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Deep Insight Section

Mechanisms of rDNA silencing and the Nucleolar Remodelling Complex (NoRC)

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Abstract

Protein synthesis in living cells requires functional ribosomes which are composed of ribosomal proteins and ribosomal RNA (rRNA) molecules. rRNA is transcribed from tandemly repeated ribosomal DNA (rDNA) which is organised into a nuclear compartment termed the nucleolus in S-phase cells. It is essential that rDNA transcription is properly regulated in order to meet the cell's requirements for ribosomes and hence protein synthesis without wasting metabolic energy. In the last twenty years many proteins involved in regulating this process have been identified, suggesting that most organisms contain multiple protein complexes that regulate rDNA packaging and transcription. Importantly, it has become clear that errors in the function of these proteins can permit aberrant cellular growth, including in several classes of cancer. In this review, I discuss the history of how protein complexes such as the Nucleolar Remodelling Complex (NoRC) were discovered, using examples from humans and from model research organisms from different biological groups. I will discuss recent discoveries of the critical roles of rDNA-binding complexes in nucleolar assembly, the widespread occurrence of regulatory non-coding RNAs which interact with these complexes, and the pathways which regulate rDNA transcription in response to cellular energy status. Finally, I will review the growing evidence that misregulation of rDNA transcription not only allows the growth of cancerous cells, but can trigger oncogenesis itself.

Introduction

All living cells have an essential requirement to transcribe rRNA genes (rDNA) to produce rRNA for use in ribosome synthesis. Ribosome production is necessary to support translation of mRNA and consumes a significant proportion of the energy available to a typical living cell. rDNA transcription, which is performed in eukaryotes by RNA polymerase I, must be correctly regulated in order to ensure that the cellular requirement for ribosomal subunits is met. Studies in many groups of organisms have revealed the existence of multiple interacting pathways which ensure this regulation by up- or down-regulate total rRNA synthesis via activation or silencing of rDNA, respectively. It has also become clear that dysregulation of these pathways is associated with many pathologies, and in mammals is associated with, and often prognostic for, the occurrence of cancer. Control of transcription at rDNA has also been used as a paradigm for understanding eukaryotic gene expression in general.

In this deep insight, key mechanisms that determine how rDNA transcription is controlled will be discussed. There is a particular focus on the pathways which act in mammalian cells, including those which have been implicated in tumourigenesis. However, reference is also made to some of the many insights into rDNA regulation revealed in other model biological systems, such as mutagenesis screens for loss of rDNA silencing in yeast, and some of the many epigenetic aspects of rDNA regulation discovered in hybrid plants of the *Arabidopsis* genus. As rRNA synthesis, the organisation of rDNA into nucleoli and the subsequent formation of ribosomes are all highly complex topics, this account will necessarily deal

only with certain key discoveries, together with some highlights of the recent literature. The reader will be referred to many excellent reviews from the last few years for further details where appropriate.
In terms of organisation, this deep insight will begin with a general discussion of how RNA polymerase I acts to generate nascent rRNA molecules as precursors to ribosome biosynthesis, before turning to how this process is controlled. I will discuss the characteristics of rDNA regulation networks with particular biological or medical significance from different systems, before turning to aspects of how rDNA transcription has been linked to cancer.
a) the mammalian NoRC complex and other complexes which interact with or oppose its activity;
b) the links between rRDNA transcription and cellular energy status;
c) other multi-protein complexes in plants and yeast which shed light on other aspects of rRNA transcription control;
d) misregulation of rDNA regulation complexes and their links with cancer and other pathologies.

rDNA chromatin and the organisation of the nucleolus

Ribosomal RNA (rRNA) synthesis occurs in the nucleolus which assembles during the packaging and transcription of ribosomal DNA. In the nucleolus, tandemly repeated 45S ribosomal RNA genes (or rDNA) are transcribed to form 45S nascent pre-rRNA (Ballal et al., 1977). Each pre-rRNA is cleaved and processed to form the 18S, 5.8S and 25S rRNA molecules which are essential for the formation of ribosomes (Shaw and Jordan, 1995). The nucleolus is not bound by any membrane but self-assembles during the processes of rRNA transcription and subsequent processing (Mélèse and Xue, 1995). Nucleoli are stable enough to be extracted from culture cells (Andersen et al., 2002; McKeown et al., 2008) and studies in plants and animals have used mass spectrometry to identify proteins involved in the production of rRNA (Andersen et al., 2002; Pendle et al., 2005). Such studies have suggested that in many organisms nucleoli have also acquired additional cellular functions in stress response, splicing and small RNA biosynthesis (Pendle et al., 2005; Raška et al., 2006; Boisvert et al., 2007; Boulon et al., 2010), although these will not be considered further here.
In most eukaryotes, the rDNA locus consists of several hundred tandemly repeated rRNA genes, separated by linker regions. These are located at one or more large loci, which are also termed nucleolar organizing regions (NORs). The tandem repetition of rRNA genes is necessary as at least 20-40 rRNA genes are required to meet the production levels of rRNA in a typical dividing cell (Sollner-Webb et al., 1985). Furthermore, most eukaryotes contain many more rRNA genes than this (usually in the range of 50-500) and under most conditions, only a subset of the total are actively transcribed while the others are silenced by a dosage-control mechanism (Dammann et al., 1993; Russell and Zomerdijk, 2005). A consequence of this is that rRNA synthesis can be up- or down-regulated either by varying the number of active genes or by altering the transcription rate per gene, although the relative importance of these two pathways may vary between organisms (Dammann et al., 1993; French et al., 2003). Both processes are controlled by cellular signalling pathways of some complexity (Schmelzle and Hall, 2000; Stefanovsky et al., 2001; Grummt, 2003; Kim et al., 2003; Moss, 2004).
The inactive rRNA genes are maintained in a transcriptionally silent, inactive state which requires the interaction of many cellular processes (McStay and Grummt, 2008). This silencing ensures that the cell's energy is not expended on unnecessary rRNA synthesis, that rRNA genes which have accumulated mutations or become pseudogenic are not transcribed, and that the activity of other RNA polymerases within the rDNA is prevented (Dammann et al., 1993). As we will see, preventing aberrant rRNA transcription may also be an important barrier to tumour formation in mammals, and failure to repress aberrant may trigger oncogenesis under some circumstances. Finally, classic studies demonstrated that the cells of hybrid organisms generally silence the entire rDNA inherited from one parental species and activate that from the other (Navashin, 1934). This phenomenon is now known as nucleolar dominance and has been used to illustrate many of the pathways that regulate rDNA activity.
A key issue for understanding rRNA transcription is therefore to understand the mechanistic basis of the switch between transcriptionally active and transcriptionally repressed rDNA. It was shown several decades ago that rDNA exists in two distinct conformations (that is, physical states within the nucleus), and that these can be stably inherited through mitosis (Conconi et al., 1989; Birch and Zomerdijk, 2008). The two forms of rDNA correspond to rRNA genes which are organised into different forms of chromatin, the DNA-protein superstructure into which DNA is 'packaged' following binding by histone octamers (Prior et al., 1983).
The two forms of rDNA chromatin can be distinguished on the basis of their differential accessibility to the DNA-crosslinking drug, psoralen.

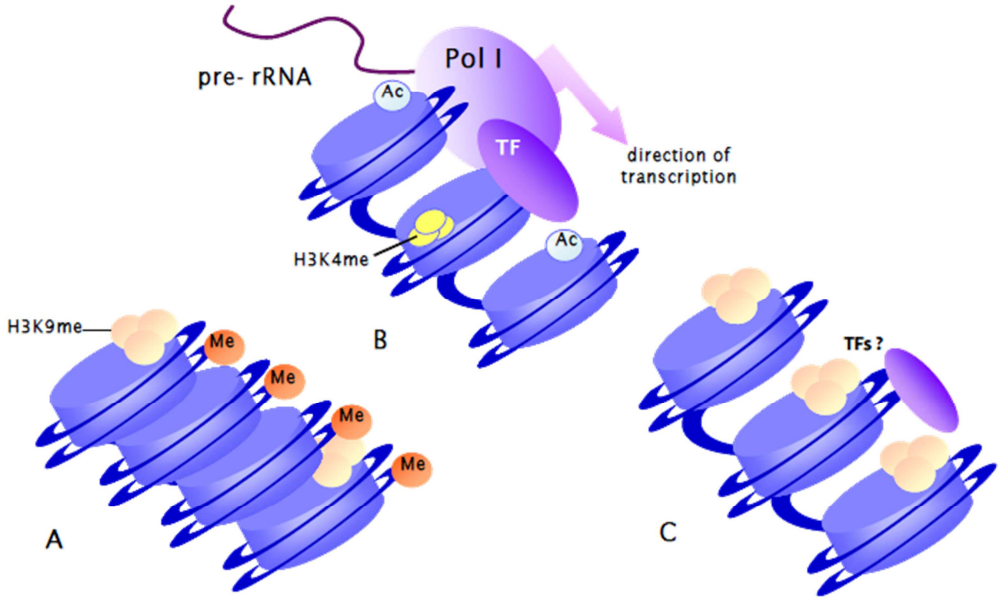

Figure 1. Generalized model for the three chromatin states in which eukaryote rRNA genes may form. A) constitutively silent rDNA repeats organised into heterochromatin; B) rDNA repeats organised into euchromatin and actively transcribed by RNA Pol I (shown as a schematic core polymerase core and transcription factor; see text for details); C) 'poised' rDNA accessible to transcription machinery but not actively transcribed.

One form has a highly compact psoralen-inaccessible organisation corresponding to densely-staining heterochromatin, and which is expected to be refractory to rRNA gene transcription.

The other is a more dispersed, psoralen-accessible form which corresponds to the lightly-stained euchromatin and is expected to be permissive for transcription (Conconi et al., 1989; Dammann et al., 1993). This suggests a basic conceptual model in which the activation level of rRNA genes depends upon a switch between two different chromatin states, which either repress transcription by Pol I or induce it, respectively. This also corresponds with electron microscopy investigations which confirm that rDNA can be present in one of two forms within the cell, one of which is indeed densely compacted in the manner of heterochromatin, while the second is less dense in the manner of euchromatin.

Under the microscope, heterochromatin is visible within nucleoli as fibrillar centres or as 'knobs' arranged around the nucleolar periphery, while euchromatin may be present throughout the body of the nucleolus. Many lines of evidence support the supposition that the compacted heterochromatic rDNA is typically inactive, while that which more loosely organised is euchromatic and likely to be undergoing transcription (Raška et al., 2006). Understanding the features of these two chromatin states, and the protein complexes which induce transitions between them, is thus essential for understanding how rRNA transcription is controlled (Gerbi et al., 2003). Various reviews describe our current understanding of the importance of nucleolar organisation and its relationship with rDNA packaging, rRNA gene transcription and pre-rRNA processing in different taxa (Nierras et al., 1997; Raška et al., 2006; McStay and Grummt, 2008; Shaw and McKeown, 2011; Shaw and Brown, 2012).

Early evidence on the nature of the molecular differences between active and inactive rRNA genes was suggested by the chemical manipulation of nucleolar dominance by aza-dC and trichostatin A, DNA methylation and histone deacetylation inhibitors, respectively (Reeder, 1985; Thompson and Flavell, 1988; Pikaard, 2000). As these pharmacological treatments altered the extent of nucleolar dominance, it was concluded that both DNA methylation and histone modification might differ between rDNA chromatin states in a manner related to the control of their transcription (Chen and Pikaard, 1997; Pikaard, 1999; McStay, 2006). Elucidating the details of what these modifications might be has been greatly accelerated by the development of the chromatin immunoprecipitation (ChIP) technique. For example, it has been shown that mammalian rRNA genes can be associated with either of two sets of covalent chromatin modifications, which correlate with their level of transcriptional activity (Santoro et al., 2002). DNA of silenced rRNA genes is highly methylated at cytosine residues, and is bound by histone octamers which are methylated at H3K9 (Figure 1A). Active rRNA genes are instead distinguished by DNA hypomethylation, and are bound to histone octamers incorporating H3K4 marks and widespread acetylation of many H3 and H4 lysine

residues (Figure 1B). It has also been proposed that rDNA can also exist in a third, intermediate state, in which the chromatin of the rRNA genes is decondensed but they remain transcriptionally silent (Figure 1C).
This is characterised by the simultaneous presence of histone modifications associated with euchromatin and heterochromatin on different sites and may correspond to a 'poised' euchromatic state (McKeown and Shaw, 2009; Xie et al., 2012).
Much research has therefore concentrated on determining what roles these chromatin modifications play at the molecular level, and to what extent they are causal for determining the activity of the rRNA genes with which they associate. Such studies have made use of many different biological systems and have exploited genetic, biochemical and cell biological techniques, including cell culture models for different human cancers and other diseases.
These studies have demonstrated that while the proteins which control rDNA chromatin typically vary between different groups of eukaryotic organisms, the regulatory networks in which they act also have various key features in common. In the following section, the control of rDNA transcription in humans (and certain model systems) will be described.

rDNA silencing I - control of human RNA Pol I transcription by protein complexes

In eukaryote, 45S pre-rRNA is synthesised by a dedicated transcriptional system centred on the multimeric protein complex, RNA Polymerase I (hereafter RNA Pol I). RNA Pol I only catalyzed the transcription of rDNA, which is in turn not transcribed by any other polymerase system under normal conditions. For a general review of the biochemical structure of RNA Pol I, see (Vannini, 2013). In addition to the core subunits of RNA Pol I itself, its polymerase activity requires the action of several other proteins and protein complexes, many of which have regulatory potential.
These RNA Pol I cofactors and transcription factors (TF) allow RNA Pol I to effect transcription at active rRNA genes and to determine the level of this.
In humans, the principal RNA Pol I TFs consist of the Upstream Binding Factor (UBF), the promoter selectivity factor (SL1, (Comai et al., 1992)) and the transcription termination factor (TTF-I, (Grummt, 2003)). These complexes remain associated with rDNA during the cell cycle, even during mitosis when rRNA gene transcription is silenced but are maintained in an inactivate state until transcription resumes in telophase (O'Mahony and Rothblum, 1991). Of these proteins, TTF-I is essential for activating rDNA transcription as its binding initiates an open chromatin conformation (Längst et al., 1997; Längst et al., 1998). This open chromatin structure is stabilised by the HMG-box protein, UBF, which binds ubiquitously across the entire rDNA locus (Roussel et al., 1993). The importance of UBF for supporting RNA Pol I-transcription is suggested by the fact that it largely supplants the histone octamer-based nucleosome as the basic subunit of chromatin at active rRNA genes (Zatsepina et al., 1993; O'Sullivan et al., 2002; Mais et al., 2005). UBF plays a particularly critical role at the rRNA gene promoter, where it serves as a scaffold for the binding of RNA Pol I transcription factors and other processing proteins (Mais et al., 2005; Prieto and McStay, 2007). As this facilitates RNA Pol I promoter escape (Panov et al., 2006), UBF therefore orchestrates the level of rRNA transcription at active rRNA genes (O'Mahony and Rothblum, 1991; O'Sullivan et al., 2002; Chen et al., 2004; Sanij et al., 2008). UBF activity is itself tightly regulated (Sanij and Hannan, 2009), including by post-translational modifications, which control how UBF reactivates rRNA transcription after it has temporarily ceased during mitosis (Voit et al., 1999; Meraner et al., 2006). Recent work has demonstrated that the control of RNA Pol I activity is both necessary and sufficient for the formation of the nucleolus in human cells (Grob et al., 2014).
RNA Pol I function also requires a complex termed FACT, (**fa**cilitates **c**hromatin **t**ranscription), which can be co-precipitated with RNA Pol I (Birch et al., 2009). In contrast to UBF which regulates the initiation of transcription, FACT is specifically required for the efficient passage of polymerases through nucleosomes. In this way, FACT facilitates transcription by RNA Pol I, II and III and is thus essential for transcriptional elongation throughout the nucleus (its relationship with other RNA polymerases present in plants remains to be ascertained). FACT contains two core subunits, SSRP1 and Spt16. In accordance with the essential nature of FACT for transciption, cells in which either subunit is down-regulated display reduced transcription at the 3' regions of the rRNA genes (Birch et al., 2009).
Both initiation and elongation can be co-ordinately regulated independently of UBF and FACT by another large (2-3 MDa) complex, this time involved in maintaining an open chromatin structure throughout active rDNA repeats. This third key complex has been termed B-WICH and its core subunits include WSTF, SNF2h and myosin1 (Percipalle et al., 2006); the complex takes its name from the Williams syndrome transcription factor (WSTF). B-WICH was first reported to be required for efficient transcriptional activity of RNA Pol II.

When its subunits are knocked down, the abundance of Pol III transcripts is also reduced, indicating that its role is not specific for control of rDNA activity (Cavellán et al., 2006; Percipalle et al., 2006). WSTF is also a component of other transcriptional complexes, leading to pleiotropic phenotypes when it is disrupted, and most of these also includes ncRNA molecules (Cavellán et al., 2006). The B-WICH complex appears to be required for the recruitment of some, but not all, of the histone acetyltransferases present at active rDNA (Vintermist et al., 2011) which presumably explains its mode of action.

The B-WICH complex physically interacts with the Cockayne's syndrome protein (CSB) protein, which also has multiple roles in controlling RNA Pol II transcription and DNA repair. CSB is itself required for RNA Pol I transcription, including performing ATP-dependent chromatin remodelling at active rDNA repeats (Lebedev et al., 2008).

CSB can additionally act in an ATP-independent manner within another complex, termed CSB IP/150. This complex also includes XPG (a protein disrupted in certain cases of the human condition, Xeroderma Pigmentosum), RNA Pol I itself, and a transcription factor complex, TFIIH (Bradsher et al., 2002). TFIIH was originally described as a Pol II-specific TF, but has subsequently been shown to activate Pol I transcription in yeast and mouse as well (Iben et al., 2002).

In human cells, CSB IP/150 performs this activation by recruiting a further histone acetyltransferase, PCAF, which leads to transcriptional initiation at rRNA genes which have already adopted an open, poised chromatin state (Shen et al., 2013). Curiously, CSB/TFIIH also recruits a histone methyltrasferase, G9a, which induces H3K9me2, which is generally considered a repressive chromatin mark (Yuan et al., 2007). The association of CSB with RNA Pol I within CSB IP/150 appears to be disrupted by mutations in the other components of the complex (Bradsher et al., 2002). This may be involved in the onset of Cockayne Syndrome, a recessive disorder associated with premature aging and neural degeneration.

As further evidence of the complexity of RNA Pol I transcriptional control, a further such complex has been reported from human cells rather recently (in 2012).

This time, the complex was associated with the establishment of 'poised' or transcription-ready rRNA genes and was named 'nucleosome remodelling and deacetylation' complex (NuRD) (Xie et al., 2012). When cellular growth is attenuated and transcription at rDNA reduced, NuRD is enriched at rRNA gene promoters which are unmethylated, associated with RNA Pol I transcription factors, yet kept silent by the positioning of a key nucleosome. Notably, this complex is also tightly regulated with respect to the growth status of the mammalian cells in question, via pathways as yet unknown. The trend established by UBF and other complexes is that rDNA activity is regulated by the activity of multi-protein complexes, many with chromatin-modifying activities. These complexes, and doubtless more which remain to be discovered, are presumed to ensure RNA Pol I transcription occurs at an appropriate level via multiple, subtle interactions. This has been argued to be the main mode of rRNA synthesis in mammals under normal circumstances (Stefanovsky and Moss, 2006).

Control of rDNA transcription II - the role of silencing complexes

While UBF determines the activity of RNA Pol I at rRNA genes with a suitably open chromatin organisation, it is not responsible for determining the proportion of rRNA genes which are organised in this transcriptionally-permissive manner. In human cells, the principal determinant of the ratio of active and inactive rRNA genes is the multi-protein complex NoRC (**n**ucle**o**lar **r**emodelling **c**omplex) which silences rRNA genes the transcriptional of which is not required. NoRC was discovered in the 1990s and was initially described as a complex of Snf2h/Smarca5 and the large (205 kDa) DNA-binding protein, Tip5 (transcription termination factor 1 (Ttf1)-interacting protein 5) - see Figure 2.

At the sub-cellular level, NoRC was found to colocalise at the NORs.

Since its discovery, it has been established that NoRC is essential both for blocking RNA Pol I transcription at these inactive rDNA loci, and moreover for catalyzing the assembly of rRNA genes into a heterochromatic conformation (Strohner et al., 2001).

In other words, it regulates the level of rDNA transcription by decreasing the ratio of active:inactive rRNA genes, and is therefore complementary to the Pol I transcription factors which reduce or increase transcription at rRNA genes which are already active. NoRC therefore seems to play an antagonistic role to complexes such as B-WICH and CSB IP/150 (see above), although whether these complexes are capable of directly regulating NoRC is unclear.

It has been suggested that complexes associated with euchromatic and heterochromatic rDNA might be recruited to rDNA at different stages during nuclear division, as active and inactive rRNA genes undergo replication at different timepoints (Yuan et al., 2007).

The possibility of cross-talk occurring between these complexes merits further investigation.

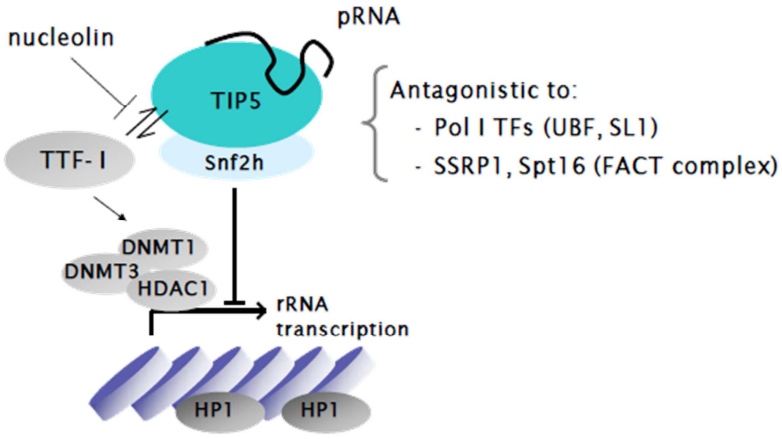

Figure 2. Predicted model for the control of rDNA silencing by the Nucleolar Remodelling Complex (NoRC). NoRC consists of Tip5 and Snf2h. Tip5 is recruited to rRNA by transcription of a non-coding RNA termed pRNA (bold line). Binding of NoRC to rRNA genes leads to TTF-I dependent recruitment of chromatin remodelling enzymes (gray) and repression of rRNA transcription. Binding of NoRC to rDNA may require, or be stabilised by, Heterochromatin Protein 1 (HP1) and acetylated H4K16 residues (not shown).

The key role of NoRC in control of human rDNA transcription was first indicated by *in vitro* experiments showing that it was able to induce nucleosome sliding (of the sort required for rRNA gene silencing) along isolated DNA. This activity was dependent upon ATP, and the N-terminal tail of histone H4 (Strohner et al., 2001), indicating a direct affect upon nucleosome organisation. Based on its chromatin-remodelling capabilities and colocalisation with UBF in the nucleolus, NoRC was therefore recognised as a candidate regulator of rDNA transcription control. This hypothesis was proven shortly afterwards with the demonstration that association of NoRC with rDNA repeats caused rRNA genes to become heterochromatic and transcriptionally silent (Santoro et al., 2002). The biochemical nature of this heterochromatic DNA was indicated by the discovery that methylated rDNA genes could be immunoprecipitated in conjunction with the two components of NoRC (Tip5, Snf2). These proteins are additionally co-immunoprecipitated with hypoacetylated and hypermethylated histones, and with heterochromatin protein 1 (HP1). This indicated again that NoRC was a complex specifically associated with methylated, inactive rDNA, and that the rRNA genes in this rDNA was likely to be organised into heterochromatin. NoRC was therefore established as a key element of the heterochromatic fraction of rDNA previously identified in microscopy-based studies (Santoro et al., 2002).

The study of Santoro et al. furthermore determined that the role of NoRC in heterochromatic rDNA was likely to be causal rather than correlative. Over-expression of Tip5 caused a transfected rDNA reporter plasmid to become resistant to cleavage by the methylation-sensitive restriction enzyme HpaII, indicating that NoRC was able to induce DNA methylation *in vivo* (Santoro et al., 2002). As expected, treatment with the DNA methylation inhibitor 5-azacytidine reversed this effect (Santoro et al., 2002). NoRC is therefore responsible for inducing the methylation of rRNA gene promoters in mammalian cells. Importantly, the NoRC component Tip5 also acts as a binding site for the transcription termination factor TTF-I (Németh et al., 2004) which had previously been shown to be essential for repressive chromatin remodelling at the promoter of silenced rRNA genes (Längst et al., 1997; Längst et al., 1998). It is therefore believed that NoRC establishes and maintains the heterochromatic organisation of inactive rDNA repeats via recruitment of TTF-I and other enzymes which induce DNA methylation and the deposition of repressive histone modifications.

These include H3K9 methylation and H4 hypoacetylation (Santoro et al., 2002) and may be mediated by Tip5 binding to H4K16ac via a bromodomain, which appears to stimulate recruitment of chromatin-remodelling enzymes such as HDAC1, DNMT1, DNMT3, and SNF2h to the rDNA (Zhou and Grummt, 2005), as shown in Figure 2. As an additional level of control, the binding of NoRC can be countered by an abundant nucleolar protein, nucleolin, which reduces the ability of TTF-I to bind to its target terminator region and thus reduces Tip5 recruitment (Cong et al., 2012).

Although initially described as a complex of proteins, it was subsequently discovered that NoRC also contains an essential RNA component. A non-coding RNA is produced from transcription of the rRNA gene promoter, termed pRNA, which binds to Tip5 and is required for targeting of the complex to the rDNA promoter. pRNA binding mediates DNMT3b association with the rRNA gene promoter (Bierhoff et al., 2011).

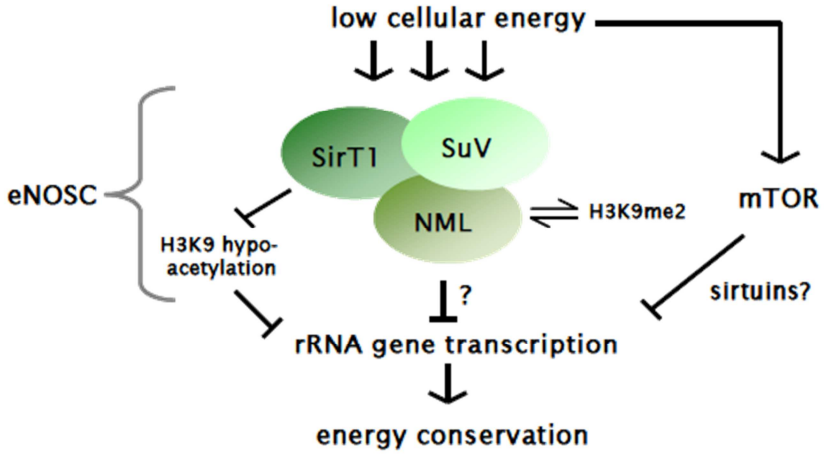

Figure 3. The regulation of rRNA gene transcription in response to cellular energy status. The eNOSC complex consists of NML (nucleomethylin), SuV (SUV39H1) and SIRT1 (sirtuin 1), and responds to signals indicating low cellular energy status by silencing rRNA genes and repressing ribosomal RNA production. At least one other pathway, involving mammalian Target Of Rapamycin (mTOR) operates in parallel. For details, and possible interaction of NMP with NMNAT1, see text.

pRNA-binding is dependent upon the acetylation of Tip5 by an interacting acetyltransferase, MOF (Males Absent on the First), which occurs only on lysine residue K633 and is essential for the gene-silencing activity of NoRC (Zhou et al., 2009). The pRNA also acts to allow the binding of poly(ADP-ribose)-polymerase-1 (PARP1) which is essential for NoRC to silence RNA Pol I and subsequent heterochromatinization of silent rDNA (Guetg et al., 2012).

rDNA silencing III - regulation of RNA Pol I silencing by cellular energy status

The manner in which the numerous complexes described above interact to control RNA Pol I activity remains a difficult issue, but the overall purpose of this complexity is clearly to allow rDNA activity to be attuned to the environment. One cellular condition to which rDNA appears to be particularly sensitive is the energetic status of the cell, which can (in mammals) regulate rRNA transcription by several different signalling pathways. This is unsurprising given the high demand that rRNA synthesis makes on the cell's ATP reserves, sometimes estimated as half of the cell's energetic output (Sollner-Webb et al., 1985; Grummt and Voit, 2010). Therefore, rDNA transcription must occur at a level suitable to meet the translational demand of the cell, while also taking account of the ATP available in the cell at that time.

The importance of this link, and its wide-ranging impacts on the healthy functioning of an organism, have been most widely established by studies of the budding yeast (*Saccharomyces cerevisiae*) protein, Silent information regulator 2 (Sir2). Sir2 is an NAD^+-dependent protein deacetylase which is sensitive to cellular energy status (Fritze et al., 1997). Amongst other targets, Sir2 is able to deacetylate histones, removing H3-acetyl marks associated with open, transcriptionally active chromatin and potentially allowing the addition of H3-methyl marks, which are associated with transcriptionally inactive heterochromatin, Sir2 is known to be able to regulate rDNA transcription by RNA Pol I, and it has been proposed that this is linked to its histone/protein deacetylation capabilities (Fritze et al., 1997; Smith et al., 1998; Straight et al., 1999; Blander and Guarente, 2004; Machín et al., 2004; Ford et al., 2006). The ramifications of the role of Sir2 and related proteins (the so-called sirtuins) for human health is a topic of intense scientific debate, as several lines of evidence suggest that Sir2 is a key regulator of ageing and longevity in yeast, nematodes and metazoans. This may occur through mimicking the effects of caloric restriction. It has been claimed, for example, that increased cellular dosage of Sir2p in *S. cerevisiae* can expand yeast life span by suppressing genotoxic recombination between rDNA repeats of the sort discussed below (Lin et al., 2000). Here I will focus specifically on the role of Sir2 and related proteins in the direct control of transcription at the rDNA locus. For the details of the ongoing debate over the more controversial claims made for these proteins, the reader is referred to dedicated commentaries (Finkel et al., 2009; Lempiäinen and Shore, 2009; Imai and Guarente, 2010; Sebastián et al., 2012).

Sirtuins share the common feature that they use NAD+ as a cofactor in the deacetylation of peptide targets, which may make them particularly suitable as sensors of cellular energy levels. The closest human homolog of ScSir2p is SIRTUIN 1 (SIRT1), which binds throughout rDNA repeats regardless of

their transcriptional state, although the majority of it is distributed in other parts of the nucleoplasm (Michishita et al., 2005).The importance of the links between rDNA transcriptional activity and cellular metabolic status was underlined by the discovery that SIRT1 is part of a protein complex termed eNoSC within human cells (Murayama et al., 2008). This complex contains the H3K9me2-binding protein, Nucleomethylin (NML), SIRT1 and SUV39H1 (Figure 3). NML had previously been shown to be at least partially localised in the nucleolus (Andersen et al., 2005) and was identified by MS-ChIP to be bound to H3K9me2 at transcriptionally inactive rDNA (Murayama et al., 2008). *In vivo* over-expression of NML reduced the accumulation of nascent rRNA, an effect ablated by knock-down of SIRT1 (Murayama et al., 2008). The authors of this study proposed a model of 'co-ordinate binding' of NML and SIRT1 occurring specifically at silenced rRNA genes, with SIRT1 triggering H3K9 hypoacetylation and subsequent methylation at the same site. Methylation of hypoacetylated H3K9 was found to be at least partially due to the methyltransferase activity of SUV39H1, which also participates in the same complex. This suggests a model in which SUV39H1 and SIRT1 compete for modification of the same lysine residue. Many aspects of the regulation of SIRT1 within eNoSC remain unclear: two potentially significant points are that nucleomethylin (NML) interacts with a NAD+ synthesis enzyme called NMNAT1, which also contributes to the silencing of rDNA (Song et al., 2013) and physically associates with SIRT1 (see Figure 3); and that SIRT1 may be able to acetylate SUV39H1 in a manner that disrupts their binding. As a further complication, there is some evidence that NML - which has a methyltransferase-like domain - may act via methylation of some downstream target (Murayama et al., 2008). Although most commonly associated with core histones, many other proteins can be subject to methylation. eNoSC binding at rDNA is increased in HeLa cells under conditions that reduce available cellular energy (glucose starvation) and this has been found to protect such cells from energy deprivation-induced apoptosis. As the discovers of eNoSC point out, rDNA-silencing may be critical for this although interaction with other apoptosis-inducing pathways cannot be excluded (Murayama et al., 2008). eNoSC does not, however, appear to act on, or alter the methylation of, p53.

Other human sirtuins have different sub-cellular distributions, including the cytoplasm and mitochondria, while SIRT6 and SIRT7 are also nuclear. The only evidence for strong nucleolar enrichment was for SIRT7 (Michishita et al., 2005). SIRT7 is also of considerable interest for regulation of mammalian rDNA as it can also function as an activator of RNA Pol I (Ford et al., 2006). Although still relatively understudied, it has been shown that SIRT7 physically interacts with UBF and remains stably associated with both UBF and rDNA during mitosis (Grob et al., 2009; Tsai et al., 2012). After mitosis, SIRT7 is essential for the resumption of rRNA gene transcription during telophase, following a phosphorylation-induced conformation change performed by an unknown kinase (Grob et al., 2009). SIRT7 acts *in vivo* as an NAD^+-dependent deacetylatase of the transcription-permissive histone modification H3K18Ac at gene promoters, thus reducing transcription (Barber et al., 2012). Misregulation of this activity has been linked with several aspects of tumour progression in human cells and in mice, and a significant increase in SIRT7 expression in breast cancer samples has been reported (Ashraf et al., 2006; Barber et al., 2012). No link between these correlations and the control of RNA Pol I has been reported, and the control of rDNA by SIRT7 has been suggested to be cell- or tissue-specific (Barber et al., 2012), although links between rDNA misregulation, SIRT7 and oncogenesis could easily remain to be discovered. Likewise, the requirement for NAD+, which varies in availability with a cell's energy status, may represent a significant link between to control of rRNA transcription in the same way that has been claimed for SIRT1.

In several eukayotic cells, another energy-sensing pathway based around signalling by Target of Rapamycin (TOR), which is a major component in the nutrient signalling machinery (Figure 3, right hand side), has been argued to control to the transcription of rRNA (Schmelzle and Hall, 2000; Kim et al., 2002). This pathway controls RNA Pol I transcription via chromatin remodelling at rDNA (Tsang et al., 2003), perhaps directly as TOR itself binds to rRNA gene promoters (Tsang et al., 2010). In yeast, TOR assists rDNA stability by increasing Sir2p association with rDNA (Ha and Huh, 2011) and also affects Rrn3p levels (Philippi et al., 2010). Whether mammalian TOR (mTOR) is able to interact with any of the sirtuins is not currently clear (Blagosklonny, 2010). Interestingly, the NuRD complex may also be regulated with regard to cellular energy status, as ATP is required to reverse the associated silencing (Xie et al., 2012). Again, the biological significance of this fact remains to be fully established.

The discovery of the presence of these multiple complexes poses several intriguing questions. One which remains to be resolved is the issue of how the different complexes which regulate rDNA function in human cell lines interact with one another. Another is why such a complex system is required to accomplish this regulation. The authors of Murayama et al., 2008 suggest an intriguing model in which NoRC is required for initiating silencing

(following its binding to the transcriptional terminator element of rDNA via TTF-I - see Strohner et al., 2001) - but it is eNoSC, which appears to bind ubiquitously to rDNA throughout the nucleolus, that ensures chromatin silencing is propagated. This suggests coordinate regulation by different complexes might occur, or that different complexes might act to reinforce each other's activities in order to ensure that regulation of rRNA transcription and silencing are both robust and responsive.

Roles for histone modification in controlling RNA Pol I transcription

As noted above, the control of rDNA by NoRC is initiated when the complex binds to nucleosomes containing H4K16ac via a bromodomain in the Tip5 protein (Zhou and Grummt, 2005). This leads to the subsequent recruitment of multiple histone deacetylases and histone methyltransferases which induce a heterochromatic organisation at the locus and suggests a model in which different histone modifications direct various stages of euchromatin and heterochromatin formation at the rDNA locus. As testament to this, pharmacological inhibition of histone-modifying enzymes commonly leads to altered rRNA expression levels and nucleolar morphology, and changes to nucleolar dominance. The association between H4 and NoRC is not the only instance in which a major regulatory cascade requires binding to histones. H3/H4 dimer was identified as a core component of the major *S. cerevisiae* RNA Pol I TF, Upstream Activating Factor (UAF) (Keener et al., 1997), in addition to four non-histone protein components. Reduction of H3 synthesis inhibited rRNA transcription and was associated with reduced efficiency of multiple processes including initiation/elongation, and rRNA processing, although in some cases these were indirect effects (Nomura et al., 2004; Tongaonkar et al., 2005; Jones et al., 2007).

In mammalian cells, too, classic biochemical studies have shown that the H1 linker histone acts as a binding site for the abundant rDNA-associated protein, nucleolin (Erard et al., 1988) which is itself now known to have nucleosome chaperone activity (Angelov et al., 2006). Nucleolin has an affinity for unmethylated rRNA gene promoters and appears to ensure deposition of H3K4me3 and other histone modifications associated with a transcription-permissive state. On the other hand, nucleolin depletion causes H3K9me2 to accumulate (Cong et al., 2012). The B-WICH complex also triggers altered rDNA activity, this time by recruitment of histone acetyltransferases (Vintermist et al., 2011). Hence, suitably modified nucleosomes also have roles as downstream effectors of chromatin regulation at rDNA, acting to reinforce the open chromatin structure induced by nucleolin, and thereby helping to maintain rRNA transcription at active rDNA repeats. As other histone chaperones have also been shown to control the activity of rDNA in human cells (Kuzuhara and Horikoshi, 2004) and more recently in Arabidopsis (Li and Luan, 2010), histone chaperone activity may have more general roles in controlling the balance between transcribed and silenced rRNA genes.

Although covalent modifications at the H3K4 and H3K9 residues are well-known regulators of transcription and other cellular processes (Iizuka and Smith, 2003), additional histone modifications may also control rRNA transcription or other nucleolar functions, and it is possible that some are enriched or specific in the nucleolus (McKeown and Shaw, 2009). In mammals, for example, RNA Pol I transcription is encouraged via H3K56 acetylation (Chen et al., 2012), and as noted above H3K18 acetylation may be involved in regulation of rDNA by SIRT7.

Active histone demethylation has also been implicated in rDNA control: JHDM1B is an evolutionarily conserved protein demethylase which regulates animal growth and may act as a tumour suppressor in mice (see Frescas et al., 2007 and references therein). JHDM1B binds to human rRNA genes in a stable manner reminiscent of UBF, and silences rRNA expression via demethylation of H3K4me3. This effect has an absolute requirement for its JmjC domain and appears to be a direct effect as H3K9me2 remains unaffected.

Reduced expression of JHDM1B is associated with increased rRNA synthesis and accelerated cell growth, and has been reported to occur in brain tumour cells (Frescas et al., 2007).

Epigenetic control of RNA Pol I silencing and the importance of feedback

In the preceding sections, some of the protein complexes which regulate the silencing of rDNA in mammalian and yeast cells have been described. It has however been made clear that important details about how the different regulatory components interact with each other remain to be determined. An important conceptual contribution to this problem comes from research in plant hybrids, which has suggested that epigenetic regulators are able to establish 'self-reinforcing loops' at active and inactive rRNA genes.

The details of how these loops are established, and their wider relevance, are discussed in the following section.

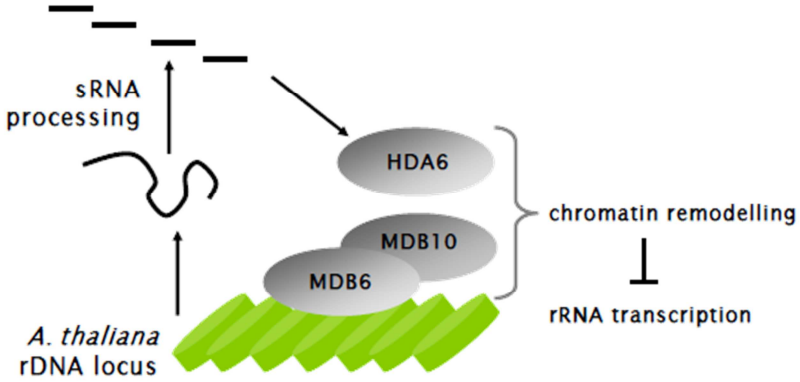

Figure 4. The regulation of rRNA gene transcription in the model plant, *Arabidopsis thaliana*. As in human cells, recruitment of rRNA silencing machinery is effected by production of non coding RNA from the rDNA repeats (e.g. 24 nt-siRNAs; short black lines); these lead to recruitment of DNA methyltransferases and methyl-binding domain proteins (MDB6, MDB10) and histone deacetylases (HDA6) which maintain silencing of rRNA genes by chromatin remodelling. Compared with rRNA gene regulation in humans, there is no NoRC, and no role has been reported for the plant ortholog of HP1.

In hybrid organisms, it commonly occurs that chromatin remodelling of NORs inherited from different progenitors ensures that only the rDNA inherited from one parent is transcribed while the other remains silenced (so-called nucleolar dominance). The establishment of active or repressed states at the two sets of NORs in interspecific hybrids within the Arabidopsis genus involves differential DNA methylation reinforced by remodelling of the rRNA gene promoter by a complex involving the histone deacetylase HDA6, a histone deacetylase-like HDT protein, and an H3/H4 dimer (Lawrence et al., 2004; Earley et al., 2006).

The same loop may also control differences in expression between different rRNA genes within the same NOR. *hda6* mutants show aberrant accumulation of rDNA-encoded small RNAs, leading to the suggestion that siRNA-triggered pathways might also be involved in this chromatin remodelling loop as in animals. Furthermore, two mutants defective in siRNA biogenesis (*dcl3*, *rdr2*) disrupt the normal patterns of nucleolar dominance when crossed in an inter-specific manner (Preuss et al., 2008). The promoters and intergenic spacers of the silenced rRNA genes derived from *A. thaliana* were associated with the production of 24nt-siRNAs from both DNA strands, which were lost if *DCL3* activity was ablated. In agreement with this, nucleolar dominance in Arabidopsis requires Pol IV and Pol V (Pontes 2006); exactly how these siRNA direct DRM2-mediated methylation, or even if they are causative for silencing at all, remains unclear. The model proposed by Preuss et al., 2008 was that transcription (possibly from promoter-like sequences in the intergenic spacers) directs 24-nt siRNA production from one of the rRNA alleles (Figure 4).

The demonstration that noncoding RNA (ncRNA) is involved in rDNA silencing in Arabidopsis marks a point in common between animals and plants, as a long ncRNA plays a similar role in mouse (Mayer et al., 2006; Mayer et al., 2008). On the other hand, no role has been reported for shorter RNA in the silencing of inactive rDNA in mammals (as far as is known), nor for long ncRNA in plants. A further point regarding comparisons between different eukaryote taxa is that there is no obvious ortholog or even functional analog of Tip5 in Arabidopsis, suggesting that there is no direct equivalent of NoRC in plants. Physical association with the siRNA/epigenetic machinery might be possible however - supported by colocalisation within Cajal bodies which may lie within the nucleolus (Pontes et al., 2006) and could also therefore allow physical association with rDNA. Curiously, antisense transcription of human rDNA can also occur, this time acting to promote H4K20me3, a repressive chromatin mark (Bierhoff et al., 2011). It can be concluded that effects of non-coding RNA (whether siRNAs or lncRNAs) on RNA Pol I transcription are likely to be widespread, although the details of how they act are likely to vary between species, and interact with other regulators in complex ways.

Nucleolar dominance also depends on an Arabidopsis methylcytosine binding domain protein, MBD6, being localised to silenced rRNA by the *de novo* DNA methyltransferase DRM2 (Preuss et al., 2008). This pathway also includes other effectors of RNAi (DCL3, RDR2) and leads to methylation of the sequence, and subsequent binding of MBD6 (Figure 4). This change is coincident with association with heterochromatic histone marks and DNA condensation, which are presumed to ensure that silencing spreads across the entire NOR and is maintained in a robust manner. This study also demonstrated that mutants and/or RNAi knock-downs of genes encoding many other DNA methyltransferases, RDR proteins and DCL proteins did not affect nucleolar dominance (Preuss

et al., 2008). Therefore, epigenetic regulation of rDNA activity is under the control of a particular subset of siRNA and chromatin-modifying pathways, at least in the *A. thaliana* X *A. arenosa* system being studied.

The precise role of MBD6 was not clear at this time - when knocked down, it caused siRNA levels to increase slightly, suggesting that it might be responsible for negative feedback within this loop of the system.

Presumably, the complex pathways which control rDNA silencing in Arabidopsis are necessary to ensure that its chromatin state - and hence the epigenetic regulation of rRNA transcription - is stable both temporally and spatially. Its self-reinforcing nature means that it can act across very large genomic regions (many megabases in length), and be transmitted with fidelity through nuclear divisions, regardless of cell differentiation. It should however be added that the actual *purpose* of nucleolar dominance remains unclear, although a likely hypothesis is that it prevents the production of hybrid ribosomes which might function with reduced efficiency. This leads to the important conclusion that nucleolar dominance represents a modification of endogenous rRNA control, albeit in a hybrid background, with many regulatory features preserved. However, nucleolar dominance does differ in other ways (e.g. the differing requirements for *de novo* DNA methylation (Preuss et al., 2008; Earley et al., 2010)), perhaps because it has evolved to effect a stable silencing effect, rather than allowing environmental response.

An interesting recent report identified that the rRNA genes of *Arabidopsis thaliana* are not as uniform as previously thought.

Rather, they include at least four variants forms, which show distinct features in their regulation (Pontvianne et al., 2010).

The possibility of rRNA gene variants being functionally distinct (either under wild-type conditions, or following tumourigenesis) would be an interesting question to address. In Arabidopsis, it has been shown that different H3K9 and K27 histone methyltransferases are responsible for control of expression of these rRNA gene variants in *A. thaliana* and in nucleolar dominance between different rDNA from different parents in *A. thaliana* X *A. arenosa* hybrids (Pontvianne et al., 2012).

This again suggests that silencing of rDNA during nucleolar dominance is in some respects a guide to rDNA control in non-hybrid organisms, and in other respects is different. Curiously, mutation of certain histone methyltransferases also leads to preferential replication of certainly variants (Pontvianne et al., 2012), implicating the covalent modification of histones in preventing replication slippage as well .

Silencing of aberrant RNA Pol II transcription in nucleoli

A further role for the rDNA silencing pathways discussed above came from *Saccharomyces cerevisiae* and concerned the mechanisms which ensure that sequences at the rDNA locus are not promiscuously transcribed by RNA Pol II. RNA Pol I is the most specific of the RNA polymerases found in eukaryotes, being solely responsible for transcription of rDNA. rDNA transcription is in turn specific for RNA Pol I, and is not transcribed by RNA Pol II machinery (Grummt, 2003). It has been known for some years that this is because of chromatin-related pathways which act to prevent Pol II transcription from occurring and hence ensure polymerase fidelity. Accordingly, if an rRNA gene is cloned into a different genomic location, then transcription by Pol II can instead occur.

In *S. cerevisiae*, repression of RNA Pol II transcription within the rDNA repeats requires the HDAC Sir2p (Smith and Boeke, 1997; Smith et al., 1998). The repressive activity of Sir2p requires the structural chromatin protein, condensin which causes rDNA to adopt the correct chromatin organisation and is essential for ensuring that sufficient Sir2p levels is retained at the rDNA locus (Smith et al., 1998; Machín et al., 2004). ScSir2 is a component of another rDNA-silencing complex which has been termed RENT (**re**gulator of **n**ucleolar silencing and **t**elophase exit (Straight et al., 1999)) which is anchored to rDNA by the component protein Net1 (the name derives from the essential role of RENT in coordinating mitotic exit and the resumption of rRNA transcription (Cockell and Gasser, 1999)). Yeast mutant screens have suggested that Set1p is also essential for blocking RNA Pol II transcription in the rDNA locus, this time acting via deposition of H3K4me3 which in this instance is associated with transcriptional silencing (Briggs et al., 2001). This activity is independent of Sir2, and hence of the RENT complex (Bryk et al., 2002), and presumably acts to reinforce the repression of RNA Pol II activity. The role of histone modifications in preventing RNA Pol II transcription at rDNA in mammals is not clear, although there is evidence that rDNA methylation is required (Gagnon-Kugler et al., 2009) which indicates that correct chromatin organisation is again essential.

The repression of Pol II-mediated transcription from the intergenic spacers of rDNA is another important function of HDA6 in *Arabidopsis thaliana*, and an *hda6* allele was found to produce Pol II-transcribed RNA from cryptic promoters throughout the rDNA. This in turn leads to siRNA production, which normally causes induction of heterochromatin but does not appear to do so in this

case (Earley et al., 2010). Instead, the rDNA in question was found to be associated with histone modifications permissive for transcription such as H3K9ac, H3K14ac, H4K16ac. This indicates that siRNA production does not necessarily lead to the induction of repressive, heterochromatin-forming loops (Earley et al., 2010). This lead to the authors proposing a model in which HDA6 directly regulates Pol I and Pol II transcription, completely blocking the latter within the rDNA. In the most simple scenario, it could achieve this via deacetylation of histones. In this model, DNA methylation acts in a reinforcing role, perhaps by ensuring that the HDA6 protein remains concentrated at rDNA.

Before concluding this section, it is interesting to note that correct chromatin organisation of the rDNA repeats may be required not only for regulating their transcriptional activity, but also for their stability (Peng and Karpen, 2006). This report demonstrated that, in *Drosophila melanogaster*, chromatin-regulation pathways (including an RNAi pathway involving *Dicer-2* and *Su(var)3-9*, and those responsible for dimethylation of H3K9) are necessary to prevent the occurrence of inter-repeat recombination. This ligase-mediated recombination, to which tandemly repeated DNA sequences are always prone, has the potential to liberate genotoxic circular DNA molecules, which were termed extrachromosomal circular repeat DNA (eccDNA). This may explain why so many pathways have evolved to prevent unregulated Pol II to occur at rDNA repeats. How chromatin actually prevents inter-repeat recombination between repeated DNA sequences is not clear, although in human cells an analogous process is known to involve the TTF-I complex (Guetg et al., 2010). Increased copy-number of certain rRNA gene variants is observed in histone methyltransferase mutants in Arabidopsis, which may suggest that correct histone modification also contributes to rDNA stability in plants (Pontvianne et al., 2012). In mammals, too, recent work has suggested that NoRC, or at least one of its component proteins, is important for the overall chromatin organisation of the rDNA repeats. The authors indicate that over-expression of Tip5 causes general changes to DNaseI accessibility (Zillner et al., 2013), a standard measure of chromatin compaction. This need not, however, be a direct effect. Given that a similar effect is mimicked by serum starvation, it is possible that there the eNoSC complex may also influence this process, although the direct physical association of Tip5 with the nuclear matrix reported here (Zillner et al., 2013) could be an additional way of amplifying local chromatin remodelling into an rDNA-wide effect. As an interesting aside, TTF-I may also have more general functions in maintaining heterochromatin stability throughout eukaryotic nuclei. Its ability to bind and stabilise heterochromatin repeats is for example essential for genomic integrity at centromeric repeats (Guetg et al., 2010) and suggests that its role at rDNA has evolved to exploit its chromatin remodelling capabilities.

Disruption of rDNA silencing and cancer prognosis

Nucleoli have long been observed to exhibit altered morphologies in different classes of cancer cell (Derenzini et al., 2000; Boisvert et al., 2007; Montanaro et al., 2008). In fact, such changes have been used as diagnostic and prognostic indicators since the early twentieth century (Shiue et al., 2010). The first report of such a link appears to have been by Pianese in 1896 (recorded in Donati et al., 2012) and was well-established by the later part of the twentieth century (Gani, 1976). This led to the hypothesis that there might be a correlation between tumourigenesis and the levels of rRNA/ribosome synthesis within the cell. At the molecular level, it has been noted that at least seven proto-oncogenes and/or tumour suppressors can alter overall levels of translation (Ruggero and Pandolfi, 2003). Furthermore, multiple ribosomal proteins are also found to be over-expressed in many different tumour types. Correlations between elevated rRNA levels and malignant transformation have also been observed (Ruggero and Pandolfi, 2003; White, 2008). This correlation has been shown to be consistent in a study of six different tumour types, and intriguingly tends to become stronger with advancing cancer stage (Williamson et al., 2006). rRNA has also been found to be over-expressed in many prostate cancer cell lines (Uemura et al., 2012).

Such studies provide convincing evidence that the changes to nucleolar morphology observed in many cancers are likely to be due to increased rRNA synthesis within these cells. This also seems to be correlated with enhanced translational capacity at the ribosome. However, most commentators traditionally considered this to be a simple consequence of the altered metabolic state of tumour cells following the resumption of proliferative growth (Donati et al., 2012). In other words, the activation level of the rDNA and associated nucleolar changes are analogous to the effects observed in non-cancerous cells when they are actively growing (e.g. in S-phase of the cell cycle). As cancer cells typically display elevated metabolic rates and hence have greater translational requirements compared with non-cancer cells, this could potentially explain the correlation between altered nucleolar morphology/rDNA transcription levels in cancer. Altered nucleolar organisation and levels of ribosome components have therefore been regarded as useful prognostic markers, but have not

been considered from the perspective of cancer causation.

Cancer causation and RNA Pol I misregulation

As described above, RNA Pol I activity is associated with rapid cell growth and division during cancer, leading to changes to nucleolar morphology of potential prognostic use. However, some researchers have argued that there may also be a causal relationship between misregulation of rDNA and the occurrence of cancer (Ruggero and Pandolfi, 2003; White, 2008; Montanaro et al., 2012). In support of this, a recent study by Bywater et al. has demonstrated that aberrant hyperactivation of Pol I is causally required for malignancy in several tumours (see Bywater et al., 2012; associated commentary (Hannan et al., 2012)) and many oncogenic and tumour-prevention pathways upregulate RNA Pol I transcription at rDNA. One immediate consequent of this is that RNA Pol I has emerged as a potential chemotherapy target. One drug which may have important therapeutic potential in this regard is quarfloxin, a specific RNA Pol I-inhibitor which has been investigated for use in the treatment of neuroendocrine carcinoma (Drygin et al., 2009). Quarfloxin appears to confirm the relationship between altered rRNA transcription and prognosis of certain cancer types and has also been proposed for use in clinical trials for treatment of lymphoma and leukemia.

If RNA Pol I can be causative for cancer progression, this also suggests that there should exist cellular mechanisms which regulate rRNA transcriptionas a means of tumour prevention. rRNA synthesis is generally limited by tumour supressors such as p53, ARF, pRB and PTEN via a combination of direct and indirect mechanisms (Budde and Grummt, 1999; Zhai and Comai, 2000; White, 2008; Wang et al., 2013). For example, in cell lines derived from gastric cancers which had lost expression of the tumour suppressor ZNF545, restoration of its expression suppressed cell proliferation and induced apoptosis due to inhibition of rRNA transcription. Importantly, this silencing was linked to restoration of the normal heterochromatin marks at the promoters, including HP1β binding and H3K4 hypomethylation (Wang et al., 2013). Direct regulators of RNA Pol I transcription activity act by various mechanisms, including promoter remodelling, PIC formation, and elevated elongation rates. Such regulators include known oncogenes such as AML1-ETO and the tumor suppressors p53, pRb, and p14ARF (Hannan et al., 2012). Various lines of evidence indicate that these pathways often target RNA Pol I regulators/TFs such as UBF or RRN3. For example, the Retinoblastoma protein is required for suppression of UBF (Cavanaugh et al., 1995), possibly by preventing SL-1 binding (Hannan et al., 2000). Studies such as these indicate that rDNA misregulation during tumourigenesis, either by increased transcription by RNA Pol I at active rRNA genes, re-activation of previously silenced rRNA genes, or both.

The human TF complex, c-Myc, is of particular interest in the study of eukaryote transcription as it is able to regulate RNA Pol I, II and III (Gomez-Roman et al., 2006). It has therefore been proposed to coordinate rDNA transcription with the biosynthesis of other ribosome components (reviewed (Lempiäinen and Shore, 2009)) i.e. the ribosomal proteins (translated from mRNA transcribed by RNA Pol II) and the 5S rRNA (transcribed by RNA Pol III). The gene encoding c-Myc is considered a proto-oncogene, which may be due to far-reaching effects of c-Myc on transcription. In principle, c-Myc could be able to single-handedly increase the concentration of ribosomes within a cell and so drive growth and tumourigenesis (Arabi et al., 2005; Grandori et al., 2005; van Riggelen et al., 2010). For example, rRNA upregulation in prostate cancer lines closely correlates with levels c-Myc levels. c-Myc may be particularly important for aberrant activity of the Pol I complex during tumourigenesis (Uemura et al., 2012) although it should be noted that probably induces many pleiotropic effects on other cellular processes as well. This may make it difficult to describe the precise nature of the link and is one reason why c-Myc has not been successfully targeted by chemotherapy. Identification of some of its functional partners may help to resolve these complications (Chan et al., 2011). One possible part of this network is the kinase ERK, which is able to stabilise c-Myc by phosphorylation and can also activate both UBF and RRN3 in the same way, providing simultaneous routes to rDNA activation (Stefanovsky et al., 2001). As a final point, it has been reported SIRT7, a potential rRNA gene regulator associated with tumour progression (see above) may also regulate the expression of ribosomal proteins (Barber et al., 2012). It is possible that certain other rDNA regulators, including sirtuins, might also have be able to alter cellular translation in a concerted way reminiscent of c-Myc.

Another route to aberrant rRNA transcription (this time independent of c-Myc and ERK) is mediated by the growth factor receptor ErbB2, which acts as a TF for RNA Pol I. ErbB2 is upregulated in many cancers and is associated with increased metastasis and other aggressive traits. It appears to act by increasing the binding affinity of the polymerase to rDNA at transcription sites within the nucleolus, identified by visualisation of BrUTP incorporation sites (Li et al., 2011). Co-immunoprecipitation experiments indicated that ErbB2 binds to RNA Pol

I as part of a complex with β-actin, and this binding leads to increased transcription levels *in vivo* (Li et al., 2011). Over-expression of ErbB2 lead to increased protein synthesis, indicating that RNA Pol I up-regulation can indeed remove the limiting factors on total cellular translation. rRNA gene silencing can also act to limit tumourigenesis via the tumour suppressor ARF, which triggers TTF-I nucleolar exit and thus reduced rRNA transcription. ARF and one of its target proteins, MDM2 (which has E3 ubiquitin ligase activity) compete for binding to TTF-I at overlapping sites. When levels of ARF are artificially ablated, MDM2 accumulates, binds to TTF-I at increased levels and catalyzes its ubiquitinylation. This reduces the concentration of TTF-I in the cell via by targeting it for proteosomic degradation (Lessard et al., 2012).

To understand the cellular networks that are proposed to link RNA Pol I regulation to cancer, it is also necessary to consider in more detail why misregulation might be associated with tumour progression, beyond the observation that cancer cells have high metabolic requirements. One fact which may be critical is the discovery that the key oncogene p53, which as noted above may regulate RNA Pol I activity, might also be partially controlled by it. It has been reported that the relative levels of ribosomal RNA (transcribed by RNA Pol I) and the rate of ribosomal protein synthesis can lead to changes in p53 levels in mammalian cells (Donati et al., 2011a). The likely complexity of any such interactions is demonstrated by research from the same group suggesting that Pol I can also control cellular proliferation via a p53-independent pathway. This pathway may instead involve E2F-1 (Donati et al., 2011b).

Sensing of reduced cellular energy status in the nucleolus by eNoSC has been proposed to play a key role in p53 accumulation, eventually leading to apoptosis or cell cycle arrest (Kumazawa et al., 2011). This is argued to occur via reduced rRNA synthesis which leads to release of Myb-binding protein 1a (MYBBP1A) from the nucleolus. This protein catalyzes acetylation of p53, which is a key element in causing it to accumulate and function (Kumazawa et al., 2011). Conversely, failure to down-regulate RNA Pol I could therefore block p53 hypomethylation as MYBBP1A would remain associated with the nucleolus even under pro-apoptopic conditions. This mechanism provides an attractive explanation of how p53-mediated cell cycle arrest could rely upon correct transcription of rRNA genes, and a route towards therapeutic intervention (Donati et al., 2012). It has previously been argued that hypoxia-induced acidification of human culture cells can promote the binding of rDNA to von Hippel-Lindau tumor suppressor protein (VHL) which in turn reduces the RNA Pol I-activating capacity of UBF (Mekhail et al., 2006). This would indicate that hypoxia can directly reduce ribosome production via pH-sensitive protein-protein interactions as well as via adverse affects on cellular energy status and is an additional pathway of relevance for disrupted response of the RNA Pol I machinery during tumourigenesis.

The sirtuin HDACs have also been proposed to play roles in cancer. HsSIRT1, for example, has been implicated in different forms of human cancer although determining precisely what role it is playing is not always clear (Deng, 2009). It has the potential to play a key part in cancer progression due to its interactions with p53 and other tumour supressors. The regulation of SIRT1 is correspondingly complex, as reviewed elsewhere (Liu et al., 2009), so its activities - and their significance to rDNA silencing - will only be briefly summarised here. SIRT1 is able to deacetylate p53 and physically interacts *in vivo* with several other proteins which regulate this deacetylation ability. Its expression is in turn controlled by p53 via two binding sites in its promoter, which repress its expression; SIRT1 can also repress its own expression via an autoregulatory loop as part of a complex with HIC1 (Chen et al., 2005). A particularly intriguing possibility is that SIRT1 might contribute to cancer development through a positive feedback loop on c-MYC expression. It has been proposed that this could perpetuate aberrant upregulation of c-MYC and suppression of apoptosis during colorectal cancer, for example (Menssen et al., 2012). Given its many interactions, and the fact that SIRT1 is present in in different domains of the nucleus, the issue of how these different roles are integrated remains an open question. Nor is it clear what role its control of rRNA transcription might play in its other cellular activities. For a discussion of sirtuin inhibitors as candidates for use in chemotherapy see (Liu et al., 2009).

rDNA silencing and further links to disease

A degree of care is needed as even when genes or pathways have been implicated in misregulating RNA Pol I, it is not necessarily the case that this is related to their oncogenic potential. It is likely that the significance of altered rDNA silencing may vary between cancer types and stages, and may be causative in some instances but a downstream consequence of malignant transformation in others. Such considerations should be borne in mind when evaluating the therapeutic potential of altering rRNA gene expression in any given case. Many chemotherapy agents affect RNA Pol I transcription, although of their nature most of these affect many other cellular processes as well so how critical their effects on the rDNA are remains unclear (Drygin et al., 2010).

Given what has been said previously concerning the potential genotoxic nature of recombination at repeated DNA, it is finally possible that mediators of rDNA silencing - such as those which control its organisation at the chromatin level - may also act as oncogenes under some circumstances. For example, the risk of rDNA which has deviated from their chromatin organisation producing DNA minicircles by inter-repeat recombination has been argued to have potentially carcinogenic effects (Peng and Karpen, 2007).

The NML-interacting protein NMNAT1 may also have roles in preventing DNA breakage, and is correlated with increased DNA damage in lung cancer (Song et al., 2013), although whether this is linked to its role in down-regulating rRNA transcription is uncertain. NoRC also plays a role in preventing potentially carcinogenic chromosome-breakage events during nuclear division by maintaining cellular heterochromatin (Postepska-Igielska et al., 2013). However, this has been argued to be principally due to its roles in stabilising heterochromatin at the centromeres, telomeres and associated chromosomal regions, rather than at the rDNA locus (Postepska-Igielska et al., 2013). On the other hand, general effects on genome stability may involve its association with rDNA as well as other tandemly repeated regions.

Although they will not be considered in detail here, it should be noted that misregulation of rDNA transcription has also been observed various other human pathologies, and may be causal in at least some cases. These disorders often occur due to disruption of the epigenetic pathways which regulate RNA Pol I activity and include various hypertrophies and atrophies (Hannan et al., 2012). Such diseases may share common pathways with cancer in that they are typically associated with altered cellular growth rates. Defects in components of the RNA Pol I machinery itself are also causative for a range of rare congenital pathologies collectively termed ribosomopathies (Narla and Ebert, 2010).

Summary and conclusion

Correct regulation of rDNA transcription is a key part of the integration between energy status and translational capacity. Altered rDNA transcription (and associated changes to nucleolar morphology) have been correlated with the occurrence of cancer. Research over the last twenty years has succeeded in identifying some of the key protein complexes and chromatin-remodelling pathways which control this regulation in humans and in model systems. The transcription of rDNA by RNA Pol I can be silenced by the activity of NoRC and other complexes which form the rRNA genes into a repressive chromatin organisation. At active local, the exact level of transcription depends upon complexes with TF-activity, and especially UBF. The interactions between the various complexes which can activate or silence rRNA gene transcription are still only poorly understood, but it is clear that they represent the components of a sophisticated regulatory network.

One key function is to link rRNA production to a cell's metabolic requirements and to the its energy reserves.

Perturbation of these regulatory networks is therefore detrimental for the cell's function and has been linked to various pathologies.

Elucidation of how misregulation of rDNA transcription of this kind contributes to the initiation and maintenance of cancer cell growth will be the focus of future research initiatives. Within this area, a particular question that will need to be addressed is the extent to which normalizing rRNA gene expression is able to restrict the growth of human cell lines and, if so, the nature of the molecular mechanisms which regulate this.

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