MDM2 inactivation (Shcherbik & Pestov, 2010).

RPL26 is so far unique in its ability to bind the 5' untranslated region of the p53 mRNA and enhance its translation. Its interaction with MDM2 triggers its own ubiquitination and degradation, which in turn causes downregulation of p53 mRNA translation (Ofir-Rosenfeld et al., 2008). The diverse roles of RPs in the regulation of the MDM2-p53 pathway is further supported by the finding that knockdown of RPS6 not only affects 40S ribosomal biogenesis but also enforces RPL11 mRNA translation. This leads to an enhanced interaction between RPL11 and MDM2 leading to the accumulation and activation of p53 (Fumagalli et al., 2009). Since multiple RPs have separate mechanisms for activating p53, it is plausible that they may have distinct roles in sensing different types of signals leading to activation of nucleolar stress response. In summary there is now a robust set of data demonstrating that the nucleolus and rDNA transcription indirectly play an important role in the regulation of tumor suppressors and oncogenes such as ARF, MDM2 and p53 and thus perturbation in the nucleolus are predicted to have profound effects on cellular functions that are controlled by these factors. In this manner the nucleolus can be considered as a sensitive cellular stress detector integrating various perturbations in homeostasis and converting them to appropriate responses such as cell cycle arrest and senescence.

4.3 Alterations in genome organization in and around the nucleolus are associated with senescence

The nucleus is compartmentalized into substructures that perform distinct nuclear activities (Lanctot et al., 2007). These nuclear structures include the nucleoli, nuclear envelope, nuclear bodies, nuclear matrix and chromosome territories (Cremer & Cremer, 2001). Organization and spatial location of chromosomes and their interactions with other nuclear substructures ensures that transcription is correctly regulated (Misteli, 2004). The periphery

of the nucleolus consists of satellite DNA repeats, which are proposed to play a role in the formation of perinucleolar heterochromatin (Manuelidis, 1984), and it has been suggested they serve as a distinct nuclear space with a primary function in maintaining repressive chromatin states (Nemeth & Langst, 2011; van Koningsbruggen et al., 2010). For example the inactive X chromosome (Xi) must continuously visit the perinucleolar compartment during S phase to maintain its epigenetic status (Zhang et al., 2007). Other conserved chromosomal regions have also been shown to interact with the nucleolus including a fraction of the human centromeres (Bridger & Bickmore, 1998; Leger et al., 1994; Park & De Boni, 1992). Furthermore, chromosome mobility studies demonstrated that nucleolar-associated chromatin is significantly less mobile than other genomic regions. Specifically disruption of the nucleolar structure enhanced chromatin mobility, thus implying the nucleolus plays an active role in constraining chromatin movement and maintaining the three-dimensional organization of the genome within the nucleus (Chubb et al., 2002). This is further supported by the identification of specific interactions between repetitive and non-repetitive loci within the yeast genome including specific repeated elements that interact with rRNA genes. Therefore, it has been proposed that genomic architecture is organized by restricting the mobility of these repeat elements relative to the nucleolar interaction point (O'Sullivan et al., 2009). Similar results have been obtained using live cell imaging, with frequent interactions observed between the nucleolar and non-nucleolar chromatin (Berger et al., 2008). Intriguingly, extensive disorganisation of nuclear architecture, at the level of whole chromosomes, is associated with the transition from proliferative to senescent states. The human non-rDNA bearing chromosome 18, for instance, exhibits altered spatial positioning, changing from the apical edge of the nucleus in proliferating cells to nucleoli in senescent cells (Bridger et al., 2000). It is plausible that the reorganisation of the genome as the cells enter senescence is responsible for extensive changes in the transcriptional status of the genome (Foster & Bridger, 2005). Consistent with this, we have recently found that reductions in UBF levels lead to disruptions in nucleolar structure and acrocentric chromosome organization and induces premature senescence in primary human fibroblasts (Huang S, Hannan RD, manuscript in preparation). Together, the data suggest a functional role for nucleoli in the organization of the genome and in the regulation of cellular senescence.

5. Conclusion

The nucleolus is a highly evolutionary conserved subnuclear compartment traditionally associated with rDNA transcription and ribosome-subunit production. However it is now apparent that the nucleolus is dynamic in nature and its organization, size and protein composition changes dramatically during the cell cycle and under different cellular conditions including stress. Consistent with this dynamic nature the nucleolus has now been implicated in regulating additional important cellular processes beyond ribosome-subunit synthesis, including cell-cycle control, stress responses, senescence and aging. The fundamental role the rDNA repeats play in aging of fission yeast is now overwhelming. Similarly, in higher eukaryotes, nucleolar function in coupling ribosome subunit biogenesis and cell-cycle progression, through the activity of the tumor suppressor protein p53, places the nucleolus at the centre of coordinating cellular stress response and determining cell fate such as survival and senescence. It is likely we have only begun to scratch the surface of the detail by which eukaryotes have evolved to utilise the unique subnuclear domain, the nucleolus, to control fundamental cellular process such as aging and senescence.

6. References

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University Campus STeP Ri
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51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821