Nucleolus: A Central Hub for Nuclear Functions

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Abstract:	The nucleus contains distinct nuclear bodies (NBs); nucleolus is the largest and the most studied NB, but its role in the functioning of the nucleus is far from being fully understood. The nucleolus is not surrounded by a membrane, yet it contains DNA, RNA and a set of proteins that can either be retained in the nucleolus or rapidly shuttle between the nucleoplasm, the nucleolus and the cytoplasm in response to various stimuli. The emerging evidence points to the central function of the nucleolus in organizing many nuclear functions besides RNA polymerase I transcription and ribosome biogenesis. Here we discuss the functions of the nucleolus related to the shuttling of proteins and nucleic acids between nucleolus and nucleoplasm. The functional processes affected by shuttling of nucleolar components include 3D organization of the genome, stress response, DNA repair and recombination, transcription regulation, telomere maintenance and other essential cellular functions.

Highlights

- Nucleolus is a PolI transcription factory and a place of ribosome assembly, but is also performs many other functions that are commonly referred to as non-canonical functions of nucleolus
- Nucleolus organizes the adjacent chromatin into a large-scale repressive hub underlying the spatial segregation of active and repressive chromatin compartments.
- The interphase chromosomes are attached to the nucleolus *via* nucleolus-associated domains (**NADs**).
- Protein shuttling between the nucleolus and the nucleoplasm regulates a multitude of nuclear processes including DNA repair, recombination, transcription and telomere maintenance.
- Sequestration of proteins within nucleolus allows decrease sharply their concentration within other cellular compartments.
- Perinucleolar regions contain a recombination compartment; they also participate in allelic exclusion and X chromosome silencing.

Nucleolus: a central hub for nuclear functions

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Summary

The nucleolus is the largest and the most studied nuclear body, but its role in nuclear function is far from understood. Much work on the nucleolus has focused on its role in regulating RNA polymerase I transcription and ribosome biogenesis; however, emerging evidence points to the nucleolus as an organizing hub for many nuclear functions accomplished *via* shuttling of proteins and nucleic acids between the nucleolus and nucleoplasm. Here we discuss the cellular mechanisms affected by shuttling of nucleolar components that include 3D organization of the genome, stress response, DNA repair and recombination, transcription regulation, telomere maintenance and other essential cellular functions.

Glossary

Allelic exclusion: a situation when one allele of a given gene is expressed while the other allele is transcriptionally inactive.

Hi-C: a high-throughput method to study the three-dimensional architecture of genomes based on proximity ligation.

Nucleolus-associated domains (NADs): chromatin domains that dynamically interact with nucleoli.

Nucleolar localization signals (NoLS): a short protein sequence allowing to localize a protein inside the nucleolus. NoLS do not have a consensus sequence, but they are constantly enriched with positively charged amino acids. As a result, nucleolar accumulation *via* NoLS is dependent on the overall charge of the signal region.

Protein shuttling: most nuclear proteins are not tightly associated with nuclear bodies, but rather are continuously exchanged between these bodies and the surrounding nucleoplasm. This mobility allows these proteins to quickly relocalize between different nuclear domains, leading to unprecedented plasticity of the genome functioning.

Sequestration: mobile, continuously exchanging proteins can increase their concentration inside a nuclear body under some conditions. This process referred to as protein sequestration may be driven either by appearance of novel binding sites for the protein inside nucleoli, or disappearance of binding sited outside nucleoli, or posttranslational modifications of protein (or a combination thereof). Sequestration may also be due to a decrease of protein exchange due to

tight association with a component(s) inside any subcellular structure (protein immobilization) or due to formation of protein aggregates.

Stress response: a reaction which dedicates energy and effort to acute adaptation to stress.

Topologically associating domain (TAD)**:** a genomic region where DNA sequences physically interact with each other with a higher frequency as compared to the sequences outside the TAD. In mammals, TAD boundaries are enriched in CTCF/Cohesin binding sites. Most interactions observed between gene enhancers and promoters occur within one TAD. TADs were shown to coincide with replication domains.

Introduction

The main function of the nucleolus is the synthesis and processing of rRNA and ribosome assembly. Nucleoli contain clusters of ribosomal RNA genes (rDNA) and their transcripts (rRNA) (reviewed in [1]), as well as several hundred proteins participating in many nuclear processes inside and outside the nucleolus [2]. Recent data on nucleolar proteins and the analysis of genomic DNA loci associated with the nucleolus suggest that the function of the nucleolus is not limited to ribosome biogenesis, and that the nucleolus plays a role of a central hub regulating many nuclear and cellular processes. Movement of chromatin *vs.* the nucleolus may trigger activation and silencing of gene transcription [3,4], mediate inactivation of chromatin domains [5], underlie **allelic exclusion,** and spatially organize gene recombination [6]. In a similar way, movement of proteins between the nucleolus and the nucleoplasm regulates DNA repair, RNA polymerase II transcription, telomere maintenance, **stress response** [7,8]**,** and apoptosis [9]. Dynamic changes in localization of many proteins and chromatin regulated by the nucleolus appears to modulate a multitude of functions that were not previously associated with the nucleolus. These non-canonical functions of the nucleolus were discovered quite recently; therefore, the molecular mechanisms regulating these newly found nucleoli functions and the interactions of various components within the nucleolus remain unclear.

Protein accumulation in the nucleolus

The nucleolus is a membraneless organelle that forms through phase separation in the nucleus (Box 1). Protein accumulation inside nucleoli is a consequence of affinity interactions with core nucleolar components: rDNA, RNA and nucleolar proteins [10]. This accumulation is driven by short amino acid stretches referred to as nucleolar localisation sequences (**NoLS**) [11]. **NoLS** are substantially enriched with positively charged amino acids. Although there is no **NoLS** consensus sequence, approximately 50% of amino acids inside **NoLS** are lysines and arginines that generate a positive charge essential for nucleolar localization [12–15]. Thus, charge-

dependent (electrostatic) interactions of amino acid residues with nucleolar components lead to their nucleolar accumulation. The nucleolus harbors a large number of negatively charged RNA molecules, potential targets for positively charged **NoLS** [14]. Of note, **NoLS** do not interact with DNA since its charge is neutralized by histones, bivalent ions and polyamines [16,17]. Low specificity of electrostatic accumulation *via* **NoLSs** allows a large number of proteins to be dynamically accumulated inside nucleoli. Nucleolar accumulation of proteins may also be due to their interaction with some nucleolar proteins including such as NCL (nucleolin or C23) which contains long acidic patches interacting with positively charged **NoLS** [18]. Nucleolar accumulation by **NoLS** is dynamic and can be affected by a variety of factors. For example, tumour suppressor ARF inhibits nucleolar import of TTF-I, a RNA polymerase I transcription termination factor, by binding to its **NoLS**, thus favouring TTF-I displacement from the nucleolus to the nucleoplasm [19] followed by the suppression of ribosomal RNA synthesis and cell proliferation arrest.

Protein shuttling between the nucleolus and the nucleoplasm regulates nuclear processes

Nucleolus is a highly dynamic structure involved in an active exchange of proteins between the nucleolus and the nucleoplasm (**protein shuttling)** [20,21]. In some cases, the appearance of strong retention centres outside of the nucleolus (e.g. recruitment of DNA repair machinery to DNA lesions) may lead to a decrease in the concentration of a protein within the nucleolus. Inversely, sequestration of various enzymes involved in stress response, DNA repair, cell cycle progression etc. within the nucleolus appears advantageous because it allows to keep the concentration of these proteins in the nucleoplasm relatively low under normal conditions and to increase it sharply upon stress. Excessive concentration of DNA repair enzymes and stress response factors in the nucleoplasm under normal conditions may result in a significant off-target activity. It seems thus logical to store these proteins within a dense phase-separated compartment until they become necessary to fulfil their specific functions [22].

The unique feature of the nucleolus is that it is both a storage place and a stress sensor. Some proteins are stored in the nucleolus irreversibly [23,24]. Other proteins perform major functions within the nucleolus and a moonlighting function in the nucleoplasm (Table 1). The third group of proteins have their main function in the nucleoplasm, but they are stored in the nucleolus until release (Table 2). Shuttling control is tightly connected with rDNA transcription and modulated by stresses, cell cycle progression, energy resources and various post-translational modifications. Below we discuss several examples demonstrating how the nucleolus integrates external signals and provides necessary responses to different stimuli.

DNA damage and repair. Cells are subjected to a multitude of DNA lesions resulting from oxidation, alkylation, exposure to UV light or irradiation. The response to these events may include DNA repair and cell-cycle arrest. The nucleolus plays an important role in DNA repair as it contains many DNA-binding proteins involved in DNA repair in the nucleoplasm and ribosome biogenesis in the nucleolus (Table 1, Figure 1). Switching between ribosome biogenesis and DNA repair functions is likely to occur through protein-protein interactions and posttranslational modifications regulating shuttling. For example, the apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1; APEX1; REF-1) interacts with several nucleolar proteins *via* positively charged N-terminal lysine residues. In the nucleolus, APE1 interacts with NPM1 (also called B23, nucleophosmin, NO38 or numatrin), the 47S, 18S and 28S rRNAs [25] and deletes lesions from rRNA [26]. Deacetylation of N-terminal lysine residues upon DNA damage results in APE1 relocalization in the nucleoplasm where it is an essential component of base-excision repair machinery [26]. Another example concerns RecQ-like helicases WRN (Werner syndrome) and BLM (Bloom syndrome) implicated in double strand break (DSB) and base lesion repair in the nucleoplasm [27–29]. Phosphorylation [8], acetylation [30] and ubiquitination [31] of WRN and BLM regulates their shuttling between the nucleolus and nucleoplasm. WRN possesses a

NoLS in its C-terminus and is normally located in the nucleolus where it interacts with RNA Pol I; the absence of WRN in the nucleolus reduces 18S and 28S rRNA levels [32]. When DNA repair is activated, WRN is dephosphorylated and moves from the nucleolus to the nucleoplasm [8]. This process is also modulated by acetylation [30]. In the nucleolus, BLM facilitates pre-rRNA synthesis by directly binding rDNA and interacting with RNA Pol I and DNA topoisomerase I [33–35]. BLM ubiquitination promotes its recruitment to DNA lesions while the absence of the RNF8 ubiquitin ligase results in its nucleolar sequestration [31].

Some proteins move into the nucleolus upon DNA damage: for example, a nucleoplasmic DNA damage response factor NBS1 can transiently accumulate in the nucleolus to inhibit rDNA transcription. Nucleolar localization of NBS1 depends on its interaction with the nucleolar protein Treacle (TCOF1). The Treacle-NBS1 complex acts as an adaptor for ATM kinase, which phosphorylates nucleolar proteins that regulate rDNA transcription in response to DNA damage. Activation of ATM kinase in the nucleus leads to translocation of the Treacle-NBS1 complex into the nucleolus and thus to silencing of rRNA transcription [36].

Similarly to many classical DNA repair enzymes located in the nucleolus, major nucleolar proteins NCL and NPM1 are rapidly exchanged between the nucleolus and the nucleoplasm so they can be readily available for DNA repair when necessary (Table 2, Figure 1) [7,37]. In particular, NCL translocates into the nucleoplasm and forms foci at sites of DSB repair where NCL is implicated in chromatin remodelling as a histone chaperone [38–41]. Relocalization of NPM1 from the nucleolus to the nucleoplasm is induced by DNA damaging agents such as ionizing radiation, cisplatin or etoposide treatment [42]. The relocalization of NPM1 to DSB repair foci is regulated by its phosphorylation [43]. In summary, DNA damage triggers an intense **protein shuttling** between the nucleolus and the nucleoplasm. Release of DNA repair

factors stored in the nucleolus as well as *bona fide* nucleolar proteins into the nucleoplasm stimulates DNA repair.

Telomere maintenance. Telomerase is a ribonucleoprotein complex associated with the telomere repeats which protects the ends of the chromosomes from degradation. Telomerase contains the telomerase reverse transcriptase (TERT), telomerase RNA, and several additional proteins. Telomerase is assembled in the nucleolus by several proteins including dyskerin that later becomes a part of telomerase in the nucleoplasm [44]. Dyskerin also participates in pseudouridylation of specific residues in newly synthesized ribosomal RNAs and snRNAs [45]. Telomerase may be temporarily retained there under certain conditions through its interaction with NCL [46]. Retention of telomerase within the nucleolus is favorable in case of DNA damage as loosening of telomere structure should be necessary for DSB repair at telomeres [47]. Remarkably, TERT contains three **NoLS** [48–50]. TERT in the nucleolus stimulates RNA PolI transcription and binds rDNA during oncogenic and regenerative hyperproliferation, probably *via* activation of the transcription initiation complex formation [51] (**Figure 1)**. The telomeric repeat-binding factor TRF2 also interacts with telomeres and protects them. TRF2 can be sequestered in the nucleolus by nucleolar protein NOLC1 [52], thereby affecting its function at telomeres [53]. NOLC1 overexpression and consequent relocalization of TRF2 to the nucleoli arrests cells in G_0-G_1 phase and prevents proliferation. In the nucleolus,TRF2 binds rDNA and promotes rRNA transcription [54].

Nucleolus and stress response. Although most nucleolar proteins are highly dynamic and shuttle between the nucleolus and the nucleoplasm [37,55,56], these proteins can be retained in the nucleolus during transcriptional, acidic, or heat stress. Disruption of protein traffic through the nucleolus may lead to formation of intranucleolar protein complexes. One example is a cyclin-dependent kinase inhibitor p21cip (or p21), present both in the nucleus and the cytoplasm. In the

cytoplasm, p21cip has oncogenic properties, while nuclear p21cip is a tumour suppressor. p21cip transits through the nucleolus on its way from the nucleus to the cytoplasm. DNA damage inhibits this transit and induces formation of p21cip-containing intranucleolar bodies (INoBs) [57]. These structures also contain SUMO-1 and UBC9, the E2 SUMO-conjugating enzyme, several DNA damage checkpoint proteins and cell cycle regulators Cdk2, Cyclin E, PCNA, p53 and MDM2 [58]. SUMO-1 and p21cip control the transit of proteins through the nucleolus, but when nucleolar export is disrupted by DNA damage, these proteins act as scaffolds that mediate the formation of the multiprotein complex in the nucleolus [58]. The MDM2–p53 pathway which coordinates cellular response to stress is also regulated by shuttling of the nucleolar proteins. In response to stress, several nucleolar proteins, including NCL [59], NPM [60], and nucleostemin [61], are released into the nucleoplasm, where they bind to and inhibit MDM2 resulting in p53 activation and stabilization (Figure 2).

Mammalian target of rapamycin (mTOR) pathway is another major cellular system that senses various types of stress and integrates intra- and extra-cellular cues for cell survival and degradation pathways [62]. One of the main functions of mTORC1 is activation of anabolic processes, including ribosome biogenesis and translation. Although mTOR complex 1 (mTORC1) is predominantly cytosolic and localizes around the lysosomal membrane, several members of mTORC1, including mTOR itself, can also locate to the nucleus and the nucleolus [63–69]. Nuclear mTOR protein can bind to thousands of sites in the genome [66,67] and interact with rDNA chromatin either directly [63–65] or *via* modulation of other rDNA-interacting factors [70,71]. Treatment with mTORC1 inhibitor rapamycin prevents mTOR localization to the nucleolus and interferes with the processing of ribosomal rRNAs [68]. One of the recent examples of the nucleolar activity of mTORC1 is related to the function of inhibitor of growth 1b protein (ING1b), which associates with rDNA repeats and is required for the efficient recruitment of chromatin regulator HDAC1 to the nucleoli [69]. ING1 reduces mTOR

localization to the nucleolus and promotes recruitment of HDAC1 to the nucleolar remodelling complex (NoRC) complex and to major Pol I transcription factor UBF1. Inversely, knockdown of ING1 results in the accumulation of mTOR in nucleolus and it association with UBF1.

Sequestration of proteins in the nucleus. Although most of the shuttling between the nucleolus and the nucleoplasm is reversible, recent studies indicate that the nucleolus can sequester and store various nuclear and cytoplasmic proteins including misfolded protein aggregates. These aggregates form proteinaceous particles that can promote proteotoxic stress and eventually cell death if they are not cleared by the ubiquitin-proteasome system. Protein aggregates may also form directly in the nucleoli when proteasome function is inhibited. [72]. These inclusions form so-called 'aggresomes' [23] or 'detention centers' [24] within or in a close contact with the nucleoli (Figure 2); they are rich in protein and RNA [73]. Aggresome formation correlates with cell survival [74].

Various stresses lead not only to formation to misfolded proteins, but also to the expression of specific lncRNAs from the intergenic spacer separating individual rDNA transcription units (IGS lncRNAs) [75]. IGS lncRNA transcription correlates with the formation of nucleolar detention centers [24] retaining DNA (cytosine-5)-methyltransferase 1 (DNMT1), the δ catalytic subunit of DNA polymerase (POLD1), Hsp70, RNF8, MDM2, APC2 [75], RPA16, RPA40, PES1, NOP52, RRP1B, NOM1, NOL1, SENP3, and von Hippel-Lindau (VHL) protein [24]. VHL protein degrades the hypoxia-inducible factor (HIF) in the presence of oxygen; **sequestration** of VHL enables HIF to evade destruction and activate its target genes [76]. Immobilization of these proteins inside the nucleolus is driven by specific nucleolar detention signals (NoDS) [75]. Importantly, key nucleolar proteins FBL, NPM1, and NOPP140 that do not contain these signals remain mobile and evade retention, highlighting the specificity of this nucleolar **sequestration** [24].

Dynamic association of chromatin with the nucleolus controls 3D genome organization.

Nucleolus and 3D genome organisation Nucleus may contain one or several nucleoli that occupy up to 25% of its volume. The nucleolus is largely filled with transcribed rDNA and assembling ribosomes. In each cell, only a fraction of rRNA genes is transcriptionally active and localise within nucleoli. Inactive rDNA repeats are located at the periphery of the nucleolus contributing to the creation of a perinucleolar inactive chromatin compartment [5,77,78]. The presence of heterochromatin at the nucleolar periphery is directly related to the positioning of silenced ribosomal genes in this area [79]. Silencing of rDNA repeats is mediated by NoRC that recruits histone-modifying and DNA methylating enzymes [80,81]. The NoRC recruited to the perinucleolar region may also introduce heterochromatic marks into other chromatin regions in the vicinity of the nucleolus. Interestingly, centromeric heterochromatin is frequently located close to nucleolus, and depletion of TIP5, a subunit of NoRC compromises both rDNA silencing and assembly of centromeric heterochromatin [82].

Genomic segments located within perinucleolar compartment are commonly referred to as **nucleolus-associated chromatin domains** (**NADs**) [83,84]. **NADs** contain sequences located in the short arms of acrocentric chromosomes, centromeric and pericentromeric chromatin of most chromosomes and subtelomeric regions of some chromosomes **(Figure 3)**. Besides repetitive sequences, **NADs** contain more than 1000 genes including those encoding for the T-cell receptors, olfactory receptors and two families of immunoglobulin genes. Many **NAD**-associated genes have similar features: they are tissue-specific and form large gene clusters [85]. **NADs** substantially overlap with lamina-associated domains (LADs) [86]. Only a portion of LADs identified in population studies is located close to nuclear lamina. After mitosis, LADs are

stochastically reshuffled, and LADs redistributed to the nuclear interior are frequently localised within the perinucleolar region [87].

What is the functional significance of **NADs** and how the nucleolus can regulate their functions? **NADs** are globally enriched in heterochromatin marks, including H3K27me3, H3K9me3, and H4K20me3. Gene targeting to the nucleolus and the perinucleolar region is globally correlated with reduced gene expression [88]. Localization of **NADs** in the peripheral region of the nucleolus may thus contribute to gene silencing [5]. Regulation of gene expression may involve changes in gene positioning or the pattern of their contacts with the nucleolus. Indeed, some processes are accompanied by relocalization of genomic loci *vs.* the nucleolus. For example, centromeric and pericentromeric repeats are dissociated from the nucleolus in aging cells [89]. Association with the nucleolus globally correlates with the inactive transcriptional state of RNA polymerase II-transcribed genes. Some exceptions exist: translocations of the *CCND1* and *MYC* genes to the acrocentric chromosome 14 into the locus of the immunoglobulin heavy chain (*IGH*) genes lead to their relocalization towards the perinucleolar region and activation by NCL [4]. Association of the *IGH* genes with the nucleolus may also be necessary for recombination. Thus, B-cell maturation is accompanied by relocalization of the *IGH* locus in the prenucleolar region to a specific "recombination compartment" containing an activation-induced cytidine deaminase (AID). Somatic hypermutation and class-switch recombination of immunoglobulin genes take place in these "recombination compartments" [6]. Localization of genes other than *IGH* within this compartment may significantly increase the probability of oncogenic translocations in B-cells [90]. Association of one allele of a monoallelically expressed gene with the nucleolus may participate in stochastic or imprinted **allelic exclusion** which could be the case for *TCR*, *IGH* and olfactory receptor genes [91,92].

In the interphase nucleus, each chromosome forms a distinct chromosomal territory that is likely linked to both the nuclear lamina and the nucleolus because every chromosome contains both LADs and **NADs**. Being attached to the nucleolus, the nuclear lamina and to each other [93,94], chromosomal territories constitute a unified chromatin compartment that is mechanically stretched [95] and can sense mechanical stress [96]. In this scenario, phase-separated nuclear compartments, the largest of which is the nucleolus, act as global chromatin organizers [94,96]. In particular, tethering of chromatin to the nucleolus and nuclear speckles is likely to account for the spatial segregation of repressed and active chromatin compartments [97]. These compartments likely corresponding to heterochromatin and euchromatin were identified by **Hi-C** [97]. Besides repressed and active chromatin compartments **Hi-C** and other recently developed experimental procedures allowed to identify self-interacting topologically associating domains (**TADs)** and contacts between chromosomes [94,97–99]. Interaction between the nucleolus and extra-nucleolar chromatin may stabilize weak interchromosomal contacts and serve as a centrefold for the arrangement of chromosomal territories inside the nucleus [100]. The association of specific genomic loci with the nucleolus may occur through their interaction with rDNA. Recent **HiC** experiments demonstrated that 5S and 45S gene arrays formed multiple contacts with genomic regions and genes on all chromosomes [101].

Association of chromatin with the nucleolus is mediated both by proteins and RNA. Nucleolar proteins Ki-67, NCL and NPM1 mediate the association of chromatin with the nucleolus. These proteins interact with CAF1, a chromatin assembly factor [89]. Depletion of the p150 CAF1 subunit leads to a decreased association of certain genomic loci, including the 5S rDNA, alpha satellite DNA, and the D4Z4 macrosatellite, with nucleoli in human cells [102]. Depletion of NLP, a Drosophila homolog of NPM1, resulted in de-clustering of centromeres and a decrease in centromere association with the nucleolus [103]. Normal fibroblasts and cancer cells depleted of NPM1 displayed deformed nucleoli and a striking rearrangement of perinucleolar

heterochromatin [104]. NPM1 also associates with HP1γ, core linker histones and a centromere-specific histone variant CENP-A [105]; NPM1 is required for efficient tethering of HP1γ-enriched chromatin to the nucleolus. Thus, interaction with chromatin and, in particular, heterochromatin proteins may be a major driver of chromatin anchoring to the nucleolus. CTCF, a structural protein that regulates 3D genome folding into **TADs** that often include co-regulated and co-transcribed genes (reviewed in [106,107]), may also anchor/target specific loci to the perinucleolar space. CTCF interacts with NPM1 to tether insulators to the nucleolar periphery [108]. In *Drosophila,* CTCF interacts with NLP and the nucleolar protein Modulo to position centromeres in the perinucleolar region [103].

Non-coding RNAs participate in the chromatin tethering towards the nucleolar periphery as well. For example, *Xist* and *Firre* non-coding RNAs (ncRNA) participate in X inactivation. *Xist* is responsible for the association of the inactive X chromosome with the nucleolar periphery [109]. *Xist* loss after X inactivation leads to dissociation of X chromosome from the nucleolus. Interestingly, autosomes bearing translocations with the X chromosome also become preferentially associated with the nucleolus [110]. *Firre* is expressed from a perinucleolar locus located on the X-chromosome; this locus contains CTCF and cohesin binding sites [111]. *Firre* transcription from the inactive X chromosome cooperates with CTCF binding to ensure perinucleolar positioning. Knockdown of *Firre* or its interaction partner hnRNPU interferes with the perinucleolar targeting of the inactive X chromosome. An imprinted antisense *Kcnq1ot* ncRNA is transcribed only from the paternal allele and insures the **allelic exclusion** by targeting the paternal locus to the perinucleolar space where this 1Mbp region containing ten protein-coding genes is silenced [112,113]. Thus, nucleolus plays an important role in the 3D organization of the genome. It participates in organization of chromosome compartments and territories as well as heterochromatin assembly. This process involves several nucleolar proteins and non-coding RNAs.

Concluding remarks

The key role of nucleolus as a regulatory hub is directly related to its main function, production of ribosomes. This process consumes more than a half of cell energy resources and thus should be coordinated with the actual needs of the cell in the ribosomes. Accordingly, many regulatory circuits link the production of ribosomes to the cell cycle progression which, in turn, can be blocked by stress responses. It is thus not surprising that nucleolus acquired a role of regulatory hub connecting many functional processes. The fundamental link between ribosome biogenesis and cell cycle progression is accomplished through involvement of certain proteins in cell cycle control and stress response on the one hand, and the regulation of ribosomal gene transcription and ribosome assembly on the other hand. Many of these proteins are sequestered within phase-separated nucleoli but may shuttle to nucleoplasm in response to various stimuli.

The principal role of nucleolus in the 3D organization of the genome consists in creation of a nucleation center for the assembly of heterochromatin. This nucleation is due to silenced rRNA genes. Silencing of a portion of rRNA genes is an ancient phenomenon occurring even in lower eukaryotes. In multicellular organisms, the mechanism that silenced rRNA genes could be extended to other genes that are not used in various types of differentiated cells. Further studies will help us to decrypt the amazing complexity of the nucleols (see Outstanding questions).

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Box 1

Membraneless nuclear compartments

Eukaryotic cell nucleus contains a number of membraneless compartments such as nucleoli, Cajal bodies, PML bodies, nuclear speckles etc. [114]. These compartments are dynamic associates of proteins and nucleic acids and are slightly denser than the bulk of the nucleoplasm. Each type of membraneless compartments is connected to certain functional processes that are not necessarily related to each other [115]. Accordingly, most of the compartments contain a number of various proteins including many moonlighting proteins [116]. The proteins participating in assembly of nuclear compartments frequently possess intrinsically disordered domains [116–118]. These proteins/domains may mediate weak-affinity and non-specific interactions with multiple target sites that trigger the liquid phase separation [119]. The phase-separated droplets accumulate macromolecules that possess an affinity to the components of the droplet interior. Usually a so called platform nucleates the assembly of liquid droplet nuclear compartments. This may be either a protein (e.g. PML in case of PML-bodies [120]) or a non-coding RNA (e.g. NEAT1 RNA in paraspeckles [121] or rRNA in nucleoli [122]). The nucleolus differs from other membraneless compartments in two ways. First, the nucleolus consists of at least three distinct phase-separated layers: droplets within droplets [123]. Second, the availability of pre-rRNAs that constitutes a platform for nucleolus assembly directly depends on the ongoing rDNA transcription [124]. Suppression of rDNA transcription results in full or partial disruption of nucleoli and release into cytoplasm of a various proteins sequestered within nucleoli [124]. This is likely to be an important part of the cellular stress response mechanism [125].

Figure Legends

Figure 1. *DNA repair and telomere maintenance are regulated by protein shuttling between the nucleolus and the nucleoplasm.*

Clockwise: APE1 interacts with nucleolar proteins NPM1 *via* its positively charged lysine residues. When some of these lysine residues are acetylated, NPM1 no longer interacts with APE1 and APE1 accumulates in nucleoplasm at the DNA damage sites. Nucleolar mTOR activates PolI transcription. WRN possesses a **NoLS** in its C-terminus; nucleolar localization of WRN is dependent on RNA Pol I transcription. When DNA repair is activated, WRN is dephosphorylated and moves from nucleolus to nucleoplasm. BLM can directly bind rDNA and interact with RNA Pol I. BLM switching between DNA repair and ribosome biogenesis occurs through protein-protein interactions and posttranslational modifications. TRAIP encodes a nucleolar protein that migrates to UV-induced DNA lesions in the nucleoplasm; inhibition of RNA Pol I activity also leads to TRAIP diffusion into the nucleoplasm. The telomerase complex biogenesis occurs in the nucleolus; TERT is retained in nucleoli through its interaction with NCL. Nucleolar localization of TRF2 is promoted by the nucleolar protein NOLC1. TRF2 also binds rDNA and promotes rRNA transcription. NCL forms nucleoplasmic foci at sites of DSB repair where it is implicated in chromatin remodelling while the mail pool of NCL is located in the nucleolus. Ionizing radiation, cisplatin or etoposide treatment leads to NPM1 phosphorylation and its relocalization from the nucleolus to the DSB foci in the nucleoplasm.

Figure 2. *Sequestration of nuclear and cytoplasmic proteins in the nucleolus.*

Misfolded proteins aggregate and form proteinaceous particles that are transported into the nucleolus form so-called ‘aggresomes’ or ‘detention centers’. p21Cip1 transits through the nucleolus on its way from the nucleus to the cytoplasm. DNA damage inhibits this transit and induces formation of p21Cip1-containing intranucleolar bodies (INoBs) containing SUMO-1, UBC9, Cdk2, Cyclin E, PCNA, p53 and MDM2. Transcriptional, acidic or heat stress leads to

expression of lncRNAs from the rDNA intergenic spacer. These transcripts capture and immobilize inside nucleoli several important proteins, including VHL, DNMT1, tPOLD1, Hsp70, RNF8, MDM2, APC2, RPA16 and RPA40, PES1, NOP52, RRP1B, NOM1, NOL1, and SENP3. Key nucleolar proteins FBL, NPM1, and NOPP140 retain their mobility and evade immobilization, highlighting the specificity of this nucleolar **sequestration**.

Figure 3. *Dynamic association of chromatin with the nucleolus regulates nuclear processes*
Clockwise: nucleoli-associated chromatin domains (**NADs**) represent hundreds of extended genomic loci comprising ~4% of the human genome. **NADs** contain sequences located in the p-arms of acrocentric chromosomes, centromeric and pericentromeric chromatin of most chromosomes and subtelomeric regions of some chromosomes. Association with the nucleolus globally correlates with the inactive transcriptional state of RNA polymerase II-transcribed genes, with some exceptions. Association with the nucleolus may also participate in **allelic exclusion**. Somatic hypermutation and class-switch recombination occur in the specific "recombination compartment" located in the prenucleolar region and containing AID. Inactive X chromosome is associated with the nucleolar periphery; this association is due to *Xist* and *Firre* non-coding RNAs.

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Outstanding Questions

- Accumulation and sequestration of different proteins with non-canonical functions influences or/and regulates canonical function of nucleoli. Is it possible to regulate these processes to develop novel therapeutic strategies?

- Is it possible to block stress response mediated by nucleoli in order to increase the efficacy of anticancer drugs?

- How were non-canonical functions of nucleoli modified during evolution? Was the evolution of the nucleolus (e.g., transition from bipartite to tripartite organization) connected with modification of its non-canonical functions?

- What is the functional context of DNA-DNA contacts between rDNA and the rest of the genome? How these contacts change during development, pathology or stress?

- It is unclear how transcriptionally active regions are functionally maintained within heterochromatin-rich NADs. CTCF, a protein involved in chromatin domain maintenance and associated with the nucleoli, may play a role in this process.

Rebuttal letter

Editor comments:

1.The abstract is currently over the 120 word limit that is set for Trends reviews; therefore, please consider this trimmed abstract below. Please make any changes you feel are necessary.
The abstract was trimmed. We accepted most of your corrections.

2.Much of this discussion lists interactions, but it may be unclear to general readers what the significance of these interactions are for DNA repair and ribosome biogenesis. Please consider a table that lists these interactions and please consider some of the changes below to make clear the significance of these different PTMs and protein-protein interactions are for the cell. Please make any additional changes you feel are necessary and please ensure the changes made remain scientifically accurate.
We have added two tables listing the interactions of different proteins with the nucleolus and have rewritten the section on protein shuttling for clarity.

3. The subheadings could be shortened to just name the nuclear process that is being described as the earlier subheading makes clear that proteins are shuttling during these events. Please consider the trimmed headings provided.
We now use short headings throughout the text

We have addressed remarks 4-10 in a new version of the section.

11. Please consider a final concluding sentence that best summarizes the main conclusion from this section.
A conclusion was added to the section on page 8: ***"In summary, DNA damage triggers an intense protein shuttling between the nucleolus and the nucleoplasm. Release of DNA repair factors stored in the nucleolus as well as bona fide nucleolar proteins into the nucleoplasm stimulates DNA repair."***

12. Please clarify what this fact has to do with its relocalization during DNA repair?
NPM1 relocalization upon DNA damage is indeed linked to its functions in DNA repair. This is now stated in the text on page 8: ***"In particular, NCL translocates into the nucleoplasm and forms foci at sites of DSB repair where NCL is implicated in chromatin remodelling as a histone chaperone [38–41]."***

13. Is it possible to speculate on why there is a need for both nucleolar proteins to translocate and DNA repair components to translocate to nucleolus? Is there a context dependency on when this happens? Please consider going into a bit more detail as to the significance of these exchanges for particular types of repair.
We have added a discussion on significance of shuttling of DNA repair proteins between the nucleoplasm and the nucleolus on page 6: ***"In some cases, the appearance of strong retention centres outside of the nucleolus (e.g. recruitment of DNA repair machinery to DNA lesions)***

may lead to a decrease in the concentration of a protein within the nucleolus. Inversely, sequestration of various enzymes involved in stress response, DNA repair, cell cycle progression etc. within the nucleolus appears advantageous because it allows to keep the concentration of these proteins in the nucleoplasm relatively low under normal conditions and to increase it sharply upon stress. Excessive concentration of DNA repair enzymes and stress response factors in the nucleoplasm under normal conditions may result in a significant off-target activity. It seems thus logical to store these proteins within a dense phase-separated compartment until they become necessary to fulfil their specific functions [22]. "

14. It is a bit unclear how this connects the two. Does DNA repair impact on ribosome biogenesis or is it just the co-opting of ribosome proteins during times of cellular stress from DNA damage? Please consider clarifying this link.
We believe that there is a link between cellular stress and the ribosome biogenesis. In many cases, ribosome biogenesis stops upon cellular stress and the nucleosomal proteins are reallocated to other nuclear functions. This is now stated in the Conclusions on page 16: ***"The key role of nucleolus as a regulatory hub is directly related to its main function, production of ribosomes. This process consumes more than a half of cell energy resources and thus should be coordinated with the actual needs of the cell in the ribosomes. Accordingly, many regulatory circuits link the production of ribosomes to the cell cycle progression which, in turn, can be blocked by stress responses. It is thus not surprising that nucleolus acquired a role of regulatory hub connecting many functional processes. The fundamental link between ribosome biogenesis and cell cycle progression is accomplished through involvement of certain proteins in cell cycle control and stress response on the one hand, and the regulation of ribosomal gene transcription and ribosome assembly on the other hand. Many of these proteins are sequestered within phase-separated nucleoli but may shuttle to nucleoplasm in response to various stimuli."***

15. Please clarify the major function of telomerase for general readers who may not be aware.
We have introduced a short description of telomerase functions on page 9: ***"Telomerase is a ribonucleoprotein complex associated with the telomere repeats which protects the ends of the chromosomes from degradation. Telomerase contains the telomerase reverse transcriptase (TERT), telomerase RNA, and several additional proteins."***

16. Is telomerase involved in DNA repair?
In fact, telomerase protects chromosome ends from DNA repair machinery in order to avoid chromosome fusions *via* NHEJ of telomeric regions. This is now mentioned on page 9: ***"Retention of telomerase within the nucleolus is favorable in case of DNA damage as loosening of telomere structure should be necessary for DSB repair at telomeres [45]."***

17. Does this occur in the nucleoli, independent of its function at telomerase? Please clarify.
TRF2 binds rDNA and promotes rRNA transcription independently of its function on telomeres.

18. It may be unclear how this relates back to its role in the nucleolus vs. nucleoplasm, where it interacts with telomerase. Please clarify.

We have removed the section on TRF1.

19. Since this discussion is fairly vague and the primary functions of this movement are still unknown, please consider removing this discussion from the main text. Instead, it could be discussed briefly in the concluding remarks to provide future directions for looking at the role of other proteins that shuttle between these compartments.
We have removed the part on Cajal bodies.

20.This section lists lots of examples of proteins that are sequestered into the nucleolus, but it is not clear why they are being sequestered and the role that the nucleolus plays in keeping these proteins there. Is there a general theme/function of the nucleolus in sequestering these proteins? Please consider adding to this introduction paragraph to provide more context around the function of this sequestration. Please consider the changes made to the introduction to highlight some of the major roles of the nucleolus is sequestration. Since most of the discussion revolves around aggresomes, please consider removing the subheadings and keeping the discussion together under one heading. Please ensure the scientific meaning has remained intact in this new section.
The section was rewritten, and the subheadings were removed.

21. Since this question isn't actually addressed or answered directly in the manuscript, please consider a new heading that better reflects the content of the section. For example, the heading could read: Sequestration of nuclear and cytoplasmic proteins in the nucleolus.
We changed the heading of the section as proposed.

22. Can you please cite the appropriate literature that supports this work?
We now cite literature on aggresome correlation with cell survival on page 11.

23. It may not be clear to general readers the significance of polyA RNAs vs rRNAs in this scenario.
We changed the phrase on page 11 as follows: ***"These inclusions form so-called 'aggresomes' [23] or 'detention centers' [24] within or in a close contact with the nucleoli (Figure 2); they are rich in protein and RNA [72]. Aggresome formation correlates with cell survival [73]."***

24. Is this process a direct formation as opposed to a translocation of the aggregates to the nucleoli as above?
This is indeed a distinct process. We changed the phrase on page 11 to reflect this fact: ***"Protein aggregates may also form directly in the nucleoli when proteasome function is inhibited. [71]."***

25. Please consider going into detail what the specificity of this sequestration is as it may not be clear how these proteins are different from those stated above that are retained.
The key difference is that they are not part of aggresomes and that their sequestration is dependent on the presence of a specific sequence. We changed the phrase on page 11

accordingly: ***"Immobilization of these proteins inside the nucleolus is driven by specific nucleolar detention signals (NoDS) [74]."***

26. Are functional roles known for other proteins that are retained? Is it possible to provide an over-arching function for this sequestration here?
Functional roles of sequestration were added for other proteins as well.

27. It may be unclear to general readers how this discussion relates to the above discussion on aggregates. Is p21 an aggregate? In addition, the discussion on p21 appears to more directly relevant to the discussion under DNA damage described above
We moved the discussion of p21 to the DNA repair section.

28. Please provide a concluding sentence that best encapsulates the main points of this manuscript.
The following concluding sentence was added: ***"Importantly, key nucleolar proteins FBL, NPM1, and NOPP140 that do not contain these signals remain mobile and evade retention, highlighting the specificity of this nucleolar sequestration [24]."***

29. Is it known what unique feature of the nucleolus accounts for this segregation? Please clarify, thank you.
We have extended the discussion on the role of the nucleolus is silencing of perinucleolar chromatin.

30. Can you please cite the appropriate literature that supports this work?
Relevant publications were cited.

31. How does the findings above on NADs and TADs relate to this conclusion that nucleolus interactions can stabilize these contacts? Please consider clarifying this mechanism further.
We have extended the discussion on NADs and TADS on page 14.

32. Much of this discussion appears to be directly related to the regulation of chromatin organization and function and therefore directly relevant to much of the discussion above. Please consider integrating this discussion with that above to provide readers with an overall view of how the nucleolus can support 3D genome organization, of which one major function appears to be anchoring.
The two sections were integrated.

33. Please consider adding an introductory sentence that briefly encapsulates the main function of the nucleolus that will be discussed here rather than jump into studies on NADs. This will help readers understand what the major focus of this section is on and why.
The following introductory sentence was added to the section on page 13: "What is the functional significance of **NADs** and how the nucleolus can regulate their functions?**"**

34. Please consider expanding the concluding remarks to discuss future directions for the field in the different areas that have been discussed such as in protein shuttling and chromatin organization. What are the outstanding questions that need to be addressed and how can new tools ensure they are addressed. In addition, please cite the outstanding questions box in this section.
The section was expanded, with a reference to the outstanding questions box.

Thank you for editing the MS. We have accepted most of your suggestions. As we have significantly corrected the MS, we provide a marked-up version of the corrections made in the MS.

Reviewer's comments

1.Most of the review is represented by a list of results of previous studies (e.g. DNA repair sections, telomere section, stress dependent response section) that is very difficult to follow for the non specialised reader This is the major problem with this manuscript, where a real effort to make all the available informations organic and useful for the reader is lacking. I believe that the Authors should do this effort reorganising the manuscript and providing a general introduction of the processes in which the nucleolus is involved and a sort of map/scheme showing all the functions (including canonical ones) to give a general picture of the issue..

We have reorganized the MS and completely rewritten the section on protein shuttling between the nucleolus and the nucleoplasm for clarity.

2. *Some concepts are omitted only shortly mentioning previous reviews (e.g. nucleolus and stress response, the RPs-MDM2-p53 axis). I think that for the reader's sake these concepts should be briefly explained as they provide a paradigm on how non-canonical -ribosome biogenesis unrelated- function are organised in the nucleolus). In addition, the role of non coding RNAs is only marginally considered.*

We have added a description of the nucleolus and stress response, the RPs-MDM2-p53 axis on page 10. We were unfortunately limited by a format imposed by the journal concerning an extended description of the relationship between the nucleolus and the non-coding RNAs.

page 5 Nucleolus id a liquid drop... (provide reference in the text)
We have added the reference in Box1.

Page 9 Telomerase is composed...: note that among the additional proteins that bind telomerase complex there are the 4 core proteins of the pseudouridylation complex which are the same that are highly aboundant in the nucleolus since they drive site specific, SNORNA guided uridine isomerization.
We thank the reviewer for this remark. This was added to the text on page 9 as follows:
"Telomerase is assembled in the nucleolus by several proteins including dyskerin that later becomes a part of telomerase in the nucleoplasm [44]. Dyskerin also participates in

pseudouridylation of specific residues in newly synthesized ribosomal RNAs and snRNAs [45].”

pag 13 nucleolus-associated chromatin domains (NADs): there's a typo here, then line below "p-arms of acrocentric..." please define it as the short arm (easier for the reader)
The typo was corrected

page 14: define Hi-C
HiC is now defined in the glossary on page 3

concluding remarks: this is more a summary of the review (and can be used in a presentation/general introduction)
Concluding remarks were rewritten.

Figure 1

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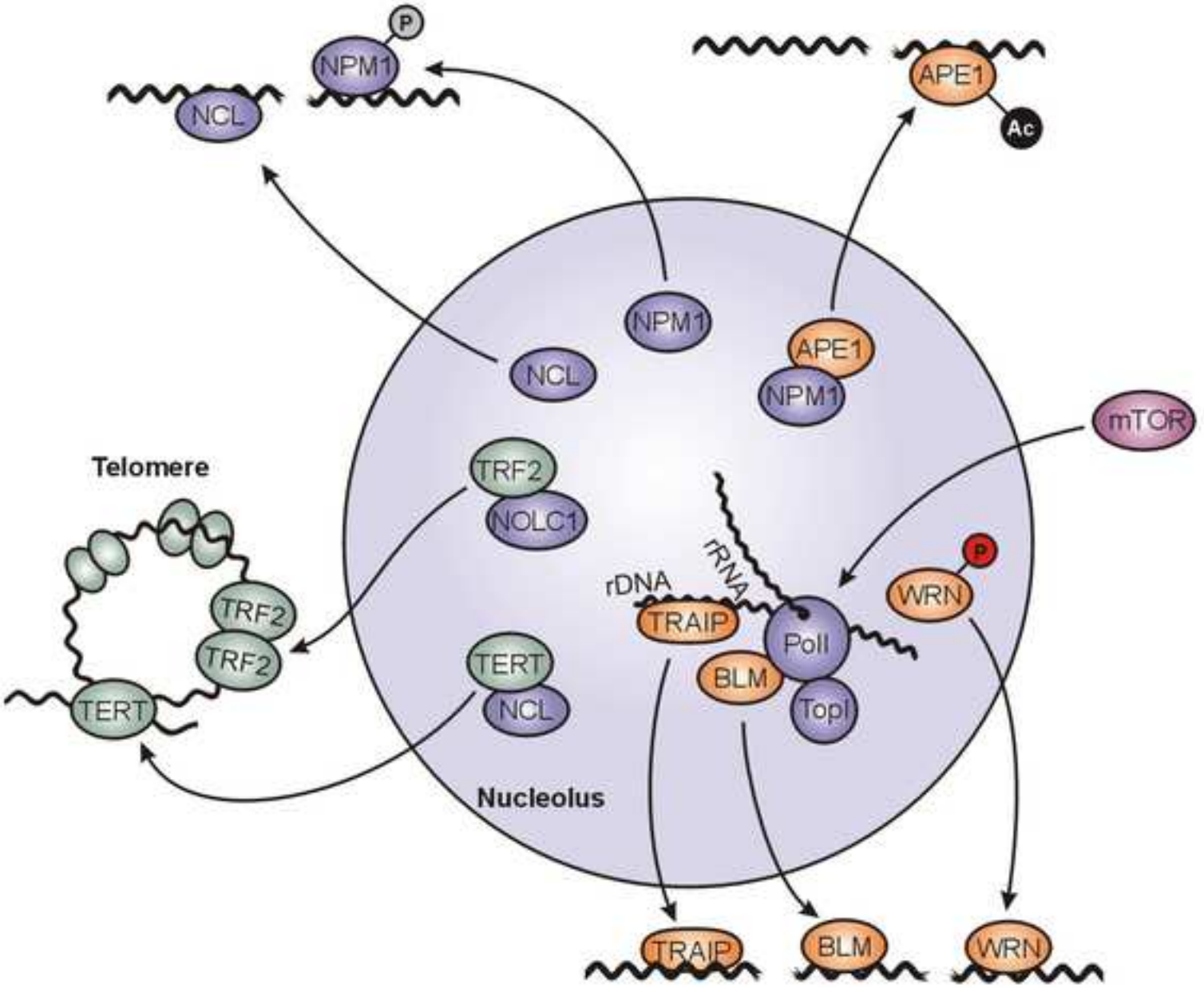

Figure 2

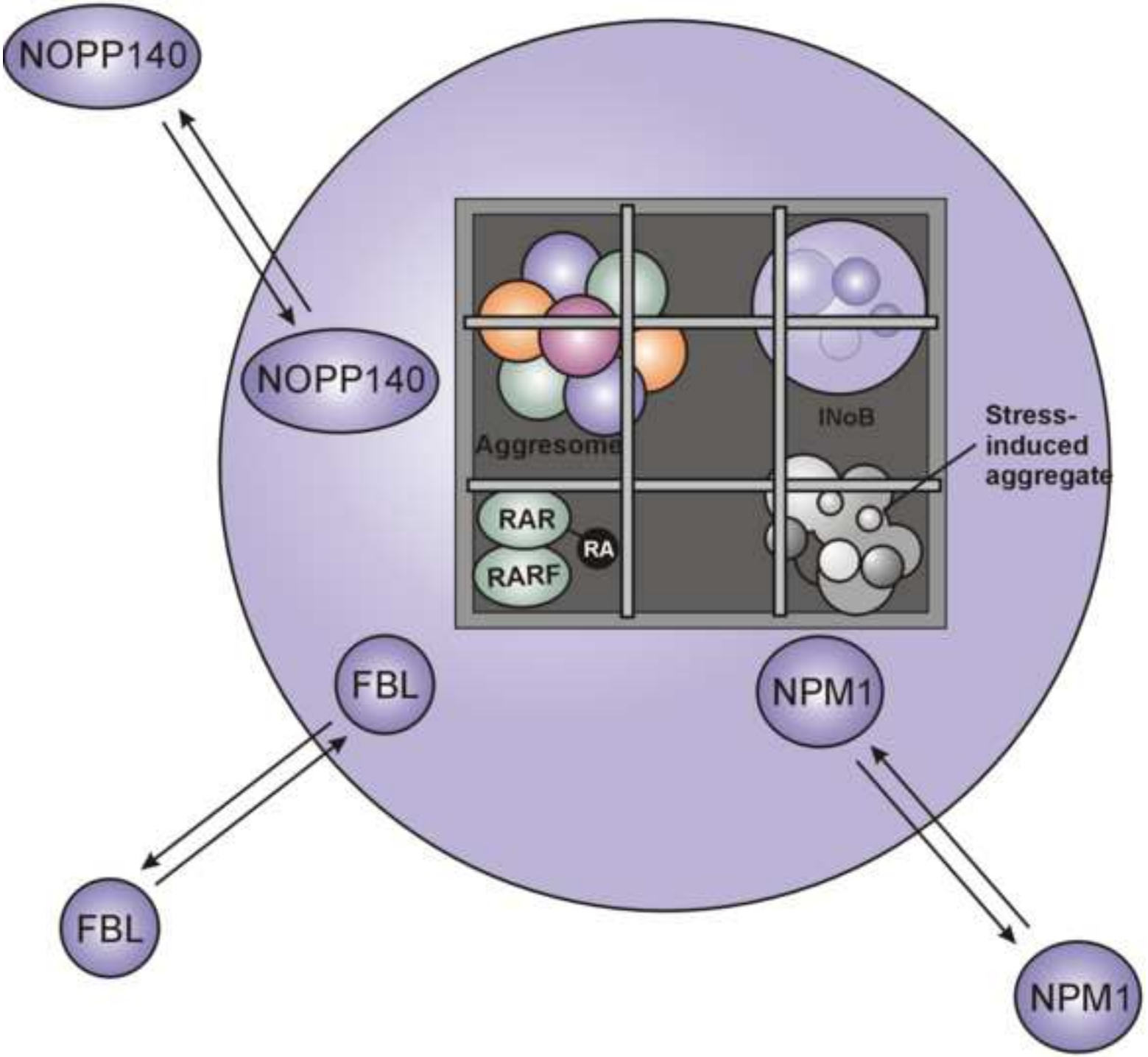

Figure 3

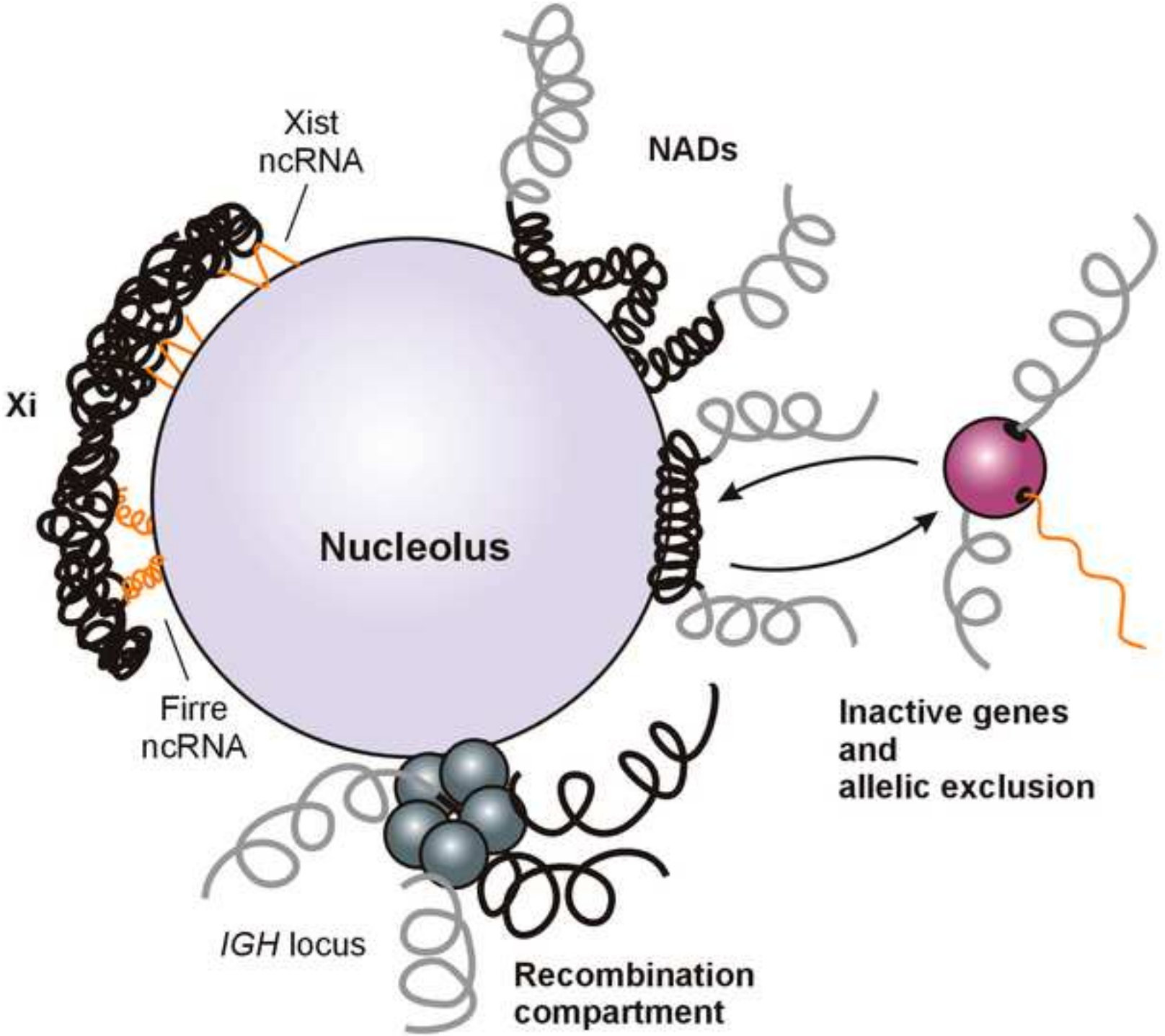

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Table 1

Table 1. Examples of nucleoplasmic proteins with a moonlighting function in the nucleolus

Protein	Function in the nucleoplasm	Function in the nucleolus	References
APE1	rDNA quality control	DNA repair	[1]
ATM	Activation of checkpoint signalling upon DSBs; vesicle and/or protein transport	rDNA silencing in the presence of persistent DSBs; large-scale reorganization of the nucleolar architecture	[2]
BLM	DNA repair	pre-rRNA synthesis	[3]
c-myc	Activation of transcription of growth-related genes	Attachment of rDNA to the nucleolar matrix *via* the IGS region	[4]
CTCF	Three-dimensional organization of chromatin, gene regulation as repressor or activator	Regulation of chromatin at the rDNA spacer promoter	[5]
Core histones	Sequestration of histones in the nucleolus	Chromatin organization	[6][7]
Ku70	Non-homologous end joining; telomere maintenance	Degradation of PICT-1; regulation of the RPL11-MDM2-p53 pathway	[8]
Lamin B2	Component of the nuclear lamina; participates in anchoring of chromatin at the nuclear periphery	Interaction with NCL and NPM; maintenance of morphology of the nucleolus; repression of rRNA transcription	[9]
p53	Tumour suppression; Induction of growth arrest or apoptosis; cell cycle regulation	Repression of rRNA transcription	[10,11]
PARP1	Poly-ADP-ribosylation of proteins; DNA repair; transcription regulation	Pre-rRNA processing; post-transcriptional modifications; pre-ribosome assembly	[12]
PIWI	Repression of transposable elements; RNA cleavage.	Restriction of expression of rDNA in heat shock conditions.	[13]
RelA	Component of a NFκB transcription factor	Triggering of an NPM-dependent apoptotic pathway.	[14]
Topoisomerase II	Resolution of topological stress upon transcription and replication; gene regulation	Three-dimensional organization of the rDNA chromatin	[15]

WRN	DNA repair; telomere stability; maintenance of DNA integrity during replication	Promoter clearance of RNA pol I; binding to the active fraction of rDNA in quiescent cells	[16,17]

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Table 2. Major nucleolar proteins with a moonlighting function in the nucleoplasm.

Protein	Function in the nucleolus	Function in the nucleoplasm	References
NCL	rRNA processing	Histone chaperone; DNA repair; regulation of gene expression	[1–4]
NPM1	rRNA processing; stress sensing	Histone chaperone; DNA repair; modulation of p300 activity; regulation of HMGA; chromatin remodelling; regulation of gene expression	[5–11]
NPM2	Nucleolus maintenance; perinucleolar heterochromatin maintenance	Histone chaperone; chromatin remodelling	[10,12,13]
Ribosomal proteins L5 and L11	Ribosome biogenesis	p53 activation in stress response	[14]
RRL11	Ribosome biogenesis; maintenance of the nucleolar structure	DNA damage response upstream regulation of p53	[15]
UBTF1 (UBF)	rRNA transcription ; maintenance of the nucleolus integrity	Recruitment of Pol II to histone gene clusters; maintenance of the genome stability	[16,17]

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Membraneless nuclear compartments

Eukaryotic cell nucleus contains a number of membraneless compartments such as nucleoli, Cajal bodies, PML bodies, nuclear speckles etc. [1]. These compartments are dynamic associates of proteins and nucleic acids and are slightly denser than the bulk of the nucleoplasm. Each type of membraneless compartments is connected to certain functional processes that are not necessarily related to each other [2]. Accordingly, most of the compartments contain a number of various proteins including many moonlighting proteins [3]. The proteins participating in assembly of nuclear compartments frequently possess intrinsically disordered domains [3–5]. These proteins/domains may mediate weak-affinity and non-specific interactions with multiple target sites that trigger the liquid phase separation [6]. The phase-separated droplets accumulate macromolecules that possess an affinity to the components of the droplet interior. Usually a so called platform nucleates the assembly of liquid droplet nuclear compartments. This may be either a protein (e.g. PML in case of PML-bodies [7]) or a non-coding RNA (e.g. NEAT1 RNA in paraspeckles [8] or rRNA in nucleoli [9]). The nucleolus differs from other membraneless compartments in two ways. First, the nucleolus consists of at least three distinct phase-separated layers: droplets within droplets [10]. Second, the availability of pre-rRNAs that constitutes a platform for nucleolus assembly directly depends on the ongoing rDNA transcription [11]. Suppression of rDNA transcription results in full or partial disruption of nucleoli and release into the nucleoplasm or cytoplasm of various proteins sequestered within nucleoli [11]. This is likely to be an important part of the cellular stress response mechanism [12].

Box references

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Nucleolus: a central hub ~~organizing~~for nuclear functions

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**equal contribution*

Keywords:

Nuclear compartmentalization, nucleolus, 3D genome organization, DNA repair and recombination, transcription

Word count: ~~3906~~3843

Summary

~~The nucleus contains distinct nuclear bodies (NBs);~~The nucleolus is the largest and the most studied ~~NB~~nuclear body, but its role in ~~the functioning of the nucleus~~ nuclear function is far from ~~being fully~~ understood. ~~The~~ Much work on the nucleolus ~~is not surrounded by a membrane, yet it contains DNA, RNA and a set of proteins that can either be retained~~has focused on its role in ~~the nucleolus or rapidly shuttle between the nucleoplasm, the nucleolus and the cytoplasm in response to various stimuli. The~~regulating RNA polymerase I transcription and ribosome biogenesis; however, emerging evidence points to the ~~central function of the~~ nucleolus ~~in~~as an organizing hub for many nuclear functions ~~besides RNA polymerase I transcription and ribosome biogenesis. Here we discuss the functions of the nucleolus related to the~~accomplished *via* shuttling of proteins and nucleic acids between the nucleolus and nucleoplasm. ~~The functional processes~~Here we discuss the cellular mechanisms affected by shuttling of nucleolar components that include 3D organization of the genome, stress response, DNA repair and recombination, transcription regulation, telomere maintenance and other essential cellular functions.

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Glossary

Allelic exclusion: a situation when one allele of a given gene is expressed while the other allele is transcriptionally inactive.

Hi-C: a high-throughput method to study the three-dimensional architecture of genomes based on proximity ligation.

Nucleolus-associated domains (NADs): chromatin domains that dynamically interact with nucleoli.

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Nucleolar localization signals (NoLS): a short protein sequence allowing to localize a protein inside the nucleolus. NoLS do not have a consensus sequence, but they are constantly enriched with positively charged amino acids. As a result, nucleolar accumulation *via* NoLS is dependent on the overall charge of the signal region.

Protein shuttling: ~~a majority of~~most nuclear proteins are not tightly associated with nuclear bodies, but rather are continuously exchanged between these bodies and the surrounding nucleoplasm. This mobility allows these proteins to quickly relocalize between different nuclear domains, leading to unprecedented plasticity of the genome functioning.

Sequestration: mobile, continuously exchanging proteins can increase their concentration inside a nuclear body under some conditions. This process referred to as protein sequestration may be driven either by appearance of novel binding sites for the protein inside nucleoli, or disappearance of binding sited outside nucleoli, or posttranslational modifications of protein (or a combination thereof). Sequestration may also be due to a decrease of protein exchange due to

tight association with a component(s) inside any subcellular structure (protein immobilization) or due to formation of protein aggregates.

Stress response: a reaction which dedicates energy and effort to acute adaptation to stress.

Topologically associating domain (TAD)**:** a genomic region where DNA sequences physically interact with each other with ~~an increased~~a higher frequency as compared to the sequences outside the TAD. In mammals, TAD boundaries are enriched in CTCF/Cohesin binding sites. Most interactions observed between gene enhancers and promoters occur within one TAD. TADs were shown to coincide with replication domains.

Introduction

The main function of the nucleolus is the synthesis and processing of rRNA and ribosome assembly. Nucleoli contain clusters of ribosomal RNA genes (rDNA) and their transcripts (rRNA) (reviewed in [1]), as well as several hundred proteins participating in many nuclear processes inside and outside the nucleolus [2]. Recent proteomics data on nucleolar proteins and the analysis of genomic DNA loci associated with the nucleolus suggest that the functions of the nucleolus are not limited by ribosome biogenesis. Indeed, chromatin non-randomly associates with the nucleolus and is organized into so-called **nucleolus-associated domains (NADs)** [3,4].function of the nucleolus is not limited to ribosome biogenesis, and that the nucleolus plays a role of a central hub regulating many nuclear and cellular processes. Movement of chromatin *vs.* the nucleolus may trigger activation and silencing of gene transcription [5,6][3,4], mediate inactivation of chromatin domains [7] underly **allelic exclusion** and spatially organize[5], underlie **allelic exclusion,** and spatially organize gene recombination [8].[6]. In a similar way, movement of proteins between the nucleolus and the nucleoplasm regulates DNA repair, RNA polymerase II transcription, telomere maintenance, **stress response** [9,10][7,8], and apoptosis [11]. All these are non-canonical functions of the nucleolus; some of them were discovered quite recently. Currently, the molecular mechanisms which allow to engage a large number of components inside nucleoli and the reasons of accumulation of multiple functions inside one organelle remain unclear. Here we discuss the role of protein and nuclear acids **shuttling** between nucleolus, perinucleolar region and nucleoplasm in mediation of non-canonical functions of the nucleolus. [9]. Dynamic changes in localization of many proteins and chromatin regulated by the nucleolus appears to modulate a multitude of functions that were not previously associated with the nucleolus. These non-canonical functions of the nucleolus were discovered quite recently; therefore, the molecular mechanisms regulating these newly found nucleoli functions and the interactions of various components within the nucleolus remain unclear.

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Mechanisms ofProtein accumulation of proteins in the nucleolus

Nucleolus is a liquid drop nuclear compartment resulting from phase separation (Box 1). Protein accumulation inside nucleoli is a consequence of affinity interactions with core nucleolar components: rDNA, RNA and nucleolar proteins [12]. This accumulation is driven by short amino acid stretches referred to as nucleolar localisation sequences (**NoLS**) [13]. **NoLS** are substantially enriched with positively charged amino acids. Although there is no **NoLS** consensus sequence, on average, ~50% of amino acids inside **NoLS** are lysines and arginines [14]. Lysines and arginines generate a positive charge essential for the nucleolar localization [14–17]. Thus, charge-dependent (electrostatic) interactions of amino acid residues with nucleolar components lead to their nucleolar accumulation. The nucleolus harbors a large number of negatively charged RNA molecules, potential targets for positively charged **NoLS** [17]. **NoLS** do not interact with DNA since its charge is neutralized in the nucleus by histones, bivalent ions and polyamines [18,19]. Nucleolar accumulation of proteins may also be due to their interaction with a major nucleolar protein NCL (nucleolin or C23), containing long acidic patches that can interact with positively charged **NoLS** [20]. Nucleolar accumulation by **NoLS** can be regulated; this allows proteins to translocate from the nucleolus to the nucleoplasm and *vice versa*. For example, tumour suppressor ARF inhibits nucleolar import of TTF-I, RNA polymerase I transcription termination factor, by binding to its **NoLS**, thus favouring TTF-I displacement from the nucleolus to the nucleoplasm [21]. This causes the suppression of ribosomal RNA synthesis followed by cell proliferation arrest.

Shuttling of proteins between the nucleolus and the nucleoplasm regulates important nuclear processes

Nucleolus is a highly dynamic structure, with proteins being actively exchanged between the nucleolus and the nucleoplasm [22,23]. In many cases, this **protein shuttling** underlies important

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nuclear processes. The equilibrium between the nucleolar and nucleoplasmic localisation of a particular protein may be influenced by posttranslational modifications. In other cases, appearance of strong retention centres outside of the nucleolus (e.g. recruitment of DNA repair machinery to DNA lesions) may be sufficient to decrease the concentration of a protein within the nucleolus. Several examples are discussed below.

DNA repair is regulated by protein shuttling between the nucleolus and the nucleoplasm.

DNA repair proteins in the nucleolus. Nucleolar proteome contains many DNA-binding proteins including those involved in DNA repair such as APE1 [24], WRN [25], ATM, ATR, MRE11, PARP1, TOPBP1, XRCC1, ZRF1 and Ku70/80 [26,27]. Many of these proteins participate both in DNA repair in the nucleoplasm and ribosome biogenesis in the nucleolus (**Figure 1**). The apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1; APE1; REF-1) is an essential component of base excision repair (BER) machinery, but it also deletes lesions from rRNA [24]. APE1 interacts with nucleolar proteins NPM1 (also called B23, nucleophosmin, NO38 or numatrin), RPLP0, SA as well as with the 47S, 18S and 28S rRNAs [28]. The N-terminus of APE1 contains several positively charged lysine residues which modulate interaction of APE1 with NPM1 resulting in APE1 localization in the nucleolus. When some of these lysine residues are acetylated, NPM1 no longer interacts with APE1, and APE1 accumulates in nucleoplasm [24]. A similar mechanism of regulated **protein shuttling** between the nucleolus and the nucleoplasm (*via* protein-protein interactions and posttranslational modifications) has been described for RecQ-like helicases WRN (Werner syndrome) and BLM (Bloom syndrome). They are implicated in double strand breaks (DSBs) and base lesions repair, resolving stalled replication forks and other forms of replication stress [27,29,30]. However, WRN possesses a **NoLS** in its C-terminus and is accumulated in the nucleolus [25]. When DNA repair is activated, WRN is dephosphorylated and moves from the nucleolus to the nucleoplasm; treatment with a

tyrosine phosphatase inhibitor maintained the nucleolar localization of WRN [10]. WRN localization can also be modulated by acetylation [31]. BLM can directly bind rDNA and interact with RNA Pol I, thus facilitating pre-rRNA synthesis in the nucleolus [32,33]. Switching between DNA repair and ribosome biogenesis is likely to occur through protein-protein interactions and posttranslational modifications. BLM interacts with DNA Topoisomerase I in the nucleolus [34] and its localization can also be regulated by ubiquitination [35]. DNA damage response factor NBS1 can transiently accumulate in nucleoli upon inhibition of rDNA transcription. Nucleolar localization of NBS1 depends on its interaction with a nucleolar protein Treacle (TCOF1). The Treacle-NBS1 complex can act as an adaptor for the ATM kinase to phosphorylate nucleolar proteins that regulate rDNA transcription in response to DNA damage. In this case, activation of ATM kinase in the nucleus leads to translocation of the Treacle-NBS1 complex into the nucleolus and thus to silencing of rRNA transcription [36]. The RING finger protein TRAIP protects genome integrity and its mutation causes primordial dwarfism [37]. TRAIP is possibly accumulated in nucleolus because of interaction with RNA PolI transcripts [38]. However, after exposure of cells to UV, it rapidly migrates to UV-induced DNA lesions in the nucleoplasm.

Nucleolar proteins in DNA repair. While classical DNA repair enzymes can be located in the nucleolus, "typical" nucleolar proteins can be imported to the nucleoplasm for DNA repair. This was described for the major nucleolar proteins, NCL and NPM1 [9]. NCL undergoes a robust yet reversible nucleoplasmic translocation and forms nucleoplasmic foci at sites of DSB repair where NCL is implicated in chromatin remodelling [39–41]. Both NCL and NPM1 are histone chaperones [42–44]. Ionizing radiation, cisplatin or etoposide treatment lead to relocalization of NPM1 from the nucleolus to the nucleoplasm [45]. This relocalization (or retention of NPM1 within the DSB repair foci) is likely to be regulated by phosphorylation. A T199-phosphorylated population of NPM1 is present at DSB foci, while the unmodified protein does not associate with

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DSBs. Replacement of endogenous NPM1 with its non-phosphorylable T199A mutant delayed DNA repair [46]. Both NCL and NPM1 are rapidly exchanged between the nucleolus and the nucleoplasm [47], therefore they can be readily available for DNA repair when necessary. The nucleoplasmic relocalization of NCL and NPM1 may be a part of general cellular **stress response** [48,49], although the functional significance of this relocalization is to be clarified.

In summary, there is an intensive cross-talk between the nucleolus and DNA repair machinery, with **protein shuttling** between the nucleolus and the nucleoplasm. This connects ribosome biogenesis and DNA repair.

Telomere maintenance is regulated by shuttling of telomerase between nucleolus and nucleoplasm. Although telomerase executes its major function in the nucleoplasm, it is assembled in the nucleolus [50] and may be temporarily retained there under certain conditions. Telomerase is composed of the telomerase reverse transcriptase (TERT), telomerase RNA, and several additional proteins. Remarkably, TERT contains three **NoLS** at positions of aa 1-15 [51], 326-620 [52] and 965-981 [53]. One of the **NoLS** coincides with the functional domain of TERT, but the nucleolar localization of TERT appears to be unrelated to its telomerase function [53]. The telomerase is retained in nucleoli through its interaction with NCL [50]. It has been proposed that retention of telomerase within the nucleolus is favorable in case of the necessity to repair DNA DSBs [54]. Of note, TERT performs some moonlight functions in the nucleolus as it binds to rDNA and stimulates RNA PolI transcription in oncogenic and regenerative hyperproliferation, probably *via* stimulation of the transcription initiation complex formation [55] (**Figure 1**). The telomeric repeat-binding factors TRF1 and TRF2 interact with telomeres and protect them. TRF2 can be sequestered in the nucleolus affecting its functions at telomeres [56]. Nucleolar localization of TRF2 is promoted by the nucleolar protein NOLC1 [57]. NOLC1 overexpression and consequent relocalization of TRF2 to the nucleoli arrests cell

~~cycle in the G_0-G_1 phase and prevents proliferation. TRF2 also binds rDNA and promotes rRNA transcription [58]. Activity of TRF1 is modulated by two nucleolar proteins: nucleostemin and guanine nucleotide binding protein-like 3-like (GNL3L). GNL3L stabilizes TRF1 [59] while nucleostemin promotes its degradation [60], inhibits TRF1 dimerization and shortens its dynamic association with the telomere [61].~~

~~Protein shuttling between the nucleolus, the nucleoplasm and the cytosol in mTOR signalling.~~ ~~The nucleolus functions in response to endogenous and environmental signals is connected with another major cellular system that senses various types of stress, the mammalian target of rapamycin (mTOR) pathway, which acts as a central hub integrating intra- and extra-cellular cues into cell survival and degradation pathways [62]. Although mTOR complex 1 (mTORC1) is predominantly cytosolic and localizes around lysosomal membrane, several members of mTORC1, including mTOR itself, can be as well found in the nucleus and the nucleolus [63–69]. One of the main functions of mTORC1 is activation of anabolic processes, including ribosome biogenesis and translation.~~ The nucleolus is a membraneless organelle that forms through phase separation in the nucleus (Box 1). Protein accumulation inside nucleoli is a consequence of affinity interactions with core nucleolar components: rDNA, RNA and nucleolar proteins [10]. This accumulation is driven by short amino acid stretches referred to as nucleolar localisation sequences (**NoLS**) [11]. **NoLS** are substantially enriched with positively charged amino acids. Although there is no **NoLS** consensus sequence, approximately 50% of amino acids inside **NoLS** are lysines and arginines that generate a positive charge essential for nucleolar localization [12–15]. Thus, charge-dependent (electrostatic) interactions of amino acid residues with nucleolar components lead to their nucleolar accumulation. The nucleolus harbors a large number of negatively charged RNA molecules, potential targets for positively charged **NoLS** [14]. Of note, **NoLS** do not interact with DNA since its charge is neutralized by histones, bivalent ions and polyamines [16,17]. Low specificity of electrostatic accumulation *via* **NoLSs** allows a large

number of proteins to be dynamically accumulated inside nucleoli. Nucleolar accumulation of proteins may also be due to their interaction with some nucleolar proteins including such as NCL (nucleolin or C23) which contains long acidic patches interacting with positively charged **NoLS** [18]. Nucleolar accumulation by **NoLS** is dynamic and can be affected by a variety of factors. For example, tumour suppressor ARF inhibits nucleolar import of TTF-I, a RNA polymerase I transcription termination factor, by binding to its **NoLS**, thus favouring TTF-I displacement from the nucleolus to the nucleoplasm [19] followed by the suppression of ribosomal RNA synthesis and cell proliferation arrest.

Protein shuttling between the nucleolus and the nucleoplasm regulates nuclear processes

Nucleolus is a highly dynamic structure involved in an active exchange of proteins between the nucleolus and the nucleoplasm (**protein shuttling)** [20,21]. In some cases, the appearance of strong retention centres outside of the nucleolus (e.g. recruitment of DNA repair machinery to DNA lesions) may lead to a decrease in the concentration of a protein within the nucleolus. Inversely, sequestration of various enzymes involved in stress response, DNA repair, cell cycle progression etc. within the nucleolus appears advantageous because it allows to keep the concentration of these proteins in the nucleoplasm relatively low under normal conditions and to increase it sharply upon stress. Excessive concentration of DNA repair enzymes and stress response factors in the nucleoplasm under normal conditions may result in a significant off-target activity. It seems thus logical to store these proteins within a dense phase-separated compartment until they become necessary to fulfil their specific functions [22].

The unique feature of the nucleolus is that it is both a storage place and a stress sensor. Some proteins are stored in the nucleolus irreversibly [23,24]. Other proteins perform major functions within the nucleolus and a moonlighting function in the nucleoplasm (Table 1). The third group of proteins have their main function in the nucleoplasm, but they are stored in the nucleolus until

release (Table 2). Shuttling control is tightly connected with rDNA transcription and modulated by stresses, cell cycle progression, energy resources and various post-translational modifications. Below we discuss several examples demonstrating how the nucleolus integrates external signals and provides necessary responses to different stimuli.

DNA damage and repair. Cells are subjected to a multitude of DNA lesions resulting from oxidation, alkylation, exposure to UV light or irradiation. The response to these events may include DNA repair and cell-cycle arrest. The nucleolus plays an important role in DNA repair as it contains many DNA-binding proteins involved in DNA repair in the nucleoplasm and ribosome biogenesis in the nucleolus (Table 1, Figure 1). Switching between ribosome biogenesis and DNA repair functions is likely to occur through protein-protein interactions and posttranslational modifications regulating shuttling. For example, the apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1; APEX1; REF-1) interacts with several nucleolar proteins *via* positively charged N-terminal lysine residues. In the nucleolus, APE1 interacts with NPM1 (also called B23, nucleophosmin, NO38 or numatrin), the 47S, 18S and 28S rRNAs [25] and deletes lesions from rRNA [26]. Deacetylation of N-terminal lysine residues upon DNA damage results in APE1 relocalization in the nucleoplasm where it is an essential component of base-excision repair machinery [26]. Another example concerns RecQ-like helicases WRN (Werner syndrome) and BLM (Bloom syndrome) implicated in double strand break (DSB) and base lesion repair in the nucleoplasm [27–29]. Phosphorylation [8], acetylation [30] and ubiquitination [31] of WRN and BLM regulates their shuttling between the nucleolus and nucleoplasm. WRN possesses a **NoLS** in its C-terminus and is normally located in the nucleolus where it interacts with RNA Pol I; the absence of WRN in the nucleolus reduces 18S and 28S rRNA levels [32]. When DNA repair is activated, WRN is dephosphorylated and moves from the nucleolus to the nucleoplasm [8]. This process is also modulated by acetylation [30]. In the nucleolus, BLM facilitates pre-rRNA synthesis by directly binding rDNA and interacting with RNA Pol I and DNA

topoisomerase I [33–35]. BLM ubiquitination promotes its recruitment to DNA lesions while the absence of the RNF8 ubiquitin ligase results in its nucleolar sequestration [31].

Some proteins move into the nucleolus upon DNA damage: for example, a nucleoplasmic DNA damage response factor NBS1 can transiently accumulate in the nucleolus to inhibit rDNA transcription. Nucleolar localization of NBS1 depends on its interaction with the nucleolar protein Treacle (TCOF1). The Treacle-NBS1 complex acts as an adaptor for ATM kinase, which phosphorylates nucleolar proteins that regulate rDNA transcription in response to DNA damage. Activation of ATM kinase in the nucleus leads to translocation of the Treacle-NBS1 complex into the nucleolus and thus to silencing of rRNA transcription [36].

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Similarly to many classical DNA repair enzymes located in the nucleolus, major nucleolar proteins NCL and NPM1 are rapidly exchanged between the nucleolus and the nucleoplasm so they can be readily available for DNA repair when necessary (Table 2, Figure 1) [7,37]. In particular, NCL translocates into the nucleoplasm and forms foci at sites of DSB repair where NCL is implicated in chromatin remodelling as a histone chaperone [38–41]. Relocalization of NPM1 from the nucleolus to the nucleoplasm is induced by DNA damaging agents such as ionizing radiation, cisplatin or etoposide treatment [42]. The relocalization of NPM1 to DSB repair foci is regulated by its phosphorylation [43]. In summary, DNA damage triggers an intense **protein shuttling** between the nucleolus and the nucleoplasm. Release of DNA repair factors stored in the nucleolus as well as *bona fide* nucleolar proteins into the nucleoplasm stimulates DNA repair.

Telomere maintenance. Telomerase is a ribonucleoprotein complex associated with the telomere repeats which protects the ends of the chromosomes from degradation. Telomerase contains the telomerase reverse transcriptase (TERT), telomerase RNA, and several additional proteins.

Telomerase is assembled in the nucleolus by several proteins including dyskerin that later becomes a part of telomerase in the nucleoplasm [44]. Dyskerin also participates in pseudouridylation of specific residues in newly synthesized ribosomal RNAs and snRNAs [45]. Telomerase may be temporarily retained there under certain conditions through its interaction with NCL [46]. Retention of telomerase within the nucleolus is favorable in case of DNA damage as loosening of telomere structure should be necessary for DSB repair at telomeres [47]. Remarkably, TERT contains three **NoLS** [48–50]. TERT in the nucleolus stimulates RNA PolI transcription and binds rDNA during oncogenic and regenerative hyperproliferation, probably *via* activation of the transcription initiation complex formation [51] (**Figure 1)**. The telomeric repeat-binding factor TRF2 also interacts with telomeres and protects them. TRF2 can be sequestered in the nucleolus by nucleolar protein NOLC1 [52], thereby affecting its function at telomeres [53]. NOLC1 overexpression and consequent relocalization of TRF2 to the nucleoli arrests cells in G_0-G_1 phase and prevents proliferation. In the nucleolus,TRF2 binds rDNA and promotes rRNA transcription [54].

Nucleolus and stress response. Although most nucleolar proteins are highly dynamic and shuttle between the nucleolus and the nucleoplasm [37,55,56], these proteins can be retained in the nucleolus during transcriptional, acidic, or heat stress. Disruption of protein traffic through the nucleolus may lead to formation of intranucleolar protein complexes. One example is a cyclin-dependent kinase inhibitor p21cip (or p21), present both in the nucleus and the cytoplasm. In the cytoplasm, p21cip has oncogenic properties, while nuclear p21cip is a tumour suppressor. p21cip transits through the nucleolus on its way from the nucleus to the cytoplasm. DNA damage inhibits this transit and induces formation of p21cip-containing intranucleolar bodies (INoBs) [57]. These structures also contain SUMO-1 and UBC9, the E2 SUMO-conjugating enzyme, several DNA damage checkpoint proteins and cell cycle regulators Cdk2, Cyclin E, PCNA, p53 and MDM2 [58]. SUMO-1 and p21cip control the transit of proteins through the nucleolus, but

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when nucleolar export is disrupted by DNA damage, these proteins act as scaffolds that mediate the formation of the multiprotein complex in the nucleolus [58]. The MDM2–p53 pathway which coordinates cellular response to stress is also regulated by shuttling of the nucleolar proteins. In response to stress, several nucleolar proteins, including NCL [59], NPM [60], and nucleostemin [61], are released into the nucleoplasm, where they bind to and inhibit MDM2 resulting in p53 activation and stabilization (Figure 2).

Mammalian target of rapamycin (mTOR) pathway is another major cellular system that senses various types of stress and integrates intra- and extra-cellular cues for cell survival and degradation pathways [62]. One of the main functions of mTORC1 is activation of anabolic processes, including ribosome biogenesis and translation. ~~mTORC1 affects nucleolar transcription through its downstream effector p70 S6 kinase 1, which in turn phosphorylates a number of its own effectors important for translation initiation [70]. Remarkably, nuclear mTOR protein can bind to thousands of sites in the genome [66,67] and interact with rDNA chromatin either directly [63–65] or *via* modulation of other rDNA interacting factors [71,72]. Treatment with mTORC1 inhibitor rapamycin prevents mTOR localization to the nucleolus and interferes with the processing of ribosomal rRNAs [68]. One of the recent examples of the nucleolar activity of mTORC1 is related with the function of inhibitor of growth 1b protein (ING1b), which associates with rDNA repeats and is required for the efficient recruitment of chromatin regulator HDAC1 to the nucleoli [69]. ING1 reduces mTOR localization to the nucleolus and promotes recruitment of HDAC1 to the NoRC complex and to major Pol I transcription factor UBF1. Reversibly, knockdown of ING1 results in the accumulation of mTOR in nucleolus and it association with UBF1.~~

~~Temporary repositioning of Cajal bodies into the nucleolus.~~ ~~Cajal bodies are highly mobile structures moving throughout the nucleoplasm, but they have also been detected within the~~

nucleolus in human breast carcinoma cells, brown adipocytes and hepatocytes of hibernating dormice [73,74]. Several observations show that Cajal bodies have the ability to move to and from the nucleolar periphery and within the nucleolus [75]. The functional significance of these unusual shuttling of one membraneless compartment in and out of another compartment is currently unknown.

Does the nucleolus serve as a nuclear «prison»?

Recent studies indicate that the nucleolus can be used for temporary storage or even exclusion from circulation of various nuclear and even cytoplasmic proteins.

Intranucleolar bodies and aggresomes. Misfolded proteins are cleared from cells by the ubiquitin-proteasome system, preventing cell death induced by the proteotoxic stress. When this system is inhibited or impaired, misfolded proteins aggregate and form proteinaceous particles that are transported into the perinucleolar region to form so-called 'aggresomes' [76] or 'detention centers' [77] within or in a close contact with the nucleoli (**Figure 2**). These inclusions are rich in proteins and RNAs, but, surprisingly, nucleolar aggresomes do not contain rRNAs, an abundant component of the nucleoli, but rather polyadenylated RNAs (polyA RNAs) [78]. Protein aggregates may also be formed in the nucleoli of proteasome inhibitor-treated cells [79]. The aggresome formation correlates with cell survival. Aggresomes are cleared from cells by aggrephagy, a selective autophagic process. Disruption of the protein traffic through the nucleoli may also lead to formation of intranucleolar protein complexes. p21cip (or p21) is a cyclin-dependent kinase inhibitor present both in the nucleus and the cytoplasm. In the cytoplasm, p21 has oncogenic properties, while nuclear p21 is a tumour suppressor. p21Cip1 transits through the nucleolus on its way from the nucleus to the cytoplasm. DNA damage inhibits this transit and induces formation of p21Cip1-containing intranucleolar bodies (INoBs) [80]. These structures also contain SUMO-1 and UBC9, the E2 SUMO-conjugating enzyme,

several cell cycle regulators and DNA damage checkpoint proteins Cdk2, Cyclin E, PCNA, p53 and MDM2 [81]. It seems that SUMO-1 and p21 regulate the transit of proteins through the nucleolus, but when nucleolar export is disrupted by DNA damage, these proteins play the role of scaffold that mediate formation of multiprotein complex in the nucleolus [81].

Sequestration of transcription factors in the nucleolus. TATA-binding protein (TBP)-related factor 2 (TRF2) is a RNA polymerase II transcription factor. During interphase, TRF2 is located almost exclusively in the nucleolus in HeLa and Cos cells and is rapidly released from the nucleolus upon inhibition of RNA Pol I transcription [82]. Another example of **sequestration**, Retinoic acid receptor (RAR), interacts with retinoic acid (RA) which is widely used for treatment of various cancers such as acute promyelocyte leukemia. However, tumour cells become resistant to this drug through modulation of expression levels of a specific repressor known as the RA resistance factor (RaRF). RaRF directly interacts with RAR and sequesters it into the nucleolus in response to RA, thereby contributing to loss of RA sensitivity [83].

Stress-dependent retention of proteins in the nucleolus. The role of the nucleolus in the stress response has been reviewed recently [84,85]. Without intention to repeat the analysis presented in these reviews, we shall address here only topics closely related to the subject of our discussion. Although most of nucleolar proteins are highly dynamic and shuttle between the nucleolus and the nucleoplasm in living cells [47,86,87], some proteins can be captured and retained inside the nucleolus under stress conditions. Transcriptional, acidic or heat stress leads to expression of specific lncRNAs from the intergenic spacer separating individual rDNA transcription units (IGS lncRNAs) [88]. These transcripts capture and immobilize inside nucleoli several important proteins, including von Hippel-Lindau (VHL) protein, DNA (cytosine-5)-methyltransferase 1 (DNMT1), the δ catalytic subunit of DNA polymerase (POLD1), Hsp70, RNF8, MDM2, and APC2 [88], RPA16 and RPA40, PES1, NOP52, RRP1B, NOM1, NOL1, and

SENP3 [77]. Importantly, key nucleolar proteins FBL, NPM1, and NOPP140 retain their mobility and evade retention, highlighting the specificity of this nucleolar **sequestration** [77]. The IGS lncRNA transcription correlates with the formation of detention centers [77]. The functional role of nucleolar **sequestration** is described for the VHL protein which degrades the hypoxia-inducible factor (HIF) in the presence of oxygen. **Sequestration** of VHL enables HIF to evade destruction and activate its target genes [89].

Dynamic association of chromatin with the nucleolus regulates nuclear processes.

Nucleolus and the 3D genome organisation. One or several nucleoli present in the nucleus occupy a large volume (up to 25% of the nucleus), mostly in the central part of the nucleus; this volume is largely filled with transcribed rDNA and assembling ribosomes. In each cell, only a fraction of rRNA genes is transcriptionally active and localises within nucleoli. Inactive rDNA repeats are located at the periphery of the nucleolus contributing to the creation of perinucleolar inactive chromatin compartment [7,90,91]. Genomic segments located within this compartment are commonly referred to as **nucleolus-associated chromatin domains** (**NADs**) [3,4]. **NADs** contain sequences located in the p-arms of acrocentric chromosomes, centromeric and pericentromeric chromatin of most chromosomes and subtelomeric regions of some chromosomes **(Figure 3)**. Besides repetitive sequences, **NADs** contain more than 1000 structural genes including TCR genes, olfactory receptor, defensin genes as well as two families of immunoglobulin genes out of six. Many **NAD**-associated genes have similar features: they are tissue-specific and form large gene clusters [92]. **NADs** substantially overlap with lamina-associated domains (LADs) [93]. In each cell, only a portion of LADs identified in population studies is actually located close to nuclear lamina. After mitosis, LADs are stochastically reshuffled and LADs redistributed to the nuclear interior are frequently localised within the perinucleolar region [94]. In the interphase nucleus, each chromosome forms a distinct chromosomal territory probably linked to both nuclear lamina and the nucleolus as every

~~chromosome contains both LADs and **NADs**. Furthermore, different chromosomal territories interact with each other [95,96]. Being attached to the nucleolus, the nuclear lamina and to each other, chromosomal territories constitute a unified chromatin compartment that may be mechanically stretched and can sense mechanical stress [97]. In this scenario, the phase-separated nuclear compartments, the largest of which is the nucleolus, play a role of global chromatin organizers [96,97]. In particular, tethering of chromatin to nucleolus and nuclear speckles is likely to account for the spatial segregation of repressed and active chromatin compartments revealed by Hi-C studies [98]. Chromosome Conformation Capture (3C) and its genome-wide variant Hi-C provided a way to measure contact probabilities between chromosomal segments. Hi-C analyses led to identification of the active and inactive chromosomal compartments, self-interacting topologically associating domains (**TADs**) and contacts between chromosomes with an increasing resolution. Comparison between **TADs** and **NADs** revealed that they were organized in a similar way, but **NADs** colocalized mostly with the inactive chromosomal compartments. The short-range contacts occurred preferentially between sequences located within the same and neighbouring **NADs**, and the long-range contacts (over >50 Mb distance) occurred preferentially between sequences located outside **NADs** [99]. Interaction between the nucleolus and extra-nucleolar chromatin may stabilize weak interchromosomal contacts and serve as a centrefold for arrangement of chromosomal territories inside the nucleus [100]. The association of specific genomic loci with the nucleolus may occur through their interaction with rDNA. Recent HiC experiments demonstrated that 5S and 45S arrays formed multiple contacts with genomic regions and genes on all chromosomes. Interestingly, no direct physical interaction between the 5S and 45S rDNA repeats was observed [101].~~

~~***Association with the nucleolus regulates genome functioning.*** **NADs** are globally enriched in heterochromatin marks, including H3K27me3, H3K9me3, and H4K20me3. Gene targeting to the~~

nucleolus and the perinucleolar region is correlated with reduced gene expression [102]. Localization of **NADs** in the peripheral region of the nucleolus may thus contribute to gene silencing [7]. Regulation of gene expression may involve changes in gene positioning or the pattern of their contacts with the nucleolus. Indeed, some processes are accompanied by relocalization of genomic loci *vs.* the nucleolus: centromeric and pericentromeric repeats are dissociated from the nucleolus in aging cells [99]. Association with the nucleolus globally correlates with the inactive transcriptional state of RNA polymerase II transcribed genes. Some exceptions exist: translocations of the *CCND1* and *MYC* genes to the acrocentric chromosome 14 into the locus of the immunoglobulin heavy chain (*IGH*) genes lead to their relocalization towards the perinucleolar region and activation by the nucleolar protein NCL [6]. Association of the *IGH* genes with the nucleolus may also be necessary for recombination. Thus, B cell maturation is accompanied by relocalization of the *IGH* locus in the prenucleolar region to a specific "recombination compartment" containing an activation induced cytosine deaminase (AID). Somatic hypermutation and class switch recombination of immunoglobulin genes take place in these "recombination compartments" [8]. Localization of genes other than *IGH* within this compartment may significantly increase the probability of oncogenic translocations in B cells [103]. Association of one allele of a monoallelically expressed gene with the nucleolus may participate in stochastic or imprinted **allelic exclusion** which could be the case of *TCR*, *IGH* and olfactory receptor genes [104,105].

Nucleolar proteins, such as Ki 67, NCL and NPM1, mediate association of chromatin with the nucleolus. These proteins interact with CAF1, a chromatin assembly factor [99]. Depletion of the p150 CAF1 subunit led to a decreased association of certain genomic loci, including the 5S rDNA, alpha satellite DNA, and the D4Z4 macrosatellite, with the nucleoli in human cells [106]. Depletion of the nucleoplasmin-like protein NLP, a Drosophila homolog of NPM1, resulted in de clustering of centromeres and a decrease in centromere association with the nucleolus [107].

Normal fibroblasts and cancer cells depleted of NPM1 displayed deformed nucleoli and a striking rearrangement of perinucleolar heterochromatin [108]. NPM1 also associates with HP1γ, core, linker histones and a centromere-specific histone variant CENP-A [109]; NPM1 is required for efficient tethering of HP1γ-enriched chromatin to the nucleolus. Thus, interaction with chromatin and, in particular, heterochromatin proteins may be the major driver of chromatin anchoring to the nucleolus.

The nucleolus is also an essential platform for parental chromatin organization in murine oocytes [110]. Enucleolated oocytes exhibit abnormal heterochromatin distribution. In the nucleoli-containing oocytes, heterochromatin is anchored to the nucleoli and forms a ring-like structure, whereas in enucleolated oocytes, the heterochromatin forms chromocenter-like foci. Oocytes deficient in the major protein of the nucleoplasmin family, NPM2, showed disorganization of perinucleolar chromatin, and expression of NPM2 alone sufficed to reconstitute the nucleolar structure in enucleolated embryos. Thus, NPM2 participates in anchoring and organization of chromatin in oocytes similarly to NPM1 in somatic cells.

CTCF, a structural protein that regulates 3D genome folding into **TAD**s that often include co-regulated and co-transcribed genes (reviewed in [111]), may also anchor/target specific loci to the perinucleolar space. CTCF interacts with NPM1 to tether insulators to the nucleolar periphery [112]. In *Drosophila*, CTCF interacts with NLP and the nucleolar protein Modulo to position centromeres in the perinucleolar region [107].

Non-coding RNAs participate in the chromatin tethering towards the nucleolar periphery. For example, *Xist* non-coding RNA (ncRNA) is responsible for the association of the inactive X chromosome with the nucleolar periphery [113]. *Xist* loss after X inactivation leads to dissociation of X chromosome from the nucleolus. Interestingly, autosomes bearing

~~translocations with the X chromosome also become preferentially associated with the nucleolus [114]. *Firre* ncRNA also participates in X inactivation. *Firre* is expressed from a perinucleolar locus located on the X chromosome; this locus contains CTCF and cohesin binding sites [115]. *Firre* transcription from the inactive X chromosome cooperates with CTCF binding to ensure perinucleolar positioning. Knockdown of *Firre* or its interaction partner hnRNPU interferes with the perinucleolar targeting of the inactive X chromosome. An imprinted antisense *Kcnq1ot* ncRNA is transcribed only from the paternal allele and insures the **allelic exclusion** by targeting the paternal locus to the perinucleolar space where this 1Mbp region containing ten protein-coding genes is silenced [116,117].~~

Although mTOR complex 1 (mTORC1) is predominantly cytosolic and localizes around the lysosomal membrane, several members of mTORC1, including mTOR itself, can also locate to the nucleus and the nucleolus [63–69]. Nuclear mTOR protein can bind to thousands of sites in the genome [66,67] and interact with rDNA chromatin either directly [63–65] or *via* modulation of other rDNA-interacting factors [70,71]. Treatment with mTORC1 inhibitor rapamycin prevents mTOR localization to the nucleolus and interferes with the processing of ribosomal rRNAs [68]. One of the recent examples of the nucleolar activity of mTORC1 is related to the function of inhibitor of growth 1b protein (ING1b), which associates with rDNA repeats and is required for the efficient recruitment of chromatin regulator HDAC1 to the nucleoli [69]. ING1 reduces mTOR localization to the nucleolus and promotes recruitment of HDAC1 to the nucleolar remodelling complex (NoRC) complex and to major Pol I transcription factor UBF1. Inversely, knockdown of ING1 results in the accumulation of mTOR in nucleolus and it association with UBF1.

Sequestration of proteins in the nucleus. Although most of the shuttling between the nucleolus and the nucleoplasm is reversible, recent studies indicate that the nucleolus can sequester and store various nuclear and cytoplasmic proteins including misfolded protein aggregates. These

aggregates form proteinaceous particles that can promote proteotoxic stress and eventually cell death if they are not cleared by the ubiquitin-proteasome system. Protein aggregates may also form directly in the nucleoli when proteasome function is inhibited. [72]. These inclusions form so-called 'aggresomes' [23] or 'detention centers' [24] within or in a close contact with the nucleoli (Figure 2); they are rich in protein and RNA [73]. Aggresome formation correlates with cell survival [74].

Various stresses lead not only to formation to misfolded proteins, but also to the expression of specific lncRNAs from the intergenic spacer separating individual rDNA transcription units (IGS lncRNAs) [75]. IGS lncRNA transcription correlates with the formation of nucleolar detention centers [24] retaining DNA (cytosine-5)-methyltransferase 1 (DNMT1), the δ catalytic subunit of DNA polymerase (POLD1), Hsp70, RNF8, MDM2, APC2 [75], RPA16, RPA40, PES1, NOP52, RRP1B, NOM1, NOL1, SENP3, and von Hippel-Lindau (VHL) protein [24]. VHL protein degrades the hypoxia-inducible factor (HIF) in the presence of oxygen; **sequestration** of VHL enables HIF to evade destruction and activate its target genes [76]. Immobilization of these proteins inside the nucleolus is driven by specific nucleolar detention signals (NoDS) [75]. Importantly, key nucleolar proteins FBL, NPM1, and NOPP140 that do not contain these signals remain mobile and evade retention, highlighting the specificity of this nucleolar **sequestration** [24].

Dynamic association of chromatin with the nucleolus controls 3D genome organization.

Nucleolus and 3D genome organisation Nucleus may contain one or several nucleoli that occupy up to 25% of its volume. The nucleolus is largely filled with transcribed rDNA and assembling ribosomes. In each cell, only a fraction of rRNA genes is transcriptionally active and localise within nucleoli. Inactive rDNA repeats are located at the periphery of the nucleolus

contributing to the creation of a perinucleolar inactive chromatin compartment [5,77,78]. The presence of heterochromatin at the nucleolar periphery is directly related to the positioning of silenced ribosomal genes in this area [79]. Silencing of rDNA repeats is mediated by NoRC that recruits histone-modifying and DNA methylating enzymes [80,81]. The NoRC recruited to the perinucleolar region may also introduce heterochromatic marks into other chromatin regions in the vicinity of the nucleolus. Interestingly, centromeric heterochromatin is frequently located close to nucleolus, and depletion of TIP5, a subunit of NoRC compromises both rDNA silencing and assembly of centromeric heterochromatin [82].

Genomic segments located within perinucleolar compartment are commonly referred to as **nucleolus-associated chromatin domains** (**NADs**) [83,84]. **NADs** contain sequences located in the short arms of acrocentric chromosomes, centromeric and pericentromeric chromatin of most chromosomes and subtelomeric regions of some chromosomes **(Figure 3)**. Besides repetitive sequences, **NADs** contain more than 1000 genes including those encoding for the T-cell receptors, olfactory receptors and two families of immunoglobulin genes. Many **NAD**-associated genes have similar features: they are tissue-specific and form large gene clusters [85]. **NADs** substantially overlap with lamina-associated domains (LADs) [86]. Only a portion of LADs identified in population studies is located close to nuclear lamina. After mitosis, LADs are stochastically reshuffled, and LADs redistributed to the nuclear interior are frequently localised within the perinucleolar region [87].

What is the functional significance of **NADs** and how the nucleolus can regulate their functions? **NADs** are globally enriched in heterochromatin marks, including H3K27me3, H3K9me3, and H4K20me3. Gene targeting to the nucleolus and the perinucleolar region is globally correlated with reduced gene expression [88]. Localization of **NADs** in the peripheral region of the nucleolus may thus contribute to gene silencing [5]. Regulation of gene expression may involve

changes in gene positioning or the pattern of their contacts with the nucleolus. Indeed, some processes are accompanied by relocalization of genomic loci *vs.* the nucleolus. For example, centromeric and pericentromeric repeats are dissociated from the nucleolus in aging cells [89]. Association with the nucleolus globally correlates with the inactive transcriptional state of RNA polymerase II-transcribed genes. Some exceptions exist: translocations of the *CCND1* and *MYC* genes to the acrocentric chromosome 14 into the locus of the immunoglobulin heavy chain (*IGH*) genes lead to their relocalization towards the perinucleolar region and activation by NCL [4]. Association of the *IGH* genes with the nucleolus may also be necessary for recombination. Thus, B-cell maturation is accompanied by relocalization of the *IGH* locus in the prenucleolar region to a specific "recombination compartment" containing an activation-induced cytidine deaminase (AID). Somatic hypermutation and class-switch recombination of immunoglobulin genes take place in these "recombination compartments" [6]. Localization of genes other than *IGH* within this compartment may significantly increase the probability of oncogenic translocations in B-cells [90]. Association of one allele of a monoallelically expressed gene with the nucleolus may participate in stochastic or imprinted **allelic exclusion** which could be the case for *TCR*, *IGH* and olfactory receptor genes [91,92].

In the interphase nucleus, each chromosome forms a distinct chromosomal territory that is likely linked to both the nuclear lamina and the nucleolus because every chromosome contains both LADs and **NADs**. Being attached to the nucleolus, the nuclear lamina and to each other [93,94], chromosomal territories constitute a unified chromatin compartment that is mechanically stretched [95] and can sense mechanical stress [96]. In this scenario, phase-separated nuclear compartments, the largest of which is the nucleolus, act as global chromatin organizers [94,96]. In particular, tethering of chromatin to the nucleolus and nuclear speckles is likely to account for the spatial segregation of repressed and active chromatin compartments [97]. These compartments likely corresponding to heterochromatin and euchromatin were identified by **Hi-C**

[97]. Besides repressed and active chromatin compartments **Hi-C** and other recently developed experimental procedures allowed to identify self-interacting topologically associating domains (**TADs)** and contacts between chromosomes [94,97–99]. Interaction between the nucleolus and extra-nucleolar chromatin may stabilize weak interchromosomal contacts and serve as a centrefold for the arrangement of chromosomal territories inside the nucleus [100]. The association of specific genomic loci with the nucleolus may occur through their interaction with rDNA. Recent **HiC** experiments demonstrated that 5S and 45S gene arrays formed multiple contacts with genomic regions and genes on all chromosomes [101].

Association of chromatin with the nucleolus is mediated both by proteins and RNA. Nucleolar proteins Ki-67, NCL and NPM1 mediate the association of chromatin with the nucleolus. These proteins interact with CAF1, a chromatin assembly factor [89]. Depletion of the p150 CAF1 subunit leads to a decreased association of certain genomic loci, including the 5S rDNA, alpha satellite DNA, and the D4Z4 macrosatellite, with nucleoli in human cells [102]. Depletion of NLP, a Drosophila homolog of NPM1, resulted in de-clustering of centromeres and a decrease in centromere association with the nucleolus [103]. Normal fibroblasts and cancer cells depleted of NPM1 displayed deformed nucleoli and a striking rearrangement of perinucleolar heterochromatin [104]. NPM1 also associates with HP1γ, core linker histones and a centromere-specific histone variant CENP-A [105]; NPM1 is required for efficient tethering of HP1γ-enriched chromatin to the nucleolus. Thus, interaction with chromatin and, in particular, heterochromatin proteins may be a major driver of chromatin anchoring to the nucleolus. CTCF, a structural protein that regulates 3D genome folding into **TADs** that often include co-regulated and co-transcribed genes (reviewed in [106,107]), may also anchor/target specific loci to the perinucleolar space. CTCF interacts with NPM1 to tether insulators to the nucleolar periphery [108]. In *Drosophila*, CTCF interacts with NLP and the nucleolar protein Modulo to position centromeres in the perinucleolar region [103].

Non-coding RNAs participate in the chromatin tethering towards the nucleolar periphery as well. For example, *Xist* and *Firre* non-coding RNAs (ncRNA) participate in X inactivation. *Xist* is responsible for the association of the inactive X chromosome with the nucleolar periphery [109]. *Xist* loss after X inactivation leads to dissociation of X chromosome from the nucleolus. Interestingly, autosomes bearing translocations with the X chromosome also become preferentially associated with the nucleolus [110]. *Firre* is expressed from a perinucleolar locus located on the X-chromosome; this locus contains CTCF and cohesin binding sites [111]. *Firre* transcription from the inactive X chromosome cooperates with CTCF binding to ensure perinucleolar positioning. Knockdown of *Firre* or its interaction partner hnRNPU interferes with the perinucleolar targeting of the inactive X chromosome. An imprinted antisense *Kcnq1ot* ncRNA is transcribed only from the paternal allele and insures the **allelic exclusion** by targeting the paternal locus to the perinucleolar space where this 1Mbp region containing ten protein-coding genes is silenced [112,113]. Thus, nucleolus plays an important role in the 3D organization of the genome. It participates in organization of chromosome compartments and territories as well as heterochromatin assembly. This process involves several nucleolar proteins and non-coding RNAs.

Concluding remarks

~~Proteins can be sequestered into the nucleolus affecting their functions. At the same time, other proteins and RNA can be freely exchanged between the nucleolus and the nucleoplasm. This shuttling is important for many processes, including transcription regulation, telomere maintenance, DNA recombination and repair and apoptosis. Recent data also point to the emerging role of the nucleolus in organizing the genome folding and transcription by sequestering specific sequences to perinucleolar regions. Further studies will help us to decrypt the amazing complexity of this nuclear body.~~

The key role of nucleolus as a regulatory hub is directly related to its main function, production of ribosomes. This process consumes more than a half of cell energy resources and thus should be coordinated with the actual needs of the cell in the ribosomes. Accordingly, many regulatory circuits link the production of ribosomes to the cell cycle progression which, in turn, can be blocked by stress responses. It is thus not surprising that nucleolus acquired a role of regulatory hub connecting many functional processes. The fundamental link between ribosome biogenesis and cell cycle progression is accomplished through involvement of certain proteins in cell cycle control and stress response on the one hand, and the regulation of ribosomal gene transcription and ribosome assembly on the other hand. Many of these proteins are sequestered within phase-separated nucleoli but may shuttle to nucleoplasm in response to various stimuli.

The principal role of nucleolus in the 3D organization of the genome consists in creation of a nucleation center for the assembly of heterochromatin. This nucleation is due to silenced rRNA genes. Silencing of a portion of rRNA genes is an ancient phenomenon occurring even in lower eukaryotes. In multicellular organisms, the mechanism that silenced rRNA genes could be extended to other genes that are not used in various types of differentiated cells. Further studies will help us to decrypt the amazing complexity of the nucleols (see Outstanding questions).

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Box 1

Membraneless nuclear compartments

Eukaryotic cell nucleus contains a number of membraneless compartments such as nucleoli, Cajal bodies, PML bodies, nuclear speckles etc. [118]. These compartments are dynamic associates of proteins and nucleic acids and are slightly denser than the bulk of the nucleoplasm. Each type of membraneless compartments is connected to certain functional processes that are not necessarily related to each other [119]. Accordingly, most of the compartments contain a number of various proteins including many moonlighting proteins [120]. The proteins participating in assembly of nuclear compartments frequently possess intrinsically disordered domains [120–122]. These proteins/domains may mediate weak-affinity and non-specific interactions with multiple target sites that trigger the liquid phase separation [123]. The phase-separated droplets accumulate macromolecules that possess an affinity to the components of the droplet interior. Usually a so-called platform nucleates the assembly of liquid droplet nuclear compartments. This may be either a protein (e.g. PML in case of PML bodies [124]) or a non-coding RNA (e.g. NEAT1 RNA in paraspeckles [125] or rRNA in nucleoli [126]). The nucleolus differs from other membraneless compartments in two ways. First, the nucleolus consists of at least three distinct phase-separated layers: droplets within droplets [127]. Second, the availability of pre-rRNAs that constitutes a platform for nucleolus assembly directly depends on the ongoing rDNA transcription [128]. Suppression of rDNA transcription results in full or partial disruption of nucleoli and release into cytoplasm of a various proteins sequestered within nucleoli [128]. This is likely to be an important part of the cellular stress response mechanism [129].

Box 1

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Eukaryotic cell nucleus contains a number of membraneless compartments such as nucleoli, Cajal bodies, PML bodies, nuclear speckles etc. [114]. These compartments are dynamic associates of proteins and nucleic acids and are slightly denser than the bulk of the nucleoplasm. Each type of membraneless compartments is connected to certain functional processes that are not necessarily related to each other [115]. Accordingly, most of the compartments contain a number of various proteins including many moonlighting proteins [116]. The proteins participating in assembly of nuclear compartments frequently possess intrinsically disordered domains [116–118]. These proteins/domains may mediate weak-affinity and non-specific interactions with multiple target sites that trigger the liquid phase separation [119]. The phase-separated droplets accumulate macromolecules that possess an affinity to the components of the droplet interior. Usually a so called platform nucleates the assembly of liquid droplet nuclear compartments. This may be either a protein (e.g. PML in case of PML-bodies [120]) or a non-coding RNA (e.g. NEAT1 RNA in paraspeckles [121] or rRNA in nucleoli [122]). The nucleolus differs from other membraneless compartments in two ways. First, the nucleolus consists of at least three distinct phase-separated layers: droplets within droplets [123]. Second, the availability of pre-rRNAs that constitutes a platform for nucleolus assembly directly depends on the ongoing rDNA transcription [124]. Suppression of rDNA transcription results in full or partial disruption of nucleoli and release into cytoplasm of a various proteins sequestered within nucleoli [124]. This is likely to be an important part of the cellular stress response mechanism [125].

Figure Legends

Figure 1. *DNA repair and telomere maintenance are regulated by protein shuttling between the nucleolus and the nucleoplasm.*

Clockwise: APE1 interacts with nucleolar proteins NPM1 *via* its positively charged lysine residues. When some of these lysine residues are acetylated, NPM1 no longer interacts with APE1 and APE1 accumulates in nucleoplasm at the DNA damage sites. Nucleolar mTOR activates PolI transcription. ~~The ING1/HDAC1 complex reduces nucleolar localization of mTOR and represses transcription.~~ WRN possesses a **NoLS** in its C-terminus; nucleolar localization of WRN is dependent on RNA Pol I transcription. When DNA repair is activated, WRN is dephosphorylated and moves from nucleolus to nucleoplasm. BLM can directly bind rDNA and interact with RNA Pol I. BLM switching between DNA repair and ribosome biogenesis occurs through protein-protein interactions and posttranslational modifications. TRAIP encodes a nucleolar protein that migrates to UV-induced DNA lesions in the nucleoplasm; inhibition of RNA Pol I activity also leads to TRAIP diffusion into the nucleoplasm. The telomerase complex biogenesis occurs in the nucleolus; TERT is retained in nucleoli through its interaction with NCL. ~~Activity of TRF1 is modulated by interaction with GNL3L that stabilizes TRF1 while NSM promotes its degradation, inhibits TRF1 dimerization and shortens its dynamic association with the telomere.~~ Nucleolar localization of TRF2 is promoted by the nucleolar protein NOLC1. TRF2 also binds rDNA and promotes rRNA transcription. NCL forms nucleoplasmic foci at sites of DSB repair where it is implicated in chromatin remodelling while the mail pool of NCL is located in the nucleolus. Ionizing radiation, cisplatin or etoposide treatment leads to NPM1 phosphorylation and its relocalization from the nucleolus to the DSB foci in the nucleoplasm.

Figure 2. *Sequestration of nuclear and cytoplasmic proteins in the nucleolus.*

Misfolded proteins aggregate and form proteinaceous particles that are transported into the nucleolus form so-called ‘aggresomes’ or ‘detention centers’. p21Cip1 transits through the

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nucleolus on its way from the nucleus to the cytoplasm. DNA damage inhibits this transit and induces formation of p21Cip1-containing intranucleolar bodies (INoBs) containing SUMO-1, UBC9, Cdk2, Cyclin E, PCNA, p53 and MDM2. Transcriptional, acidic or heat stress leads to expression of lncRNAs from the rDNA intergenic spacer. These transcripts capture and immobilize inside nucleoli several important proteins, including VHL, DNMT1, tPOLD1, Hsp70, RNF8, MDM2, APC2, RPA16 and RPA40, PES1, NOP52, RRP1B, NOM1, NOL1, and SENP3. Key nucleolar proteins FBL, NPM1, and NOPP140 retain their mobility and evade immobilization, highlighting the specificity of this nucleolar **sequestration**.

Figure 3. *Dynamic association of chromatin with the nucleolus regulates nuclear processes*
Clockwise: nucleoli-associated chromatin domains (**NADs**) represent hundreds of extended genomic loci comprising ~4% of the human genome. **NADs** contain sequences located in the p-arms of acrocentric chromosomes, centromeric and pericentromeric chromatin of most chromosomes and subtelomeric regions of some chromosomes. Association with the nucleolus globally correlates with the inactive transcriptional state of RNA polymerase II-transcribed genes, with some exceptions. Association with the nucleolus may also participate in **allelic exclusion**. Somatic hypermutation and class-switch recombination occur in the specific "recombination compartment" located in the prenucleolar region and containing AID. Inactive X chromosome is associated with the nucleolar periphery; this association is due to *Xist* and *Firre* non-coding RNAs.

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