

Chromatin: linking structure and function in the nucleolus

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Abstract The nucleolus is an informative model structure for studying how chromatin-regulated transcription relates to nuclear organisation. In this review, we describe how chromatin controls nucleolar structure through both the modulation of rDNA activity by convergently-evolved remodelling complexes and by direct effects upon rDNA packaging. This packaging not only regulates transcription but may also be important for suppressing internal recombination between tandem rDNA repeats. The identification of nucleolar histone chaperones and novel chromatin proteins by mass spectrometry suggests that structure-specific chromatin components remain to be characterised and may regulate the nucleolus in novel ways. However, it also suggests that there is considerable overlap between nucleolar and non-nucleolar-chromatin components. We conclude that a fuller understanding of nucleolar chromatin will be essential for understanding how gene organisation is linked with nuclear architecture.

Abbreviations

DNMT	DNA methyltransferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HMT	histone methyltransferase
MS	mass spectrometry
NOR	nucleolar organiser region
rDNA	ribosomal DNA
rRNA	ribosomal RNA
UBF	upstream binding factor.

Introduction

The defining characteristic of eukaryotes is the double membrane-bound nucleus. Within the nucleus, DNA is packaged into the proteinaceous ‘super-structure’, chromatin, by association with histones, RNA molecules and other proteins. Organisms can be understood in terms of their physical structure and of the genetic information that they transmit, and chromatin links these aspects by arranging DNA into a structure which both organises and regulates its transcription. So essential is this connection between form and function that chromatin has been described as ‘a, if not the, hallmark of eukaryotic life’ (Benecke 2006).

The packaging of DNA into chromatin produces a nucleus that is structured yet dynamic, and at least partially self-organising (Misteli 2001; Lamond and Sleeman 2003). This self-organisation may be regulated by covalent modification of histones (Kimura et al. 2005; Martin and Zhang 2005; Fuchs et al. 2006) or result from the interplay of chromatin regions (Espada and Esteller 2007). It also leads to the formation of many sub-nuclear domains and

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bodies (Spector 2003), perhaps through the aggregation of functionally related proteins (Hancock 2004). Such structures highlight the roles of chromatin in linking nuclear function to the emergence of higher-order organisation.

The nucleolus, the site of rDNA transcription, rRNA maturation and ribosome production, is the largest such nuclear domain (reviewed Lyon and Lamond 2000). It is also the site of many other RNA-processing reactions and viral maturation and function (Raska et al. 2006; Boisvert et al. 2007; Andersen et al. 2002; Hiscox 2007), and nucleolar alterations occur in many pathologies and are, for example, of diagnostic importance in cancer (Maggi and Weber 2005). The question of how such structures form has been posed as a dichotomy between ‘self-organisation’—purely due to the biochemical processes—and regulation by ‘watchmakers’—external structure-imposing elements (Kurakin 2005). As nucleoli are reformed as a result of rDNA transcription, a self-organizing model seems appropriate (Melese and Xu 1995; Hernandez-Verdun et al. 2002), but with external inputs playing a fine-tuning role. In this review, we describe the central place of chromatin in this ‘modulated self-organization’ through direct structural effects on rDNA, and subsequent control of its transcription, and discuss how these regulate the emergence of nucleolar structure. The roles chromatin may play in other nucleolar functions are also considered.

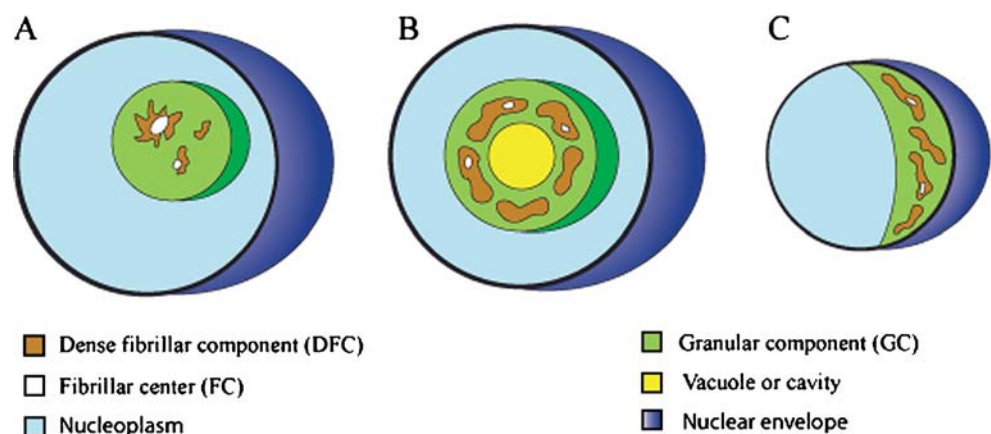
“Born at the junction of form and function”—structure and role of the nucleolus

Within the nucleolus, ribosomal RNA (rRNA) genes, organised within ribosomal DNA (rDNA) as tandem repeats at nucleolar organiser regions (NORs) and separated by linker regions, are transcribed by RNA polymerase I (Pol I) to produce pre-rRNA. These transcripts are processed into three of the ribosomal RNAs and complexed with 5S RNA,

probably transcribed elsewhere in the nucleus, and ribosomal proteins imported from the cytoplasm to form pre-ribosomes (Fig. 1). Nucleoli show considerable substructure in thin section transmission electron microscopy (TEM), which has traditionally been described as comprising lightly staining fibrillar centres (FCs) of about 0.1–1.0 μm in size, surrounded by or appressed to dense fibrillar component (DFC), which is usually more heavily stained than the rest of the nucleolus. The remaining parts of the nucleolus appear in the TEM to be filled with granules (called the granular component—GC); the granules are assumed to represent mainly pre-ribosomal particles in various stages of maturation (Shaw and Jordan 1995; Raska et al. 2006; Hernandez-Verdun 2005). There has been considerable debate about what the EM ultrastructure corresponds to in functional terms (Raska et al. 2006), and in particular where the transcribing genes are located. Different investigators favour either within the DFC or at the border between FC and DFC (Gonzalez-Melendi et al. 2000; Raska 2003). In fact, in spite of efforts to fit nucleolar ultrastructure into this simple tripartite model, there is considerable variation in organization between kingdoms, species, cell types and individual cells (see Fig. 1). In plants, the DFC is typically much more extensive than in animals, and often does not stain any more intensely than the GC; this distinction is also difficult to make in *S. cerevisiae*. Nucleoli may be associated with the nuclear membrane (as in *S. cerevisiae*), heterochromatic ‘knobs’, or other nuclear bodies.

Pol I transcription begins as telophase ends. Small clusters of potentiated rRNA genes become active and associate with factors from the ‘pre-nucleolar bodies’ (PNBs) which contain a subset of nucleolar proteins and RNAs that had been dispersed on entry into mitosis, a process known as nucleologenesis. As pre-rRNA processing is activated by transcription-dependent interactions between a Pol I TF and elements of the processing machinery (Kopp et al. 2007), it is possible that transcrip-

Fig. 1 Substructure of nucleoli. **a** animal nucleolus; **b** plant nucleolus; **c** yeast nucleolus



tion drives nucleogenesis by stimulating processing as well as by direct control of rRNA synthesis. Inactive rDNA is visible as peripheral knobs or internal foci, or may be interspersed with active rDNA within the nucleolus (Shaw et al. 1995; Carmo-Fonseca et al. 2000; Kalmorova et al. 2007); rDNA in peripheral knobs can be recruited as active genes when required (Highett et al. 1993).

Nucleolar structure as an evolutionary adaptation of eukaryotes

There is a strong correlation between nucleolar size and rate of rDNA transcription. Formation of nucleoli also requires Pol I: when constructs containing *S. cerevisiae* rRNA genes connected to Pol II promoters are transferred to different locations in the genome and transcribed, full nucleoli do not form, although ribosomes are produced (Oakes et al. 1999). However, there is evidence to suggest that the form of the nucleolus is an evolutionary adaptation to increase efficiency of ribosome synthesis, rather than being purely a consequence of transcription. For example, *X. laevis* oocyte or mammalian nucleoli can be dissociated without stopping rDNA transcription (Gonda et al. 2003; Yu et al. 2006), suggesting that transcription-driven self-assembly cannot fully explain nucleolar structure. Similarly, transcription occurs without formation of nucleoli in Archaea and other prokaryotes (Omer et al. 2000). rRNA transcription also occurs in the absence of visible nucleoli in certain simple eukaryotes and yeast strains with few rRNA genes (Nierras et al. 1997). Archaeal transcription is controlled in a relatively simple manner by DNA-binding Hmt proteins, which are thought to have evolved into histones during the emergence of eukaryotes, when most chromatin-remodelling machinery also evolved (Ouzounis and Kypides 1996, Malik and Henikoff 2003). So the evolution of nucleoli may also have depended upon the evolution of more sophisticated mechanisms of chromatin regulation.

The emergence of the nucleolar structure is controlled by chromatin

During nucleogenesis not all NORs are necessarily activated. If many are present, some will typically be entirely inactive, whilst only a subset of the genes on the others will be transcribed. Only rarely are all rRNA genes active simultaneously (Sogo et al. 1984). This activation can vary during differentiation as seen in comparisons of primary and adventitious onion roots (Hasterok and Maluszynska 2000). rRNA genes are assumed to be identical in sequence but differ in their chromatin organisation, which can render them active or inactive (Grummt

and Pikaard 2003). They can also exist in a poised or potentiated state in which their chromatin is open and available for transcriptional activation, but remains untranscribed (reviewed by Huang et al. 2006). Thus, there are at least three different chromatin states, with active genes in an open, accessible arrangement, inactive genes in a condensed arrangement and potentially active genes with a more dynamic nucleosomal arrangement than inactive genes (Stefanovsky and Moss 2006; Jones et al. 2007). During mitosis NORs are compacted into a condensed organisation, although they are still ~tenfold less compact than most the chromosome (Heliot et al. 1997), and so appear as secondary constrictions in metaphase chromosomes (McClintock 1934). NORs which were active in the preceding cell cycle can be recognised by positive AgNOR staining due to the retention of proteins involved in transcriptional activity (Roussel et al. 1994).

The level of Pol I association with these chromatin states may depend upon the level of cytosine methylation of the rDNA, remodelling of promoter chromatin, differences in histone variants and modifications at the promoters and coding sequences (reviewed by Grummt and Pikaard 2003). rDNA can retain the resulting levels of Pol I association through mitosis, allowing epigenetic inheritance of activity state (Conconi et al. 1989; Roussel et al. 1996; Grummt 2007). The importance of chromatin state is seen particularly well in the phenomenon of nucleolar dominance in hybrid organisms in which the NORs of one genome are repressed by another (Santoro 2005; Preuss and Pikaard 2007). In a given hybrid, the same genome will be dominant whether maternal or paternal, although this can be modulated by other loci, which could have chromatin-modifying roles (Chen et al. 1998; Pontes et al. 2003; Lewis et al. 2004). Some mechanisms of nucleolar dominance are also involved in the control of rDNA transcription in non-hybrid organisms. rRNA genes can show different levels of cytosine methylation, (in *Xenopus* Bird et al. 1981; and wheat, Flavell et al. 1988), and this can be an essential determinant of nucleolar dominance, as inhibition of such methylation can reduce the strength of nucleolar dominance (reviewed Chen and Pikaard 1997). rDNA methylation may affect rDNA by recruiting DNMTs to methylated promoters (Majumder et al. 2006).

However, rDNA methylation is only one aspect of chromatin which can be involved in nucleolar dominance (Pikaard 1999). It is not involved in nucleolar dominance in *X. laevis* (La Volpe et al. 1983) and tobacco (in which rDNA is hypomethylated; Kovarik et al. 2000), and DNA is barely methylated in *Drosophila*. In these cases, other aspects of chromatin organisation must be involved (Pennock and Reeder 1984; Avramova 2002), especially different histone modifications. This is well demonstrated by reversion of nucleolar dominance following histone

methyltransferase inhibition in onion (de la Torre et al. 1991). HMT inhibition can also increase rDNA methylation, reduce histone acetylation and cause chromatin to condense (Thompson and Flavell 1988; Eden et al. 1998; Jones et al. 1998; Lim et al. 2000) suggesting that these mechanisms operate in a concerted manner. Association of active DNA with permissive marks may also involve repackaging of transcribed regions with covalently modified replication-independent histone variants. This mechanism was first described in rDNA but is involved in wider aspects of epigenetic inheritance (Henikoff and Ahmad 2005; Schwartz and Ahmad 2005).

Chromatin controls nucleolar structure by regulating transcription, and by overall rDNA packaging

Above, it was described how active and inactive rRNA genes differ in DNA methylation, histone variant incorporation and histone modification. These processes also control Pol II transcription (reviewed by Kouzarides 2007 and Rando 2007). However, the protein complexes which establish them are not all shared between Pol I and Pol II, and this may allow rRNA genes to be regulated separately from those transcribed by other polymerases. Well-characterised examples of these include NoRC in mammals, the HDT1/HDA6 complex in *Arabidopsis*, and Sir2p in *S. cerevisiae* (Table 1). Although from different kingdoms and apparently unrelated, Pol I transcription factors do share common components such as TATA binding protein (TBP) (see, e.g. Grummt 2003). In *S. cerevisiae*, another interesting feature is that histone H3/H4 dimers can regulate transcription not through chromatin organisation but as components of a Pol I-associated complex, upstream activating factor (UAF; Keener et al. 1997), and reduced H3 synthesis causes dramatic reductions in Pol I transcription (Tongaonkar et al. 2005). This indicates additional direct roles for histones in control of rRNA synthesis.

The role of vertebrate UBF is particularly interesting as it demonstrates that rDNA can direct nuclear reorganisation both through transcription and independently of it. UBF binds across rDNA loci throughout the coding sequence and in the intergenic spacers. UBF-binding sequences transferred elsewhere in the genome are able to recruit UBF and other factors to form ‘pseudo-NORs’ which are transcriptionally inactive, but resemble some aspects of NORs (reviewed Moss et al. 2007). They form secondary constrictions at mitosis (O’Sullivan et al. 2002; Mais et al. 2005), and recruit factors required for rDNA transcription and processing (Prieto and McStay 2007). UBF also acts in such an ‘architectural’ manner in amplified rDNA in *X. laevis* oocytes (Mais and Scheer 2001) but can also function as a transcription factor (TF), possibly by

facilitating promoter escape (Panov et al. 2006). Perhaps because it is able to function both as a TF and as a determinant of DNA organisation, control of UBF has emerged as a major factor in rDNA regulation. For example, it undergoes cell cycle-specific acetylation which is necessary for Pol I binding (Meraner et al. 2006).

In contrast to rDNA control in vertebrates, no protein with the DNA-binding activity of UBF has yet been found in other organisms. Instead, studies of nucleolar dominance in plants suggest that rDNA organization and activity are controlled by a combination of DNA methylation, histone modification, and chromatin remodelling. These factors feed back upon each other to produce a ‘self-reinforcing loop’, which controls rRNA expression within species as well as in hybrids (Lawrence and Pikaard 2004; McStay 2006). In a hybrid of two *Arabidopsis* species, nucleolar dominance was shown to involve H3K9me2 at inactive, hypermethylated NORs and H3K9ac/H3K4me/H3K14ac and histone H4 tetra-acetylation of inactive, hypomethylated NORs (Lawrence et al. 2004). This required histone deacetylation by HDA6. Point mutations in HDA6 released transgenic and endogenous repetitive elements from silencing and caused H4 hyperacetylation and H3K4 hypermethylation at the 25S rRNA gene resulting in low levels of DNA hypomethylation and chromatin decondensation. Knock-down of HDA6 caused a complete loss of nucleolar dominance, and DNA hypomethylation and increased H3K9ac, H3K14ac, H3K4me3 and H4 tetra-acetylation of previously suppressed NORs (Earley et al. 2006). Purified HDA6 counteracted HAT-mediated H3K14, H4K5 and H4K12 acetylation in vitro, suggesting a direct effect, with other changes in modification occurring downstream or through histone replacement. Aufsatz et al. (2002) proposed that this also required establishment of methylation by other proteins, as HDA6 acts at symmetrical cytosine sites rather than the asymmetric sites most prone to de novo methylation.

Whilst histone deacetylases from several species have been shown to regulate rDNA and to be located in the nucleolus, there is much less evidence for nucleolar histone acetyltransferases. Those known to act in the nucleolus also function at Pol II-transcribed genes, e.g. the H4K16-specific Sas2p/PCAF of yeast (Meijsing and Ehrenhofer-Murray 2001). Additionally, certain enzymes acetylate other proteins to control nucleolar activity. Tip60 upregulates transcription by acetylating UBF (Halkidou et al. 2004), whilst the MYST-family acetyltransferase, Esa2p, has the opposite effect in *S. cerevisiae*, contributing to Sir2-mediated gene silencing (Clarke et al. 2006). As many organisms have multiple unattributed HATs in their genomes it remains possible that some are rDNA-specific.

rDNA transcription underlies nucleolar-chromatin organisation, but conversely, NORs can also be regulated by chromatin at other loci. Centromeric heterochromatin is

Table 1 Chromatin-remodelling proteins and other complexes regulate nucleolar function in different organisms

Organism	Protein/complex	Modification targeted	Outcome
<i>S. cerevisiae</i>	RENT (Net1p, Sir2p (an HDAC), Fob1p, Cdc14), which binds throughout rDNA ^{a, b}	Sir2p—deacetylation target(s) unknown; causes loss of H3K4me, regulates total H3 content ^c	Gene inactivation. Inhibits rDNA recombination
	Rpd3-Sin3 HDAC	Net1p ^b —binds rDNA rDNA H4 acetylation; sets chromatin in form competent to transcribe RNA Pol II in some mutants ^c	Gene activation ^d Gene activation; may be inhibited by TOR in association with altered nucleolar morphology ^f
<i>D. melanogaster</i>	siRNA-mediated heterochromatinisation	H3K9me2 in nucleolus and flanking repeats	Loss of pathway causes rDNA recombination, and detachment into dispersed foci ^g
Vertebrates (<i>H. sapiens</i> , <i>M. musculus</i> , <i>X. laevis</i>)	UBF(Upstream Binding Factor: binds throughout rDNA; occluded from upstream control element by DNA methylation ^h	Reorganisation of rDNA into left-handed chromatin-like structure ⁱ	Decondensation ^j which allows loading of RNA Pol I ⁱ and recruitment of pre-rRNA processing components ^k
	Runx2	Prevents H3K4me2 and H4 hypoacetylation	Maintains mitotic rDNA as open but inactive ^l
	MBD3	rDNA gene promoter demethylation ^m	Allows RNA Pol I binding and transcription
	NoRC	Targets HDAC1 to rDNA promoters ⁿ . Mediates subsequent DNA methylation ^o	Histone H4 hypoacetylation, RNA Pol I repression; delayed rDNA replication ^p
	Nuclear myosin1, WSTF	Bind RNA Pol I on rDNA; may act to associate the chromatin-remodelling complex, WICH ^q	Increases rate of transcription through chromatin templates ^q
<i>A. thaliana</i>	Complex including HDA6, H3/H4 and the plant-specific HDT1 (HDT2, 3 and 4 are also nucleolar, and may have roles in transcriptional repression ^r)	HDA6 acetylates H3K9, K14 and H4. HDT1 mediates gene silencing perhaps by H3K9 deacetylation. H3/H4 are RNA Pol I components ^s	Loss of HAD6 causes DNA methylation, aberrant rRNA expression and NOR decondensation ^t
	Linker histones	Stabilise DNA methylation patterns ^u	Ensure fidelity of epigenetic inheritance of activation states
	MBD proteins ^v	Roles unknown	

Core components of the RNA Pol I machinery are conserved, but in many organisms, lineage-specific chromatin-remodelling proteins and complexes regulate rRNA synthesis. In higher eukaryotes, these are typically specific for rDNA regulation although some have other functions

^a Huang and Moazed (2003)

^b Straight et al. (1999)

^c Li et al. (2006b)

^d Shou et al. (2001)

^e Oakes et al. (2006)

^f Tsang et al. (2003)

^g Peng and Karpen (2007)

^h Santoro and Grummt (2001)

ⁱ Mais et al. (2005)

^j Chen et al. (2004)

^k Prieto and McStay (2007)

^l Young et al. (2007)

^m Brown and Szyf (2007)

ⁿ Zhou et al. (2002)

^o Santoro and Grummt (2005)

^p Li et al. (2005)

^q Percipalle et al. (2006)

^r Wu et al. (2003)

^s Probst et al. (2004)

^t Earley et al. (2006)

^u Wierzbicki and Jerzmanowski (2005)

^v Thorstensen et al. (2006)

involved in yeast nucleogenesis (Pluta et al. 1995) and exogenous heterochromatic NORs alter mitotic interactions of endogenous NORs in rye (Caperta et al. 2002). Heterochromatin on other chromosomes can also affect NORs: introduction of heterochromatic B-chromosomes from rye into wheat lines caused endogenous NORs to become more compacted and less active (Morais-Cecilio et al. 2000). It is known that heterochromatin is involved in maintaining overall nuclear organisation in *Drosophila* (Csink and Henikoff 1996) and it is therefore possible that heterochromatin could regulate nucleolar organisation in a similar manner.

Another good example of the potential importance in the regulation of nucleolar chromatin is seen in the Sir2 (Silent information regulator2)-like family of proteins (sirtuins). These were identified in *S. cerevisiae* as rDNA-binding proteins which use NAD⁺ reduction to fuel H3/H4 deacetylation during heterochromatin formation and, thus, repress transcription (Table 1; Gotta et al. 1997; Imai et al. 2000). Sir2p has homologues with roles in nucleolar chromatin throughout eukaryotes, although the RENT complex with which it associates is not conserved. In humans, it has recently been shown that a homologue, SIRT1, instead interacts with a complex termed eNoSC (Murayama et al. 2008). This also contains SUV39H1, which mediates the repressive histone modification, H3K9 dimethylation, and is argued to allow repression of rDNA transcription in response to falling cellular energy levels (Murayama et al. 2008).

In many organisms, sirtuin activity is linked to longevity. This could reflect the fact that sirtuins can mediate cellular energy levels, possibly in the same way as caloric restriction, which is known to increase longevity. Sir2p also controls aging in *S. cerevisiae*, perhaps by forming inactive rDNA into heterochromatin to prevent recombination, which produces small loops of rDNA termed minicircles, which are believed to be toxic to cells (Gottlieb et al. 1989; Fritze et al. 1997; Parsons et al. 2003; Kobayashi et al. 2004). Sirtuins may have roles in genome stability in other organisms (e.g. rice; Huang et al. 2007), so this mechanism may not be restricted to fungi. However, some sirtuins seem to mediate aging pathways through down-regulation of p53 (Vaziri et al. 2001). Sirtuins also act upon other substrates entirely, including tubulin and acetyl-CoA synthetase (North et al. 2003; Hallows et al. 2006). Sirtuins have been shown to interact with nucleoli to activate Pol I transcription (Ford et al. 2006) by mechanisms which remain unclear.

The level of rRNA synthesis at active genes can also be controlled by the loading rate of Pol I complexes at the promoters and by elongation efficiency (Stefanovsky and Moss 2006). Elongation control has been argued to be particularly important for controlling rRNA synthesis in

human cells as control through elongation is more significant than that of initiation; furthermore, alterations in elongation levels are sufficient to explain changes to cell growth in cancer lines (Moss et al. 2007 and references therein). Diminished capacity for transcribing to the end of the gene may also explain the reduced rRNA synthesis of *Drosophila* rDNA inserted into retrotransposable elements (Ye and Eickbush 2006). Histone modification and UBF phosphorylation may also control transcription via elongation, perhaps by accumulation of permissive histone modifications or replication-independent histones such as H3.3 in the 3'-parts of the genes. rDNA-associated histones may also be required for control of elongation (Tongaonkar et al. 2005).

The different systems which control rDNA transcription may act in varying combinations in different cells or species. For example, the *Arabidopsis* Cape Verde Island (Cvi) ecotype has 80% as many rRNA genes as Colombia ecotype (Col) but more than twice the proportion of these are hypermethylated and thus inactive (Riddle and Richards, 2002). As it is unlikely that Cvi requires less than 40% of the number of rRNA molecules that Col does, Cvi probably controls rRNA content by methylation-mediated repression of most of its rRNA genes, whilst Col has more active genes but controls their activity through other methods (Riddle and Richards 2002). These could include Pol I loading and extension rates.

The three states of nucleolar chromatin: roles for novel components?

Nucleolar chromatin is also likely to include further components of rDNA-specific enzyme complexes, nucleolar-specific histone modifications or histone variants. It is possible that such components contribute to the role of chromatin in regulating nucleolar structure as well as transcription.

Nucleolar histone variants

Histones with variant sequences are obvious candidates for nucleolus-specific chromatin components. This has already been shown in the case of a potential nucleolar H2A variant in human and possible enrichment of an H2A in carp nucleoli (Allis et al. 1982; Alvarez et al. 2006). As many organisms have multiple histone variants, it is possible that rDNA may also associate with nucleolus-specific histone variants more generally. This would parallel the association of histone variants with other nuclear domains such as centromeres, telomeres and Barr bodies (Table 2). Linker H1 histones are also enriched in the nucleolus in lily and *Arabidopsis* (Tanaka et al. 1999; Pendle et al. 2005). Linker histones repress inactive human rDNA and maintain

Table 2 Histone variants and modifications associate with nuclear structures and regions

Nuclear structure/domain	Protein/modification	Reference
Barr body (mammalian)	macroH2A, H3 hypoacetylation, K9 methylation, and DNA methylation	Constanzi et al. 2000; Boggs et al. 1996; Mermoud et al. 2002; Kohlmaier et al. 2004
Centromeres	Centromeric histone H3 variants (many organisms)	Fernandez-Capetillo et al. 2004a, b
Locations of double-stranded breaks	H2AX	Fernandez-Capetillo et al. 2004a
Euchromatic chromosome territories	H2B phosphorylation Gene-associated modifications (H3K4me, K36m; H3K9ac and H4ac). Replication-independent histones. H2Av	Fernandez-Capetillo et al. 2004b Rando 2007; Stargell et al. 1993
Heterochromatic chromosome territories	Typically enriched in H3K9/27/ K79 and H4K20 methylations, DNA methylation, with variation between species	
Nuclear periphery/pores	Ku and Sir4, which interact with NPC to silence <i>S. cerevisiae</i> genes H3 trimethylations (some animals)	Galy et al. 2000; Taddei et al. 2004 Payne and Braun 2006
Nucleolar domains	Histone variants (human/carp) Linker histone (plants)	Allis et al. 1982; Alvarez et al. 2006 Tanaka et al. 1999; Pendle et al. 2005
Telomeres	Single myb histone— <i>Z. mays</i> H3K79ac— <i>S. cerevisiae</i>	Marian et al. 2003. Ng et al. 2003
Tetrahymena micronucleus	CenH3-like histone	Cervantes et al. 2006

The complexity of nuclear organisation in higher eukaryotes typically involves specialisation of histone variants, covalent modifications and chromatin-remodelling proteins for association with particular nuclear structures. Some histones have been found enriched in nucleoli from different systems, but as yet no histone modifications are known

epigenetic control of rDNA in tobacco, indicating roles in chromatin domain stabilisation within NORs (Slusarczyk et al. 2003). Linker histones interact with nucleolin in human cells (Erard et al. 1988; see below) and compete with Pol I for upstream binding sites in yeast (Kermekchiev et al. 1997) suggesting that they could link the maintenance of chromatin domains with transcription. In contrast with the reports of nucleolar-specific histone variants, nucleolar-specific histone modifications are conspicuous by their absence (Table 2), although various H4 acetylations are enriched at replicating NORs in the plants *V. faba* and *A. thaliana* (Houben et al. 1996; Jasencakova et al. 2000). The combinatorial complexity of the histone code suggests that other modifications could have roles in NOR replication or other nucleolar functions, but these may vary between species.

Nucleolar histone chaperones

Several nucleolar proteins also act as histone-binding proteins. These include nucleolin and nucleophosmin (also called B23, NO38 and numatrin) and novel proteins such as NO29 (Sharma 2004) and the human peptidyl prolyl *cis-trans* isomerase protein, FKBP (Kuzuhara and Horikoshi 2004). Nucleolin is a DNA-binding protein which is essential for Pol I transcription (Richards et al. 2007) and

nucleolar integrity (Pontvianne et al. 2007). It is required for H1-related chromatin compaction (Erard et al. 1988) and the SWI/SNF2-remodelling of H2A/H2B dimers (Angelov et al. 2006) and, as an AgNOR protein, remains associated with NOR chromatin through the cell cycle (Roussel et al. 1994). Because it has many other functions (reviewed Mongelard and Bouvet 2007), nucleolin provides potential links between nucleolar-chromatin organisation and other nuclear processes.

Nucleophosmin (NPM) is part of the small nucleophosmin/nucleoplasmin histone chaperone family (Frehlick et al. 2007), and regulates rRNA transcription. NPM interacts with FRGY2, which can mediate the disassembly of *Xenopus* nucleoli whilst not disrupting rRNA transcription (Gonda et al. 2006), but how this links with its histone-binding activities remains unclear. Nucleophosmin associates with H3/H4 dimers via its N-terminal domain, and, via a C-terminal acidic tract, with H2A/H2B, in the assembly of nucleosomes (Okuwaki et al. 2001). It may be important for the binding and reassembly of nucleosomes disrupted during the high rates of transcription which occur at rDNA. The related NPM2 (nucleoplasmin) is also a histone chaperone, suggested by structural studies to be specific for H3/H4 tetramers (Nambodiri et al. 2004). As with nucleolin, nucleophosmin acts in many different ways—its deposition

is linked to poly-adenylation, for example (Palaniswamy et al. 2006). Hence, it too could integrate histone remodelling with a range of other nucleolar processes.

Nucleolar roles of small RNAs

In many organisms, silencing of repetitive DNA often occurs through mechanisms involving small RNA species and it has recently been shown that the siRNA pathway in *Drosophila* plays a structural role by forming rDNA into inactive heterochromatin, which blocks recombination (Peng and Karpen 2007). This occurs via H3K9 dimethylation of rDNA and inactive microsatellites. Disruption of the pathway caused DNA reorganisation, including dissociation of the nucleolus into dispersed rDNA foci. Non-nucleolar rDNA also became excised from the chromosomes. As mutation of a ligase essential for the homologous recombination pathway rescued this, siRNA-mediated heterochromatinisation seems to be involved in preventing unwanted homologous recombination.

These important roles for siRNAs in nucleolar integrity may be specific to *Drosophila*, perhaps due to the lack of DNA methylation in this organism, but it remains an intriguing possibility that small RNA pathways could be involved in regulating rDNA in other species. siRNAs are encoded between non-NOR 5S rRNA genes of *Arabidopsis* (Llave et al. 2002), and could also regulate nucleolar transcription or structure. *hda6* mutants were derepressed in transcription at centromeric repeats (Athila; May et al. 2005) through the RNA-directed DNA methylation pathway; hence the effect on NORs might also involve small RNAs (Aufsatz et al. 2002). Both nucleoli and Cajal Bodies of *Arabidopsis* are involved in maturation of small RNAs (Shaw et al. 1998; Pontes et al. 2006; Li et al. 2006a). Small RNAs are also matured in small ncRNA-processing bodies in which ASSYMETRIC LEAVES1 (AS1) and AS2 interact with the rRNA gene-regulating HDT proteins to regulate leaf development (Ueno et al. 2007), although HDTs have been proposed to be solely nucleolar (Lawrence et al. 2004) or found throughout the nucleus (Zhou et al. 2004). These structures may represent the perinucleolar microRNA processing bodies to which DCL1 localises (Song et al. 2007). The observation of a direct interaction between an ncRNA-processing pathway and nucleolar-chromatin-remodelling proteins suggests that chromatin could also play important roles in non-canonical nucleolar functions. Little is known about the roles of small RNAs in mammalian nucleoli, although miRNA-206 has been shown to bind ribosomal proteins in the GC in rat (Politz et al. 2006).

Further chromatin components identified by MS

Finally, mass spectrometry has been used to determine the proteomes of nucleoli from human and *Arabidopsis* culture

cells (Andersen et al. 2002; Scherl et al. 2002; Pendle et al. 2005; Lam et al. 2007) and has identified potential nucleolar chromatin components which remain uncharacterised. MS analyses of the human nucleolus have to date identified 5 linker histones, 5 H2A isoforms, H2A.F/Z, 6 H2B isoforms, 2 H3 isoforms, a HAT-B subunit, HDAC 1 and 2, a FACT component, a CAF1 subunit, three chromobox proteins, and a splice-variant of a bromodomain-like protein: a total of 28 out of c. 700 (www.lamondlab.com/NOPdb/). The *Arabidopsis* nucleolar proteome so far contains two linker histones, six H2A isoforms, H2A.F/Z, five H2B isoforms, three H3.1 isoforms and one H3.2, at least one H4, a FACT component, an HDAC, three putative HAT components and the four HDAC-like HDT proteins (Pendle et al. 2005; McKeown et al. unpublished data), i.e. 27 out of c. 650. Hence, the ratios of chromatin proteins in both proteomes appear to be similar.

Such studies emphasise that many chromatin components may remain to be discovered. However, current studies of the dynamics of nuclear proteins suggest that few nuclear proteins are likely to be wholly excluded from the nucleolus, so the nucleolar proteome should be thought of as part of a continuum with the nucleoplasm. Different histone isoforms may associate with nuclear regions in different proportions, for example, and understanding nucleolar chromatin will require a consideration both of instances of ‘nucleolar isoforms’ and subtler changes to the whole histone profile. Better quantitation of mass spectrometry data may provide the information necessary to elucidate this.

Conclusions

Studies of nucleoli illustrate the way in which chromatin acts at the interface between structure and function. Different organisms use different sets of chromatin-remodelling proteins to deploy components, often shared with Pol II-transcribed genes, to selectively activate a subset of rRNA genes. The level of transcription is controlled by chromatin-mediated loading of the Pol I complex at promoters and this, in turn, modulates the structure of the nucleolus. Nucleolar structure is also maintained and modulated by heterochromatinisation of inactive rDNA by siRNAs or sirtuins or both. Chromatin at other NORs or B-chromosomes may also affect transcription.

This level of regulation of chromatin structure, and the organisation of the resultant nucleolus, may be necessary to allow transcription from repeated DNA without activating silencing mechanisms. In at least some instances, it may also be connected with the maintenance of rDNA in different epigenetic states through replication. Finally, organising rRNA transcription and processing into a

defined compartment containing different sub-regions may also allow other cellular processes to take advantage of this compartmentalization, especially those requiring RNA-processing steps.

The nucleolus represents an example of the way activity can control architecture, yet also the manner in which this architecture is modulated to ensure the efficiency of overall nuclear function; this might be described as a system of ‘modulated self-organization’. In the future, it should be expected that this model will be extended to incorporate components of uncertain function, such as the HDACs and HATs identified in proteomic screens, and small RNA pathways, and to explain what role the histone-interactions of well-known proteins such as nucleolin and nucleophosmin may play. The possibility that particular histone modifications and variants may have specialised roles within the nucleolus should also be explored. In this way, the nucleolus will continue to shed light on the relationship between gene function and nuclear organisation in eukaryote cells, and hence on the relationship between structure and activity in biological systems.

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