Proteins of the Nucleolus

Danton H. O'Day • Andrew Catalano
Editors

# Proteins of the Nucleolus

## Regulation, Translocation, & Biomedical Functions

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*Editors*
Danton H. O'Day
Biology Department
University of Toronto at Mississauga
Mississauga, ON, Canada

Andrew Catalano
City College of New York
New York, NY, USA

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# Introduction

# Chapter 1
# Proteins of the Nucleolus: An Introduction

**Danton H. O'Day and Andrew Catalano**

**Abstract** This book contains 15 original review chapters each containing data that yield new, exciting and intriguing data about the emerging understanding of nucleolar structure and function in normal, stressed and diseased cells. The goal of this introduction is not to review the fine details of each chapter but to give a sampling of each and some of the information therein that gives us special insight into the nucleolus of the past, present and future. A final summary chapter will look at the contents of this volume as a whole with a view to future research.

**Keywords** Nucleolus organizer regions • Nucleolar proteins • rRNA • Ribosome • Cancer • Protein targeting • Protein translocation

## Part I: Introduction

## 1.1 Introduction

The classic or canonical structure of mammalian nucleoli indicates that they form around rDNA domains called nucleolus organizer regions (NORs). The basic organization of the nucleolus then resulted from the progressive synthesis of pre-ribosomal subunits beginning with the transcription of rRNA, its processing to form smaller

D.H. O'Day (✉)
Biology Department, University of Toronto at Mississauga,
3359 Mississauga Road, L5L1C6 Mississauga, ON, Canada
e-mail: danton.oday@utoronto.ca

A. Catalano
City College of New York, 160 Convent Ave.
Marshak Building, rm.1221 New York, NY, 10031, USA

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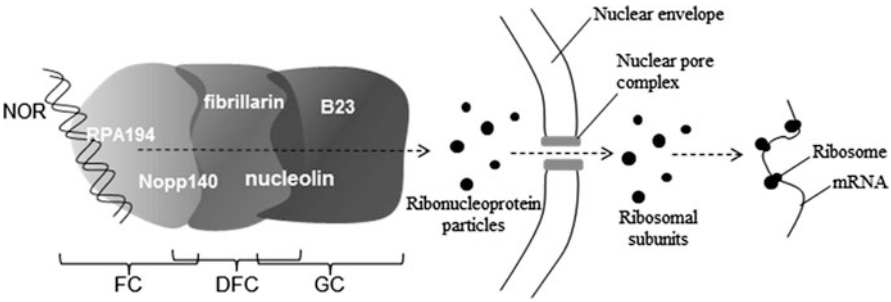


**Fig. 1.1** The diagram separating the three classic compartments of mammalian nucleoli and some of their components. *NOR* nucleolar organizer region (See text for details)

rRNAs which associated with proteins to form ribonucleoprotein particles that ultimately exited the nucleus as pre-ribosomes which would associate to form ribosomes for protein synthesis. By this model, each progressive compartment of the nucleolus represented a discrete stage of ribosomal productions. The tri-partite structure fits with the nucleoli visualized in transmission electron microscope images which consisted of a fibrillar centers (FC). dense fibrillar components (DFC) and granular component (GC). While the following figure presents a diagrammatic representation of these events and the three compartment nucleolus, in reality nucleoli are not so clearly organized but instead show multiple, overlapping compartments that fit with this general model.

Research has revealed that there are specific markers that also support the tri-partite nucleolar model with specific critical proteins localizing to specific regions (Fig. 1.1). Thus the RPA194 subunit or RNA polymerase I localizes to the FC presumably as a prelude for early events of rDNA transcription that occurs at the FC/DFC border. Nopp140 (NOLC1) also resides in the FC but also in the adjacent DFC while fibrillarin is specifically associated with the DFC. Nucleolin (C23) is detected in both the GC and DFC. While B23 (nucleophosmin localizes to the GC where the ribonucleoprotein particles are assembled before exiting via nuclear pores. While NORs organize nucleoli, knockout mutants have shown that B23 and nucleolin are each essential for nucleolar integrity. As we gain insight into this classical structure, new aspects of nucleolar organization are emerging as detailed throughout the following chapters.

The nucleolus is the largest transcriptional region in the interphase nucleus. As several chapters indicate, the visualized structure of the nucleolus depends on the technique used (i.e., transmission electron microscopy or fluorescence microscopy). Thus, the FC and DFC regions and FC/DFC border cannot be precisely defined in turn making it difficult to state exactly where each step in ribosome synthesis precisely occurs. That said pre-rRNA transcription localizes to the FC/DFC border while rRNA processing mainly occurs in the DFC. The early steps of ribonucleoprotein particle assembly occur in the GC.

While nucleolar localization signals (NoLS) have been revealed for a number of resident nucleolar proteins, many of these proteins do not contain typical NoLSs.

Furthermore, transient nucleolar proteins exist that translocate in and out of nucleoli under various normal and pathological conditions. Other proteins are carried into nucleoli or bind to existing nucleolar proteins after various post-translational modifications.

## Part II: The Nucleolus and Nucleolar Proteins

### 1.2 Functional Consequences of Nuclear and Nucleolar Architecture

The stage is set for this volume by Bártová and Stixová (Chap. 2) who graphically review the functional architecture of the nucleus and nucleolus. In the nucleus DNA replication occurs in replication foci, DNA repair is associated with repair foci, while splicing is regulated by proteins in nuclear speckles. In contrast rDNA gene transcription occurs in transcription "factories" or nucleoli. The numerous other functions of the nucleolus are detailed in subsequent chapters. A diversity of post-translational histone modifications regulate DNA replication, transcription, splicing, and repair. During interphase, specific chromosomal territories exist that are related to transcriptional activity and which change during cell differentiation, the cell cycle and under various normal physiological and pathophysiological events. The role of histone replacement as a kind of epigenetic event is also reviewed. The focus then shifts to ribosomal subunit synthesis in the nucleolus and the role of nucleolus organizer regions (NORs) that consist of "Christmas tree-like arrangements of hundreds of transcriptionally active rDNA repeats. The nucleolus is presented as dynamic structure (e.g., upstream binding factor (UBF) proteins recover quickly after photobleaching) within which a diversity of proteins (e.g., fibrillarin, nucleolin) work to synthesis and process ribosomal gene products. Ribosomal genes are modified epigenetically via histone acetylation, histone methylation, and DNA methylation and these modifications are important for nucleolar organization and gene expression. For example, loss of histone methyltransferase SUV39h function induced the substitution of H3K9me3 by H3K9me1 at clusters of centromeric heterochromatin, chromocenters and also caused the loss of heterochromatin protein 1β (HP1β) at chromocentres surrounding nucleoli. This is important because HP1 subtypes play a number of central roles (e.g., DNA damage response) that are affected by their localization. This chapter concludes with a discussion of the nucleolus as the site of the DNA damaging response and nucleolar proteins play a critical role in DNA damage stress induced by genotoxic events. For example, UV exposure causes the translocation of Werner syndrome (WRN) protein from the nucleolus to nucleoplasm with accumulated evidence suggesting it functions as part of the DNA repair machinery. In total, Bártová and Stixová show that the events of global genome organization and chromatin dynamics work together with functional genomic changes to oversee the ongoing balance between genome stability and instability.

## 1.3 rDNA and Nucleologenesis in *Drosophila*

Taking a critical, historical perspective in setting the stage for future research, DiMario et al. (Chap. 3) review nucleolar organizers and flanking heterochromatin, intergenic region function in *X-Y* chromosome pairing, as well as the basics of *Drosophila* rDNA magnification and compensation. As detailed in their review, research on *Drosophila* has contributed significantly to understanding how rDNA expression drives nucleologenesis. The fact that nucleoli in this species don't appear until the blastocyst stage of embryonic development presents a unique situation for studying nucleologenesis. DiMario et al. then focus on R1 and R2 retrotransposons and their transposition mechanism, followed by an analysis of how rDNA acts as an epigenetic regulator of genome-wide gene expression. Having set the stage so well, the authors introduce their work showing that deletion of the nucleolar protein Nopp140 (140 kDa *n*ucle*o*lar *p*hospho*p*rotein) prevents the silencing of R2 rDNA genes, an event linked to larval survival after the second instar stage of development. The two isoforms Nopp140 (Nopp140-True, the orthologue of mammalian Nopp140, and Nopp140-RGG which differs in its carboxy domain) present in *Drosophila* were used to study nucleologenesis during development. Foci of GFP-tagged Nopp140-RGG appear during the last mitosis prior to gastrulation. Data, presented in their chapter, show that Nopp140-RGG is first present in the cytoplasm of somatic cells and nuclei of pole cells (future gametes) prior to formation of foci. Since the colocalization of Nopp140-RGG and new rRNA synthesis occur in these foci, this suggests the foci are activated nucleoli forming at NOR sites. In total, the authors have provided evidence that nucleolus formation in somatic cells starts at stage 13 of embryogenesis. In pole cells, nucleologenesis starts as they begin their posterior migration also presenting a useful system for studying nucleolar formation. DiMario et al. conclude their chapter by posing eight questions that should be answered as research moves forward to understanding nucleolar function and nucleologenesis in *Drosophila*.

## 1.4 The Nucleolus of *Dictyostelium* and Other Lower Eukaryotes

The nucleolus has been studied extensively in a multitude of different organisms however little is known about the nucleolus in *Dictyostelium*. In their chapter, Catalano and O'Day discuss the relatively recent work performed which characterizes the nucleolus in this model eukaryote. *Dictyostelium* has proved an excellent model for the study of several fundamental cellular processes and human diseases. Understanding the nucleolus of *Dictyostelium* as well as other lower eukaryotes may shed light on the rules that govern nucleolar structure and function in all eukaryotes. To date, seven nucleolar proteins have been identified in *Dictyostelium*, and all display a slightly different pattern of nucleolar localization. Treatment with the rDNA transcription inhibitor actinomycin-D results in the disappearance of nucleolar eif6

and NumA1 from the nucleolus, as expected, but interestingly, also results in the nucleolar accumulation of Snf12. During mitosis nucleolar NumA1 and CBP4a redistribute throughout the nucleus, albeit with different redistribution patterns, while Snf12 and FhkA redistribute throughout the entire cell. It is also interesting to note that although there are differences in nucleolar morphology between the nucleolus of *Dictyostelium* and that of other lower eukaryotes, in most of these organisms, the nucleolus is located adjacent to the nuclear envelope. This is in contrast to the mammalian nucleolus, which is usually embedded within the nucleoplasm.

## 1.5 Human rDNA Genes: Identification of Four Fractions, Their Functions and Nucleolar Location

Originally the nucleolus was functionally identified as the site of ribosome synthesis. The rDNA genes serve to organize nucleoli through the synthesis of rRNA and the initial formation of pre-ribosomal subunits. In Chap. 5 Lyapunova et al., review the organization of rDNA genes and the diversity in different organisms, especially with regard to promoter regions and the issue of variable ribosomal gene copy number in humans. They also give insight into different techniques used to study nucleoli and ribosomal genes and why different research teams have obtained varying results over the years. The significance of copy number to the pathogenesis of a number of heritable (e.g., Down Syndrome) and non-heritable diseases (e.g., those caused by Robertsonian chromosomal translocations) is detailed. While humans have many copies of rDNA repeats they can be divided into four different fractions based on their structure, localization and function. As revealed by research carried out by these researchers on human peripheral blood lymphocytes as well work done by others, the four fractions of ribosomal repeats are: active, potentially active, inactive, and silent rDNA genes. Inactive rDNA genes are different from silent rDNA genes which usually are highly methylated. The characteristics of these four regions and their localization to different regions of the nucleolus are detailed. After covering the attributes of each of these groups and after detailing the accuracy of their methodologies, the phenotypic effects of gene dosage for active and potentially active rDNA genes is discussed. Lyapunova et al. conclude their chapter with data on the relationship of rDNA gene dosage to specific diseases including rheumatoid arthritis, schizophrenia and autism spectrum disorders.

## 1.6 Chromatin Organization and the Mammalian Nucleolus

After a review of nuclear structure—including nuclear lamina, Cajal bodies and promyelocytic leukemia (PML) nuclear bodies—and chromosome organization, Nemeth and Längst add to our understanding of the nucleolus as a ribosome factory (Chap. 6).

While years of research have revealed the structure of nucleolus-associated chromatin and the rRNA genes, it is only recently that insight is being gained into the constituents and organization of the nucleolar genome. In their chapter, Nemeth and Längst focus on the structural organization and protein components of nucleolar chromatin domains. The nucleolus is surrounded by a dense chromatin nucleolar shell that contains centromeric and pericentromeric chromosomal regions and constitutes the major part of nucleolus-associated chromatin domains (NADs). NADs form a stable and conserved compartment that has unique attributes. Although they mainly contain inactive chromosomal regions, specific satellite repeats present here may function in the assembly of the perinucleolar heterochromatin. As also discussed by others in this volume, the rDNA genes are present in three states, active, inactive and poised (i.e., ready to be activated). Epigenetics plays a role in the transcriptional regulation of the rRNA genes but little is known about how the active and poised states of rRNA genes are established, a subject this chapter addresses. It appears that several dozen epigenetic regulatory proteins are involved in regulating events from modifying histones to chromatin domain topology, each of which are detailed in the remainder of this chapter. In their conclusions, Nemeth and Längst note work that needs to be done on these topics and on the importance of RNA molecules as architectural factors in establishing and maintaining nucleolar integrity.

## 1.7 Chaperones and Multitasking Proteins in the Nucleolus

In Chap. 7, Kodiha and Stochaj focus on molecular chaperones (e.g., heat shock protein, hsc70) and multitasking proteins (e.g., B23, nucleolin and Nopp140) in the structure and function of the nucleolus during stress and disease. All major chaperone families are represented in the nucleolus proteome and nucleolar multitasking proteins (NoMPs) can serve as chaperones as well as perform other functions. In response to a heat shock a multistep process occurs involving hsc70. The actual heat shock causes it to move from the cytoplasm into the nucleus then during initial recovery it accumulates in the nucleolus before exiting at a later time. Each of these steps is temperature-dependent. How chaperones are targeted to nucleoli is still under investigation. Several nucleolar heat shock proteins possess putative NoLSs while others do not. The issue of nucleolar localization is complex because while some proteins are more or less full time residents others are transients only moving into the nucleolus under certain conditions such as stress, disease or during the cell cycle. For hsc70, a complex temperature-dependent NoLS exists in its ATP binding domain for which the authors have devised a model to explain the translocation of hsc70 in the heat shock response. Post-translational modifications are also important for nucleolar localization of hsc70 as well as B23 (also known as nucleophosmin, NPM1, numatrin, NO38) and nucleolin in interphase cells adding additional insight into how proteins are targeted to nucleoli. Shifting the focus to multitasking proteins (e.g., B23, nucleolin and Nopp140), Kodiha and Stochaj examine each of these proteins in detail with respect to their isoforms, post-translational modifications and their function as chaperones and in other cellular events. The role

of post-translational modifications in nucleolar protein function as well as in protein binding is also detailed. Their chapter reveals links between chaperones and nucleolar multitasking proteins and the role of chaperones in the nucleolus and concludes with insights into future research directions.

## Part III: Nucleolar Protein Translocation

### 1.8 Nucleolar Localization/Retention Signals

The nucleus contains several different domains and compartments, the most prominent being the nucleolus. The nucleolus is a multifunctional compartment that is usually comprised of three subcompartments. Despite the large number of known nucleolar proteins it is not well understood how these proteins accumulate in the nucleolus given that this organelle is not bound by a lipid membrane. The nuclear matrix is dynamic and as such nucleolar proteins constantly shuttle between the nucleolus and nucleoplasm. In Chap. 8 on nucleolar localization/retention signals Sheval and Musinova discuss the nature of the signals that localize proteins to the nucleolus and suggest that they act as retention signals rather than localization signals. They also show how the presence of a nucleolar retention signal (NoRS) does not necessarily imply that the protein is nucleolar and interaction via NoRSs is only part of the reason proteins localize to the nucleolus. The authors use the transient nucleolar accumulation of histone H2B as a model to investigate nucleolar protein retention. For example, GFP-fused histone H2B accumulates in nucleoli immediately after transfection but redistributes to chromatin later. This nucleolar localization is due at least in part to its NoRS and is thought to result from the overexpression of GFP-H2B, since after cell division this fusion protein does not reaccumulate in nucleoli. However this is strange given that H2B has no nucleolar function. The authors show that in fact a nuclear localization signal (NLS) within H2B, which is composed of a high proportion of basic residues, functions as an imitative NoRS. It is the basic residues alone that are responsible for nucleolar localization. In fact poly-K and poly-R are sufficient to target GFP to the nucleolus. The authors explain that since proteins diffuse quickly through the nucleoplasm it is thought that nucleolar proteins are not targeted to nucleoli, but are rather retained there, and they conclude by discussing the association of histone H2B with the nuclear matrix.

### 1.9 Nucleolar Transport of Putative GTPase GNL1 and Related Proteins

GTPases are involved in a multitude of cellular functions, many with ties to the nucleolus. In Chap. 9, “Nucleolar transport of putative GTPase GNL1 and related proteins”, the authors discuss recent findings which link GTPases to ribosomal and

nucleolar function. GTPases belong to the P-loop NTPase superfamily and are divided into two classes, TRAFAC and SIMIBI. The YawG/YIqF family, part of the TRAFAC superfamily, contains five subfamilies all of which are characterized by the circular permutation of their GTP-binding motifs, contrary to the canonical arrangement. They are thus known as cpGTPases. Members of the YIqF family are found in phylogenetically diverse organisms and show a large expansion in eukaryotes. In yeast they are all involved in ribosome biogenesis. YIqF family members are also known as YIqF Related GTPases (YRG) and are linked to ribosomal functions. The more complex the eukaryote, the greater the number of YRGs found in the organism. Another family of GTPases in humans, the HSR1-MRR1 family, contains at least four well known members, GNL1, GNL2, GNL3, and GNL3L. GNL2 and GNL3L are nucleolar, while GNL1 is localizes to the nucleolus only during the G2 phase of the cell cycle. All contain nucleolar localization signals. For GNL2, this nucleolar localization signal may also act as a nuclear localization signal. However these GNLs only localize to the nucleolus when rDNA transcription is active. The authors conclude by suggesting further studies that may elucidate the targeting of these GTPases to the nucleolus.

## 1.10 Nucleolar Protein Anchoring and Translocation

The nucleolus is a self-organizing body that is the site of ribosome biogenesis but also houses proteins involved in several other cellular functions. It contains three subcompartments, the fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC). In their chapter (Chap. 10) Staron and Girstun review how proteins translocate to the nucleolus and how they are retained there. The main idea assumes that these proteins are constantly travelling throughout the nucleus and can freely enter and leave the nucleolus. Their travelling is slower in the nucleolus and therefore they are more abundant there. This may be due to an increased number of interactions and binding to nucleolar targets what results in the relatively high protein density in the nucleolus. Physical rules and biochemical reactions govern establishing of the interaction net that actually forms the structure of the nucleolus. Nucleoli exist because of nucleolar organizing regions (NORs) which result from rDNA transcription. In fact transcription inhibition results in nucleolar dissolution and the loss of nucleolar proteins. rDNA transcription leads to synthesis and recruitment of several types of RNA and nucleolar nucleic acids recruit structural/recruiting proteins serving as platforms for other nucleolar constituents. Majority of these recruiting proteins, which are reviewed by the authors in detail, are characterised by long acidic stretches with a high amount of phosphorylated serine and threonine residues. This phosphorylation serves to regulate protein-protein interactions. Examples of such recruiting proteins include upstream-binding factor (UBF), fibrillarin, nucleolin, and nucleophosmin. Proteins that bind to the nucleolar recruiting proteins can themselves also function to recruit additional proteins and

nucleolar proteins are thus retained in the nucleolus via interactions with either DNA, RNA, recruiting proteins, or other nucleolar proteins. The authors then review the translocation of nucleolar proteins discussing specific examples of translocation induced by DNA damage (UV and ionizing radiation, which triggers changes in the nucleolar proteome), cell stress (actinomycin-D-induced transcription inhibition, camptothecin-induced topoisomerase I inhibition and hypoxia), pre-rRNA processing (via nucleophosmin), and mitosis.

## Part IV: Nucleolar Proteins and Disease

### 1.11 The Nucleolus as a Stress Response Organelle

The nucleolus is not only a ribosome factory but also houses proteins involved in several other functions including stress response. In this chapter Lindström and Latonen review the many aspects of nucleolar stress and its relation to several diseases. The authors begin with a review of the nucleolus and proteotoxic stress, and discus specifically the proteasome pathway. Proteasomes are present only in the cytosol and nucleoplasm but translocate to the nucleolus upon stress-induced inhibition of the ubiquitin/proteasome pathway. This is accompanied by the formation of nucleolar aggresomes inside or associated with the nucleolus. These aggresomes contain proteins involved in the stress response and most if not all of them are normally degraded via the proteasome pathway. Interestingly, these aggresomes also contain poly A RNA but not rRNA. The authors then show that disruption of ribosomal proteins leads to nucleolar stress as well as several diseases including cancer. A subset of ribosomal and nucleolar proteins are involved in cancer and disruption of the nucleolus triggers a p53-dependent stress response leading to cell cycle arrest. The authors also show links between the nucleolus and several neurodegenerative diseases; Alzheimer's disease is associated with a reduction in ribosomal gene expression, Parkinson's disease with nucleolar damage, and Huntington's disease with inclusions adjacent to nucleoli, similar to aggresomes induced by proteasome inhibition. Nucleolar stress can also be induced by cytostatic and antibiotic agents. For example, actinomycin-D (AM-D) treatment results in dramatic shrinkage of the nucleolus and massive redistribution of nucleolar proteins into the nucleoplasm. Such drugs are therefore used in anti-cancer treatment. The chapter concludes with a discussion of the models for p53 activation which follows nucleolar stress and the authors show that there is a tight relationship between the nucleolus and p53. For example, there exists a p53-dependent nucleolar stress checkpoint that may be activated directly by nucleolar disruption or indirectly via redistribution of nucleolar proteins to the nucleoplasm. This checkpoint may rely on the nucleolus regulating the nuclear exit of p53 (to be degraded in the cytoplasm). Alternatively, in response to the disruption of ribosome biogenesis, nucleolar and ribosomal proteins may inhibit p53-negative regulator MDM2 leading to p53 activation. Interestingly, it is possible that knockdown of ribosomal proteins represent a different

type of stress signal for the cell than that caused by inhibitors of ribosomal DNA transcription. Ribosomal proteins could thus play a wider role in MDM2 regulation than previously thought.

## 1.12 The Nucleolar Aspect of Breast Cancer

Breast cancer is the most common fatal malignancy in women and is often caused by mutation or inactivation of one of the genes Breast Cancer 1 (BRCA1), Breast Cancer 2 (BRCA2), retinoblastoma tumor suppressor (RB), or tumor suppressor p53. In Chap. 12 Yan and Tang discuss the relationship between the nucleolus and breast cancer beginning with a review of nucleolar hypertrophy. The high growth demand of breast cancer cells requires an increased production of ribosomes which is evident by an enlargement of the nucleolus referred to as nucleolar hypertrophy. The rate of ribosome biosynthesis is proportional to the rate of cell proliferation and enlarged nucleoli are thus used as a diagnostic marker for cancer. Nucleolar size is determined by staining the nucleolar organizing regions (NORs) and AgNOR staining can thus be used as a marker for breast cancer. Although p53-mutations are not a necessity of breast cancer, AgNOR regions are larger in breast cancer cells possessing a mutation of p53. Moreover p53 is found in the nucleolus in these cancers, where it inhibits rDNA transcription. The authors also point out that nucleolar disruption has been shown to activate p53. The chapter continues with a review of the tumor suppressors and oncogenes associated with breast cancer and the nucleolus. Due to the increased demand for ribosomes several proteins that upregulate rDNA transcription are associated with breast cancer and several tumor suppressors that inhibit rDNA transcription have therefore been identified. One example is the rDNA transcription inhibitor retinoblastoma (RB), the loss of which can lead to breast cancer. Accordingly, RB function is a major contributor to tamoxifen therapy resistance. Another example is the breast cancer suppressor PTEN. However of the several tumor suppressers found in the nucleolus BRCA1 is one of the most important. It associates with the ribosome synthesis regulator nucleolin, suggesting it also may regulate the synthesis of ribosomal subunits. BRCA1 interacts with p14ARF, another nucleolar breast cancer suppressor. p14ARF inhibits ribosome biosynthesis by inhibiting pre-RNA processing, directly inhibiting rDNA transcription, and activating p53. The authors also discuss the oncogenes that upregulate rDNA transcription such as CDK2, mTOR, and EGF.

## 1.13 Cysteine Proteinase Inhibitors in the Nucleus and Nucleolus in Activated Macrophages

In Chap. 13 Kopitar-Jerala reviews the cysteine cathepsin inhibitors present in the nucleus and nucleolus of activated macrophages. The chapter begins with an introduction of macrophages and cysteine proteinases. Macrophages are found in all

tissues and are involved in the host defense against pathogens. They are the primary sensors of danger in the host. M1 macrophages are involved in inflammatory responses while M2s are involved in wound healing, tumor suppression, and parasite infections. Environmental signals cause a change in macrophage physiology, mediated through transcription regulation. When activated, macrophages upregulate proteinases such as cathepsins to degrade endocytosed pathogens. Cysteine cathepsins are involved in protein degradation in lysosomes, antigen presentation, and cancer progression. The author explains the general features of cathepsins and discusses the nucleolar localization of cathepsin L in classically activated macrophages. The chapter continues with a review of the cathepsin inhibitors, cystatins, and serpins. Type 1 cystatins are intracellular (cytosol and nucleus) while type 2 are extracellular. Stefin A, a type 1 cystatin present in skin, and stefin B, which is nuclear and cytosolic, are reviewed in detail. The author discusses the inhibition of serine and cysteine proteases by serpins but explains that serpins also have non-inhibitory functions. Most serpins are cytoplasmic and nuclear, with some localizing to nucleoli. The serpin Myeloid and Erythroid Nuclear Termination (MENT) stage-specific protein associates with compact heterochromatin and interacts with DNA while Spia3g is a serpin that translocates to nucleoli upon activation of macrophages. Mammary serine protease inhibitor (Maspin) is a non-inhibitory serpin that localizes to the cytoplasm and nucleus. The chapter concludes with a discussion of papain-like cysteine cathepsins in the nucleus and nucleolus.

## 1.14 Nucleolar Proteins and Cancer: The Roles of Aurora A-Interacting Nucleolar Proteins in Mitosis and Cancer

A number of nucleolar proteins are involved in cell cycle events, so it follows that the nucleolus will play some role in diseases such as cancer. In Chap. 14, Iyer et al. provide a diversity of evidence supporting the role of Aurora A kinase as a “mitotic master regulator and ocongenic kinase”. They first review the enzyme’s function in the emerging role of nucleolar and spindle-associated protein (NuSAP) which is over-expressed in malignancies including carcinoma, glioblastoma, hepatocellular carcinoma and pancreatic adenocarcinoma. Among other functions, NuSAP expression and phosphorylation is also correlated with the aggressiveness and metastatic capability of breast cancer and melanoma serving as an indicator of poor prognosis. These and other lines of evidence suggest that NuSAP may serve as a useful reporter antigen and a potential therapeutic target. In keeping with this Iyer et al., have focused on Aurora-A, a kinase that phosphorylates Ser240 on NuSAP to alter its binding to microtubules. While the ending to this story needs to be written, Iyer et al. have gained insight into another Aurora-A substrate, Eg5 (aka Kif11, mitotic kinesin5, KSP). This highly conserved microtubule kinesin motor protein mediates centrosome separation and spindle formation during mitosis. Eg5 is required for proper mitosis and over-expression leads to tumor formation in Mice. In keeping with this, this kinesin is over-expressed in bladder cancer, blast cyst chronic myelogenous leukemia CML (BC CML) and pancreatic cancer making Eg5 a potential marker

and, via its inhibition, a potential therapeutic avenue. Actin-Related Complex 1B (Arpc1B) is a third Aurora-A kinase target linked to various malignancies including esophageal, gastric, hepatocellular, melanoma and pancreatic cancers. It is an isoform of Arpc1, one of the seven subunits that comprise the actin related protein 2/3 (Arp2/3) complex involved in nucleating actin filaments. Arpc1B is a nucleolar protein which upon phosphorylation of Thr21 by p21 activated kinase (Pak1) associates with p34 and actin, and event that may underlie the proteins role in cancer metastasis and progression. Finally evidence is presented indicating that Arpc1B is as a physiological activator of Aurora A. During mitosis, these proteins co-localize in the centrosome along with gamma-tubulin. Aurora A also phosphorylates Arpc1B at Thr21 the same target residue as Pak1. Thus Iyer et al., provide evidence that many of the aforementioned proteins can serve both as markers and therapeutic targets with recent results on Aurora kinase inhibitors providing support that inhibiting this protein has resulted in decreased cell proliferation and increased apoptosis in several cancer cell lines. They also suggest that novel approaches that prevent nucleolar proteins, that are involved in cell proliferation and other events linked to cancer, from leaving the nucleolus might be a way to control malignant cells.

## 1.15 Nucleolar Transplantation and Human Embryogenesis

One way to gain new insight into nucleolar structure and function is to examine natural biological systems where nucleoli undergo transitional states. In Chap. 3 DiMario et al. took this approach in the study nucleologenesis in the fruit fly embryo where nucleoli don't appear until numerous nuclear divisions have occurred after fertilization. In Chap. 15, Fulka et al. examine the nucleoli of mammalian oocytes and early blastomeres which possess atypical nucleoli with potentially unique functions. Referred to as nucleolus precursor bodies (NPBs) these atypical nucleoli are considered to be sites for the storage of material for normal nucleolar formation during embryonic development but there is more to this story. During oogenesis nucleoli initially show the typical tripartite structure associated with active ribosome synthesis, a critical event in egg formation. However these nucleoli undergo changes related to both normal and abnormal egg maturation. The presence of NPBs signifies a fertilizable egg that once fertilized will undergo normal development. These events includes specific changes in nucleolar organization during cleavage leading the re-acquisition of the tripartite, active nucleolus that reflects the activation of the embryonic genome. To date only nucleoplasmin 2 (NPM2) has been identified in NPBs and the function of NPBS is poorly understood. Fulka et al. take the novel approach of manipulating embryos by removing and adding NPBs to gain deeper insight into their true embryonic functions. The reader is rewarded with a detailed explanation of and the requirements for performing enucleolation as well as the controls that are required. This allows them to appreciate the technological complexity of this useful experimental approach. While early oocytes require nucleoli for maturation (germinal vesicle breakdown), removal of nucleoli from fully grown

oocytes has no effect on maturation or progression to metaphase II. Similar nucleolar removal and addition shows that nucleoli from both parental pronuclei are required for development past the morula stage. Of intriguing interest was the result that simply transplanting NPBs into the cytoplasm was sufficient to subsequently generate nucleoli within nuclei. Fulka et al. have also shown that nucleoli from somatic cells cannot substitute for the original oocyte nucleolus yielding new insight into events of nuclear transplantation as well as the special functions of oocyte nucleoli. The potential application of nucleolar transplantation in human eggs and embryos in assisted reproductive technologies is discussed before speculation about future research is presented. Clearly NPBs represent a novel nucleolar type that is involved in regulating events of embryonic development either by storing future nucleolar components or regulating genes.

## Part V: Conclusions

## 1.16 Conclusion

In the concluding chapter, the editors summarize some of the issues brought up in the various chapters with a view to future research. These chapters support the continued emergence of the nucleolus as a dynamic intranuclear region that oversees a vast diversity of events linked to normal cellular survival and the ability of cells to cope with stressful environmental and pathophysiological challenges. The over 700 nucleolar proteins function in the assembly of ribosomal subunits, they the control cell cycle, apoptosis and aging, they coordinate stress responses and play a crucial role in cancer and viral infections. Thus after years of languishing solely as a ribosome factory, the nucleolus continues to reveal its true cellular importance that will only continue to grow research continues.

# Part II
# The Nucleolus and Nucleolar Proteins

# Chapter 2
# Functional Consequences of Nuclear and Nucleolar Architecture

**Eva Bártová and Lenka Stixová**

**Abstract** The nucleus is a highly compartmentalized structure. One of the most prominent nuclear compartments is the nucleolus. The nucleus and nucleolus share many structural and epigenetic features, but these features have specific functional significance. For instance, replication proceeds in replication foci, transcription in transcription "factories" or nucleoli, and splicing is regulated by proteins accumulated in nuclear speckles. Similarly, DNA repair events are associated with specific structural characteristics and occur in repair foci consisting of accumulated DNA repair-related proteins. Based on these observations, it is increasingly clear that changes in global genome organization and chromatin dynamics occur in parallel with functional changes in the genome. These structural characteristics contribute to the balance between genome stability and instability.

**Keywords** Chromatin • DNA repair • Epigenetics • Replication • Transcription

## Abbreviations

| | |
|---|---|
| ATM | Ataxia telangiectasia mutated kinase |
| ATR | Serine/threonine kinase/ataxia telangiectasia/Rad3-related protein |
| BER | Base excision repair |
| BrdU | 5-bromo-2′-deoxyuridine |
| CAF1 | Chromatin assembly factor 1 |
| CD | Chromodomain |
| CDK2 | Cyclin-dependent kinase 2 |

E. Bártová (✉) • L. Stixová
Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i,
Královopolská 135, Brno CZ-612 65, Czech Republic
e-mail: bartova@ibp.cz

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|---|---|
| CK2 | Casein kinase 2 |
| CSD | Chromoshadow domain |
| DDR | DNAdamage responses |
| DFC | Dense fibrillar components |
| dn | Double null |
| DSB | Doubles strand break |
| FC | Fibrillar center |
| GC | Granular components |
| HDAC | Histone deacetylases |
| HP1 | Heterochormatin protein 1 |
| HR | Homologous recombination |
| NER | Nucleotide excision repair |
| NHEJ | Non-homologous end joining |
| NORs | Nucleolar organizing regions |
| PML | Promyelocytic leukaemia bodies |
| Rb | Retinoblastoma gene |
| snRNPs | Small nuclear ribonucleoprotein particles |
| SSB | Single strand break |
| TDP | Time decision point |
| TEM | Transmission electron microscopy |
| UBF | Upstream binding factor |
| wt | Wild type |

## 2.1 General Aspects of Nuclear Architecture

Chromatin consists of DNA, histones, and other non-histone proteins and is organized into several hierarchical levels (Alberts et al. 2008). An important architectural component of chromatin is the nucleosome, in which DNA is wrapped around a histone octamer $(H2A\text{-}H2B\text{-}H3\text{-}H4)^2$ (Fig. 2.1). Nucleosomes are connected by linker DNA regions associated with the structurally important histone H1, which is responsible for chromatin condensation (Fig. 2.1). Multiple histones form a 30-nm fibre, which folds to higher-organized structures, called metaphase or interphase chromosomes that consist of euchromatin and heterochromatin (Fig. 2.2).

A variety of post-translational histone modifications on N-terminal tails regulate nuclear functions as replication, transcription, splicing, and DNA repair (Fig. 2.1). For example, individual amino acids on histones can be acetylated, methylated, phosphorylated, ubiquitinated, sumoylated, or poly(ADP)-ribosylated (Kouzarides 2007). Citrullination or deimination, involving the chemical conversion of arginine to citrulline, represents another example of biochemical changes in chromatin. In addition, highly specific phosphorylation of histone H1 occurs non-randomly during the cell cycle and is regulated by threonine-specific kinases (Sarg et al. 2006). For instance, retinoblastoma-deficient [Rb (dn)] fibroblasts exhibit a higher level of H1 phosphorylation during G1 phase than do wild-type cells, likely a result of CDK2

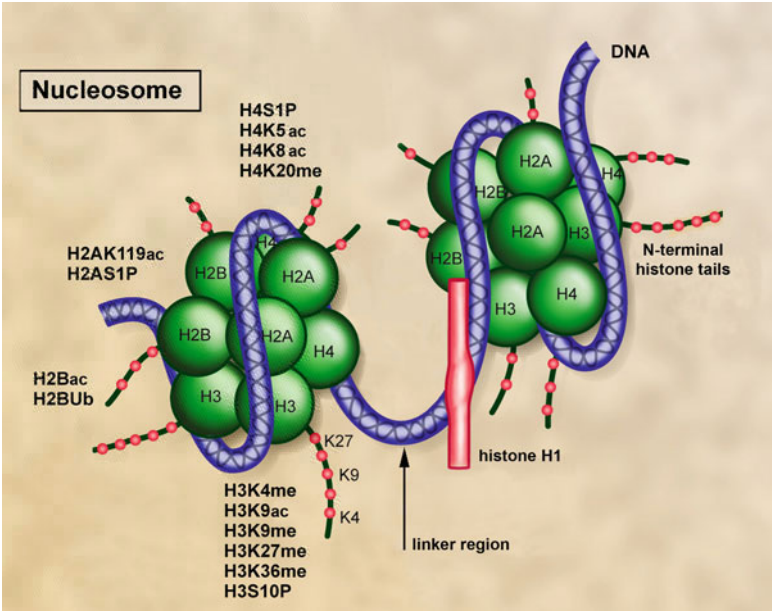


**Fig. 2.1 Nucleosome composition and post-translational modifications of histones**. Nucleosomes consist of a histone octamer formed by histones H2A, H2B, H3, and H4. Histone octamers are wrapped by 146 bp of DNA, and linker regions between individual nucleosomes occupy histone H1. Differential biochemical modification of amino acids in N-terminal histone tails regulates different nuclear processes. For example, acetylation (*ac*), methylation (*me*), phosphorylation (*P*), and ubiquitination (*Ub*) likely regulate nuclear processes, such as replication, transcription, splicing, and DNA repair. Post-translational modifications of lysine (*K*) are important for all nuclear processes

function. Moreover, Rb (dn) cells are more sensitive to micrococcal nuclease digestion, which indicates chromatin relaxation. Thus, H1 phosphorylation appears to decrease H1 affinity to linker regions (Herrera et al. 1996).

### *2.1.1 Chromosome Territories*

Interphase chromosomes are organized into so-called chromosome territories that reside in specific regions of the nucleus (Cremer and Cremer 2001; Lanctôt et al. 2007). Chromosome territories are separated by interchromatin channels or compartments that are crucial for the transport of regulatory molecules to target DNA (Fig. 2.2). Moreover, chromosomes are non-randomly organized within interphase nuclei. One very well-known example is the location of human chromosome 19, which is gene-rich, at the interior region of interphase nuclei, and the location of chromosome 18, which is gene-poor, in close proximity to the nuclear periphery

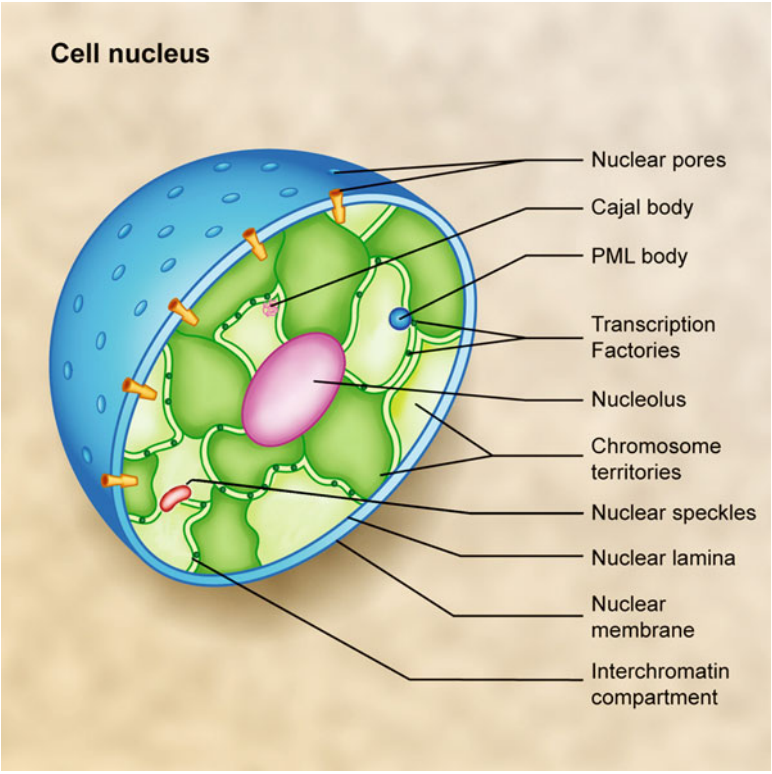


**Fig. 2.2 Schematic diagram of nuclear architecture.** The interphase nucleus of mammalian cells is highly compartmentalized. The biggest transcription factory is the nucleolus. Chromosomes occupy chromosome territories that overlap to some degree. The transport of regulatory proteins proceeds within interchromatin compartments. Nuclear processes occur in specialized domains: replication in replication foci, transcription in transcription factories, splicing in nuclear speckles, and DNA repair in DNA repair foci. PML bodies or Cajal bodies store regulatory proteins. The shape of nuclei is maintained by the nuclear membrane, and nuclear pores guarantee protein exchange between the nucleus and cytoplasm

(Croft et al. 1999). Thus, chromatin organization in three-dimensional space to some extent reflects the level of gene expression or density. For example, the transcriptionally active *c-myc* proto-oncogene and the *GFAP* gene are in a more interior location of the interphase nucleus than their transcriptionally inactive counterparts (Harničarová et al. 2006; Takizawa et al. 2008). Other work has shown that radial chromatin position is determined by local gene density rather than gene expression (Küpper et al. 2007).

Changes in the nuclear position of chromosome territories or their sub-compartments, including centromeres or telomeres, can be observed in particular cell cycle phases or during physiological processes leading to cell differentiation (Chaly and Munro 1996; Bártová et al. 2000; Essers et al. 2005). Pathophysiological events, including malignant

cell transformation or loss of A-type lamin function, are also accompanied by pronounced and highly specific structural changes in higher order chromatin structure and complex nuclear architecture. For example, A-type lamin deficiency is characterized by relocation of gene-rich chromosome 19 from the nuclear interior into nuclear blebs that are enriched in epigenetic markers associated with transcriptional activity, such as H3K4 methylation (Shimi et al. 2008). Moreover, A-type lamin deficient cells exhibit changes in peripherally positioned heterochromatin and in the compaction of chromosome territories (Galiová et al. 2008).

Several specific changes in nuclear architecture were also described in tumor cells. For example, the nuclear positions of fusion genes involved in chromosome translocation can be determined by the final structure of related derivative chromosome. Such aberrant genes are located in intermediate positions between those of the original genes (Taslerová et al. 2003). In addition, the probability of chromosome translocation increases after cell exposure to γ-irradiation, which results in close proximity of chromosome territories due to G2 cell cycle blockage (Kozubek et al. 1997; Nikiforova et al. 2000; Bártová et al. 2000).

As mentioned above, the interchromatin space in the interphase nucleus is interlaced with functionally specific structures or domains, such as nuclear speckles, nuclear bodies or foci of accumulating proteins that regulate particular nuclear processes (summarized by Ferrai et al. 2010). For example, gene expression is regulated not only by transcription factors, enhancers, and the histone code, but also by structural events. Transcriptionally active genes, which are mostly positioned on de-condensed chromatin loops, occupy so called transcription "factories" that contain Ser5-phosphorylated RNA polymerase II (Jackson et al. 1993; Ibora et al. 1996; reviewed in Chakalova and Fraser 2010). However, it is unclear whether splicing always proceeds co-transcriptionally in these functionally specific structural units. For example, some co-localization was observed for small SC35-positive nuclear speckles and transcription sites (Wansink et al. 1993), while large SC35 foci, which likely function for protein storage, were not associated with transcriptional activity (Ibora et al. 1996; Pombo and Cook 1996). Interestingly, the number of transcription factories per nucleus is cell type-specific and range between 100 and 300 in erythroblasts and lymphocytes and 2,000 in mouse embryonic fibroblasts (summarized by Chakalova and Fraser 2010).

A relationship between nuclear arrangement and transcription activity has been also demonstrated for transcriptionally active, developmentally important genes that are located on large and highly de-condensed chromatin loops extending away from compact chromosome territories. Examples include the pluripotency-specific transcription factor Oct4 (*POU5F1*, mapped to 6p21) and the entire major histocompatibity complex (MHC) region on human chromosome 6, which are highly de-condensed when transcriptionally active (Volpi et al. 2000; Wiblin et al. 2005; Bártová et al. 2008a). Indeed, fluorescence in situ hybridization (FISH) experiments have indicated that local gene density and transcriptional activity, rather than the activities of individual genes, correlate with the nuclear topography of chromosomes (Mahy et al. 2002). Thus, chromatin located outside of chromosome territories

is likely in an "open" configuration and poised for transcription (Mahy et al. 2002). Taken together, the results of these experiments clearly showed specific nuclear architecture that smooth the condition for transcription.

## 2.2 Nuclear Architecture and Epigenetic Events During Replication

Another elusive structural phenomenon is the spatial organization of DNA replication. Some studies have shown that DNA replication occurs in foci containing replication factors and proteins that regulate the cell cycle (Cardoso et al. 1993, 1997). Euchromatin loci are replicated early in S-phase, while constitutive heterochromatin replicates during late S-phase (Sadoni et al. 1999). Late-replicating chromatin (G-bands) is located preferentially at the nuclear periphery or around nucleoli, whereas early replicating euchromatic regions (R-bands) are broadly distributed but do not overlap with G-bands (Fig. 2.3; Camargo and Cervenka 1982). The distinct G- and R-bands, observed by Giemsa staining of mitotic chromosomes, appear to be distinct sub-chromosomal or nuclear domains that might, for example, reflect replication timing in interphase (Zink et al. 1998, 1999).

The correlation between replication timing and transcriptional control is underscored by the finding that biallelically expressed genes usually replicate synchronously, while monoallelically expressed or imprinted loci, similar to female X chromosomes, replicate asynchronously, with early and late replication patterns (Gilbert 2002). This has structural consequences; for example, in comparison with transcriptionally active chromosome X, the inactive female X chromosome, known as Barr body, relocates at the nuclear periphery during differentiation of human embryonic stem cells (e.g., Bártová et al. 2008b). Thus, there appears to be a correlation between transcriptional activity or inactivity and chromosome position within the interphase nucleus. It also corresponds to the early and late replication pattern, generally described for euchromatin and constitutive heterochromatin (Sadoni et al. 1999; Boyle et al. 2001). Whether replication timing determines the structure of chromatin or vice versa is still elusive. Interestingly, extensive chromatin rearrangement was observed at timing decision point (TDP) that arisen after late G1 phase when replication timing is established (Gilbert 2002). Before this point, chromosomal domains replicate randomly, but later, replication pattern is constituted even on structural level (summarized by Gilbert 2002).

### *2.2.1 Replication and Histone Signature*

Replication is also linked to the histone signature, and replacement of histones by newly synthesized proteins is functionally significant. Nucleosome assembly during DNA replication proceeds via two steps. The first step involves transfer of the parental

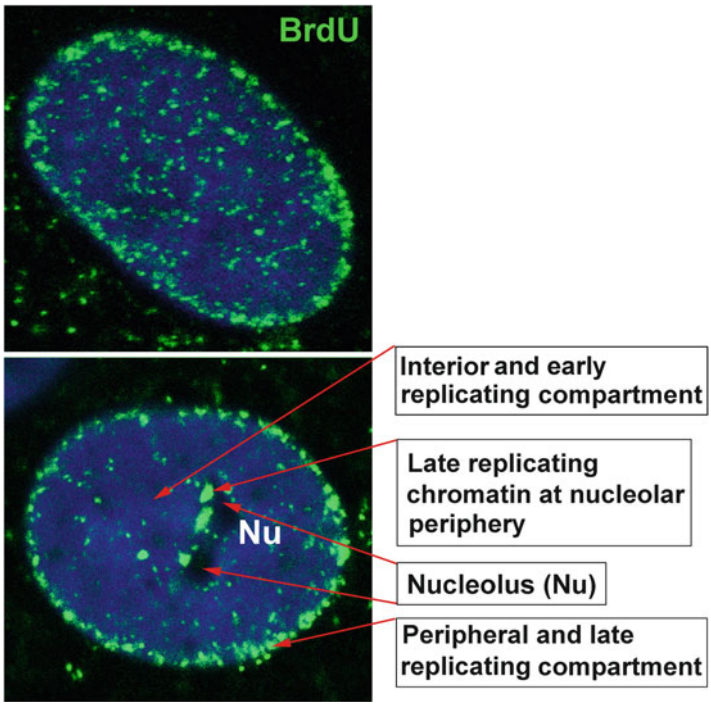


**Fig. 2.3 Nuclear pattern after BrdU incorporation.** The synthetic nucleoside bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU) is an analogue of thymidine. During the S phase of the cell cycle, BrdU is incorporated into the newly synthesized DNA of replicating cells and is detected by antibody staining. Based on the BrdU pattern, it possible to discriminate between early (located in more internal parts of nuclei) and late (green signals located at the nuclear periphery or around nucleoli [*Nu*]) replicating chromatin

nucleosome and its deposition onto a newly synthesized DNA strand. However, this recycling of parental histones only contributes to half of the chromatin of replicated DNA. The remaining histones are incorporated in a reaction known as de novo nucleosome assembly. This event is mediated by chromatin assembly factors such as the histone chaperone CAF-1, which guides soluble histones to sites of assembly at DNA replication forks (Krude and Keller 2001). Intriguingly, histone modifications do not affect the transfer of parental histones to newly replicated DNA; therefore, this represents a kind of epigenetic "inheritance." Recent studies have shown that acetylation of histone H3 at lysine 56 is important for maintaining genomic integrity and for H2A/H2B exchange during DNA replication (Clemente-Ruiz et al. 2011). The absence of H3K56 acetylation or the simultaneous knockout of CAF1 and Rtt106 factors affects the integrity of advancing replication forks, which potentiate homologous recombination (Clemente-Ruiz et al. 2011). Thus, this process also contributes to optimal DNA repair. Another histone modification, associated with replication, is H3Thr45 phosphorylation in budding yeast *Saccharomyces cerevisiae.*

Interestingly, loss of H3Thr45 phosphorylation causes replicative defects linked to replication stress (Baker et al. 2010). Based on these results, it is evident that specific epigenetic events in parallel with optimal replication-related nuclear architecture guide optimal replication timing or vice versa (Jackson and Pombo 1998).

## 2.3 General Function and Structure of Nucleoli

Synthesis of ribosomal subunits proceeds in a nuclear, non-membrane-bound compartment called the nucleolus (Olson and Dundr 2005; Chen et al. 2005). The morphology of nuclei is determined by nucleolar organizing regions (NORs) consisting of rDNA repeats, which have been mapped in humans to the short arms of acrocentric chromosomes 13, 14, 15, 21, and 22 (Worton et al. 1988; Gonzalez and Sylvester 1997. The nucleolus consists of several functionally specific compartments responsible for many events: the transcription of ribosomal genes, the production of preribosomal particles, the processing of primary transcripts into mature 18S, 5.8S, and 28S rRNA, the addition of proteins to nascent pre-ribosomes, and the incorporation of 5S rRNA, which is synthesized by RNA polymerase III away from the nucleolar compartment. Nucleoli consist of three sub-compartments that are clearly visible by transmission electron microscopy (TEM): the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The transcription of ribosomal genes is mediated by RNA polymerase I, but precisely where in the nucleolar region this occurs is a matter of debate (González-Melendi et al. 2001; Raska 2003; Scheer and Benavente 1990; Raska et al. 1995; Cmarko et al. 2000; Thiry et al. 2000). It is clear, however, that ribosomal units are assembled in the GC, and they leave the nucleus through the nuclear pores and unite once in the cytoplasm for the purpose of protein synthesis (Olson and Dundr 2005).

The specific nucleolar architecture in the region of the ~400 copies of human rRNA genes is also of functional significance. For example, nucleoli, as the largest transcription factories, are often located in the central parts of interphase nuclei (Bernstein and Allis 2005, reviewed by Russell and Zomerdijk 2005). Moreover, ribosomal genes are arranged into the structure called “Christmas tree” (Miller and Beatty 1969), characterized by location of transcriptionally active ribosomal genes on bifurcated branches. This structure has been observed for ribosomal genes in many human and plant cells (Koberna et al. 2002; Shaw et al. 2002), and decondensed branches of “Christmas tree” resemble chromatin loops with mRNA transcripts (Volpi et al. 2000).

Stable nucleolar composition is also crucial for maintaining genomic integrity. In some pathophysiological disorders, such as cancer, the amount of nucleolar proteins and silver stained NORs (AgNORs) can be significantly changed (summarized by Sirri et al. 2008). Some studies have additionally demonstrated a close relationship between the function of tumor suppressor genes or proto-oncogenes and ribosome biosynthesis (Ruggero and Pandolfi 2003). For example, ribosome synthesis is likely regulated by the *c-myc* protooncogene (Arabi et al. 2005). Reduced ribosome

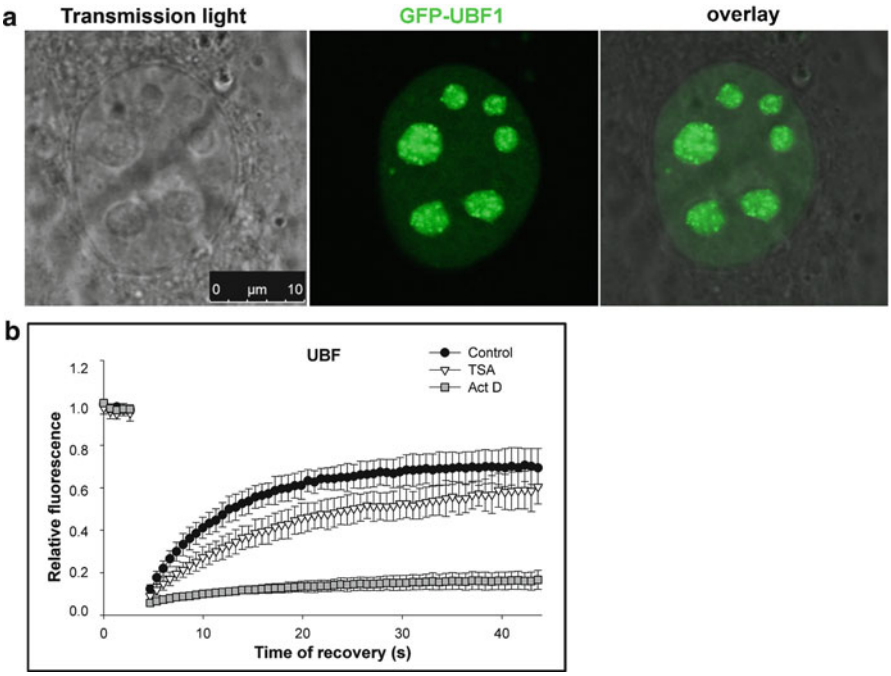


**Fig. 2.4 UBF nuclear pattern.** Upstream binding factor (*UBF*), which has two splice variants (UBF1 and UBF2), is required for transcription of ribosomal genes. (**a**) UBF1 accumulates in the nucleolar compartment (see GFP-UBF1) and is responsible for subsequent formation of 18S, 5.8S, and 28S ribosomal RNA. It acts together with the SL1 complex and TBP-associated factors. (**b**) An example of UBF1 recovery time after photobleaching (FRAP analysis) in non-treated and actinomycin D and Trichostatin A (TSA; HDAC inhibitor) treated mouse embryonic fibroblasts (Adopted from Stixová et al. 2011)

production leads to p53-dependent or independent apoptosis (David-Pfeuty et al. 2001), and interestingly, the function of several nucleolar proteins is tightly related to p53 tumor suppressor, indicating the necessity of proper nucleolar function for genome stability (Tsai and McKay 2002; reviewed by Sirri et al. 2008).

The dynamic properties of nucleolar components are also important to note. For example, nucleolar proteins rapidly associate and dissociate from nucleolar components (Dundr et al. 2002; Phair and Misteli 2000). Upstream binding factor (UBF) proteins in nucleus recover quickly after photobleaching (Fig. 2.4, Stixová et al. 2011), but this recovery can be abrogated by treating the cells with actinomycin D, an RNA polymerase I inhibitor (Stixová et al. 2011). Moreover, functional studies showed that active UBF proteins are highly acetylated in comparison with inactive UBFs (reviewed by Sirri et al. 2008).

The nucleolus contains many other key proteins. For example, fibrillarin is a component of small nuclear ribonucleoprotein particles (snRNPs) and plays an important role in processing of pre-rRNA. Fibrillarin associates with the U3, U8,