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Beyond ribosome biogenesis: noncoding nucleolar RNAs in physiology and tumor biology

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ABSTRACT

The nucleolus, the largest subcompartment of the nucleus, stands out from the nucleoplasm due to its exceptionally high local RNA and low DNA concentrations. Within this central hub of nuclear RNA metabolism, ribosome biogenesis is the most prominent ribonucleoprotein (RNP) biogenesis process, critically determining the structure and function of the nucleolus. However, recent studies have shed light on other roles of the nucleolus, exploring the interplay with various noncoding RNAs that are not directly involved in ribosome synthesis. This review focuses on this intriguing topic and summarizes the techniques to study and the latest findings on nucleolar long noncoding RNAs (lncRNAs) as well as microRNAs (miRNAs) in the context of nucleolus biology beyond ribosome biogenesis. We particularly focus on the multifaceted roles of the nucleolus and noncoding RNAs in physiology and tumor biology.

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Introduction

The nucleolus is the largest subcompartment of the nucleus and is organized around one or more transcribed ribosomal RNA gene (rDNA) repeat arrays. The volume occupancy of the nucleolus (or nucleoli) within the nucleus can be estimated to be as high as 20% (BNID 113,849 - human cancer cells, BNID 110,468 - budding yeast cells) [1]. In contrast, transcriptionally active rDNA, the central DNA component of the intra-nucleolar space, accounts for less than 1% of the genome in human cells [2]. Thus, the nucleolus represents a nuclear space with very low local DNA and chromatin concentration as it is mainly filled with RNA, proteins and ribonucleoprotein (RNP) assemblies with remarkably high estimated local RNA concentrations (around 50 mg/mL in contrast to 0.5 mg/mL in the nucleoplasm) [3]. These molecular characteristics are readily visible on fluorescence microscopy images of RNA- and DNA-stained cells. The nucleolus shows up as

a large, bright signal in the RNA channel, while it appears as a void in the DNA channel (Figure 1a).

The core function of the nucleolus is ribosomal RNA (rRNA) synthesis and co-transcriptional ribosome precursor assembly. Thus, RNAs associated with the early steps of ribosome biogenesis form the bulk of nucleolar RNA molecules. The synthesis of the 47S rRNA precursor (45S, if processed co-transcriptionally) of the mature 18S, 5.8S and 28S rRNA molecules is catalyzed by up to around 100 RNA polymerase I complexes per gene from the 13.3-kbp-long coding regions of the active rDNA units. The coding sequences are separated by intergenic spacer regions (rDNA IGS) that are more than 40 kbp long (44,838 bp rDNA reference sequence, GenBank accession KY962518) [4]. These units are largely organized in head-to-tail tandem repeat DNA arrays, which in human cells are localized in telomere-to-centromere orientation on the short arms of acrocentric chromosomes 13, 14, 15, 21, and 22 and

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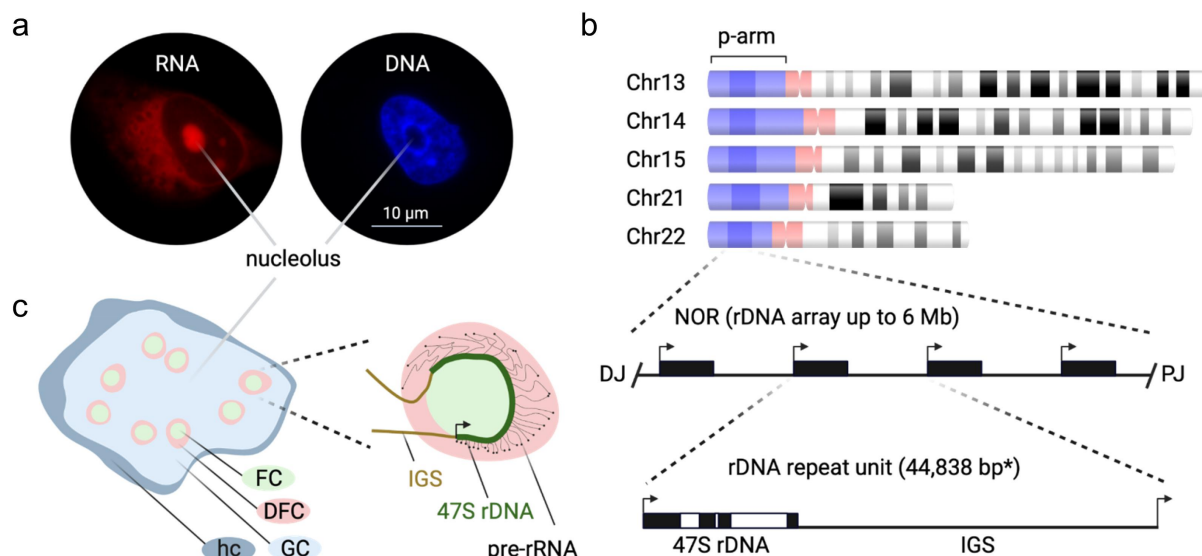


Figure 1. Structure of the nucleolus and NORs. a) the nucleolus is characterized by a high RNA:DNA ratio. A549 human lung adenocarcinoma cells were stained with PhenoVue 512 Nucleic acid stain and PhenoVue hoechst 33,342 to stain RNA and DNA, respectively. The local depletion of DNA contrasts strongly with the local enrichment of RNA and marks the nucleolar region within the nucleus. Images were acquired using a leica DMI8 thunder imager system. b) organization of NORs. Ideograms of NOR-bearing human acrocentric chromosomes and schemes of an rDNA array flanked by distal and proximal junctions (DJ and PJ) at the telomeric and centromeric sites, respectively, and a single repeat unit are shown. White boxes within the 47S rDNA mark the positions of the coding regions of the 18S, 5.8S, and 28S rRNA. IGS: intergenic spacer. *The size of the GenBank KY962518 rDNA reference sequence. The ideograms were generated using <https://www.ncbi.nlm.nih.gov/genome/tools/gdp>. c) schematic illustration of a nucleolus with the nucleolar territories fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC). Perinucleolar heterochromatin is labeled as hc. A scheme of a transcribed 47S rDNA with nascent pre-rRNA is shown on the right. Parts of the figure were created with BioRender.com.

represent the nucleolus organizer regions (NORs). The rDNA arrays are flanked by specific DNA sequences shared between the short arms. These sequences build the proximal and distal junctions (PJ and DJ) at the centromeric and telomeric sites, respectively (Figure 1b) [5,6]. In interphase cells, they are located at the nucleolar periphery. Thus, they belong to nucleolar DNA, which is comprised – besides rDNA and these sequences – of specific nucleolus-associated chromosomal domains [7,8]. As a result of pre-rRNA synthesis and processing, the nucleolar space is largely filled with 90S pre-ribosomal RNP particles at varying stages of maturation [9]. The molecular assemblies associated with the early steps of ribosome biogenesis define distinct nucleolar territories, namely the fibrillar center (FC), the dense fibrillar component (DFC) and the granular component (GC). The synthesis of the 47S rRNA precursor occurs at the interface between the FC and DFC, whereas its processing and assembly

into early ribosomal RNPs involves the DFC and GC (Figure 1c, model based on [6,10–12]). The non-homogeneous organization of this subnuclear microenvironment is underlined by the physico-chemical principles of colloid systems and the nucleolus is presently considered as a multiphase liquid condensate [13–15].

Importantly, the nucleolus with its high local enrichment of proteins that bind or enzymatically process RNA plays host to various RNA-associated biological processes other than ribosome synthesis. In this review, we first give an overview of methodological approaches to identify and analyze nucleolar RNAs, as well as the different types of RNA and their link to the nucleolus. Next, we explore the role of selected nucleolar lncRNAs and miRNAs in physiological and pathological processes focusing on tumor biology. Notably, our detailed discussion focuses on ncRNAs that neither regulate ribosome biogenesis directly nor originate from the rDNA repeat unit. For a more

in-depth look into rDNA-derived ncRNAs and other ncRNAs that control the synthesis of ribosomes, readers are directed to two comprehensive recent reviews [16,17].

Approaches for identifying nucleolar RNAs

The distribution of the required components to confined spaces within the cell increases the efficiency of many subcellular processes. Therefore, new information about the molecular composition of subcellular spaces or, from a molecular point of view, information about the subcellular distribution of biomolecules is of great importance for a better understanding of their functions. Within the nucleus, membraneless structures allow subcompartmentalization and thus the emergence of local microenvironments with distinct functionalities. To these structures belongs the nucleolus with its high local RNA content, composed mainly,

but not exclusively, of rRNA. Most nucleolar RNAs not associated with ribosome biogenesis have been identified in just about the last decade. The pioneering studies combined cell fractionation of various cell lines with RNA extraction and diverse high-throughput sequence analyses (Figure 2a).

For instance, the Pederson laboratory isolated nucleoli from L6 rat myoblasts and extracted total RNA for LNA-based microRNA profiling microarray analysis [18]. Purification of the nucleolus was controlled by microscopy and by monitoring accumulation of a small nucleolar RNA (snoRNA) and depletion of a cytoplasmic RNA in nucleolar RNA in comparison to nucleoplasmic and cytoplasmic RNA using RT-qPCR. The miChip platform [19] used in this study allowed the detection of mature, primary, and precursor miRNAs. In subsequent studies from the same laboratory, nucleolar enrichment of mRNAs was detected in L6 cells using the Affymetrix

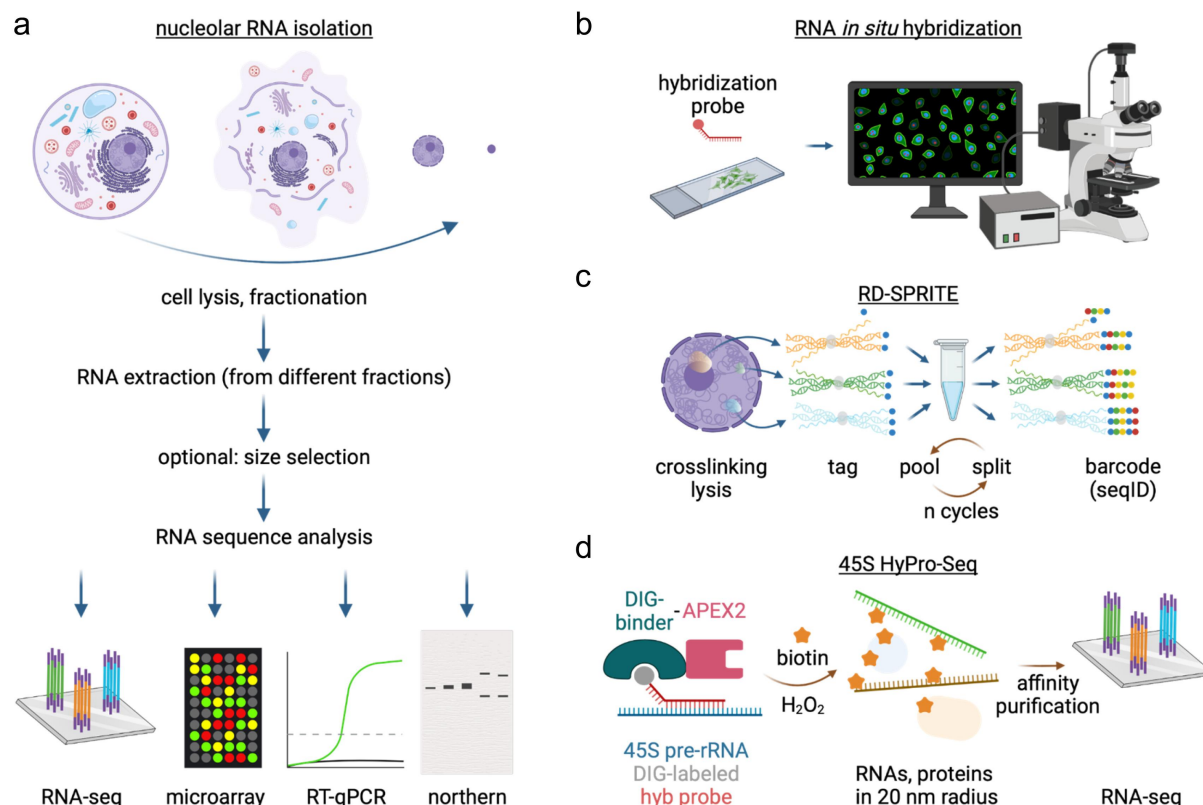


Figure 2. Overview of methods used to detect nucleolar RNAs. a) the most common methods used to date to study nucleolar RNA involve cell fractionation, RNA extraction from nucleolar and possibly other fractions, optional size selection, and subsequent analysis by high-throughput sequencing, microarray, RT-qPCR or northern blotting. b) RNA *in situ* hybridization has mainly been used at low throughput for validation of nucleolar RNA localization. c) RD-SPRITE (RNA&DNA split-pool recognition of interactions by tag extension) uses a combinatorial barcode-based sequencing approach to identify DNAs and RNAs located in close proximity to each other in nuclear space. d) 45S HyPro-Seq employs a hybridization-based approach to tag, capture and sequence RNAs within a distance radius of about 20 nm from the 45S rRNA. Created with BioRender.com.

GeneChip Rat Exon ST array platform for RNA analysis [20]. Fluorescence *in situ* hybridization (FISH, Figure 2b) completed the analyses of both selected miRNAs and mRNAs.

As part of the GENCODE section of the Encyclopedia of DNA Elements (ENCODE) project, a comprehensive assessment of the cellular compartmentalization of the transcriptome of human cells was undertaken led by the Gingeras and Guigó laboratories. Among other cellular spaces, a focus was also on elucidating the RNA content of the confined space of the nucleolus. In this project, a comprehensive RNA isolation was performed from five subcellular fractions (cytosolic, nuclear, nucleoplasmic, nucleolar and chromatin-associated) of the human myeloid leukemia cell line K562. RNA was further fractionated into long (>200 nt), small (<200 nt), polyA+, and polyA- fractions, and mature rRNA was depleted in each fraction before library preparation. RNA was subjected to Illumina sequencing (2×76 nt paired-end for long RNA, single-end 36 nt for short RNA) and subsequent bioinformatics analyses [21–23].

The Laiho laboratory embarked on an in-depth analysis of small RNA (<200 nt) content in total cellular RNA as well as in cytoplasmic, nuclear and nucleolar RNA fractions of human HeLa cervical adenocarcinoma cells and MCF7 breast adenocarcinoma cells by employing Invitrogen's NCode Multi-Species miRNA v.2 microarray platform and Applied Biosystems' TaqMan Array Human MicroRNA Panel v2.0 microarray platform. In addition, the isolated small RNAs were further separated on a denaturing polyacrylamide gel and the 10–40 nt fraction was purified and subjected to deep sequencing of the small RNAs using an Ion Torrent sequencing platform. Nucleolar enrichment of selected RNA species was validated by northern blotting. Here, the dispensability of the Dicer protein for nucleolar localization of selected miRNAs was also demonstrated by comparative analyses of RNA fractions from Dicer wild-type and Dicer knock-out HCT116 human colorectal cancer cells [24,25].

Contributing studies from the Lam, Rippe, and Liu labs showcased the effectiveness of cell fractionation for primary detection of nucleolar miRNAs [26] or lncRNAs [27,28]. In the first work, HeLa cells were fractionated and miRNA abundances in

the nucleolar fraction and total cellular RNA were determined using a highly multiplexed RT-qPCR assay. Of the 337 detectable miRNAs, 48 miRNAs displayed high nucleolus/cell ratios. The cutoff value was set to > 30% of the nucleolus/cell ratio of the *bona fide* nucleolar snoRNA U44. The 11 most abundant miRNAs for which the nucleolus/cell ratio could be reliably determined were identified as nucleolar miRNAs. Nucleolar localization was confirmed for selected nucleolar miRNAs by RNA-FISH in HeLa cells, AG06858 human fibroblasts, A549 and H1299 human lung adenocarcinoma cells, Huh-7 hepatocellular carcinoma cells, and RPE human retinal pigment epithelium cells. Moreover, RNA-FISH in primary mouse fibroblasts and the canine cell line MDCK indicated some evolutionary conservation of the nucleolar localization of these miRNAs [26]. In the second study, cell fractionations were again performed in HeLa cells, but here, total RNA, nucleoplasmic RNA, and nucleolar RNA were analyzed after mature rRNA removal by Illumina sequencing using 36 nt or 100 nt read lengths. Because approximately 90% of the sequences within the 100 nt sequence reads mapped accurately to repetitive *Alu* elements, the nucleolar localization and function of *alu*RNAs was further validated and examined here using RNA-FISH, northern blot, microinjection, and transfection experiments [27]. In the third study, mouse neuroblastoma N2a cells were subjected to cell fractionation, nucleolar RNA was isolated, mature rRNA was removed and > 200 nt fragments were subjected to 150 bp paired-end sequencing on the Illumina platform. Approximately 90% of the RNA represented lncRNA transcripts and 10% mRNA transcripts. The expression levels of the 30 most abundant lncRNAs in the nucleolar RNA were validated by RT-qPCR, and the nucleolar-specific GM17362 lncRNA, renamed LoNA, was subjected to further analyses, including confirmation of nucleolar localization by RNA-FISH [28,29].

Recently, the RNA&DNA version of the SPRITE (split-pool recognition of interactions by tag extension) method has provided new insights into the interaction map of nucleolar RNA and DNA (Figure 2c). In the SPRITE method, cells are subjected to formaldehyde crosslinking, the chromatin of isolated nuclei is fragmented by sonication, and crosslinked RNA-

DNA-protein complexes are uniquely barcoded using a split-pool method. The nucleic acid content is assessed by high-throughput sequencing. Spatial proximity maps can be defined from sequence reads with identical barcodes derived from the same interacting complex. SPRITE analyses mapped multiple contacts between the most abundant nucleolar ncRNAs, including the 45S pre-rRNA, various snoRNAs, and the RNA component of the RNase-MRP complex, along with their physical proximity to DNA sequences localized in the nucleolus [30].

Another groundbreaking spatial interactome mapping technique is HyPro-Seq [31], which maps RNA-protein and RNA-RNA proximity (Figure 2d). In this assay, cells are fixed and permeabilized by using thiol-cleavable dithio-bis(succinimidyl propionate) and 70% ethanol, respectively. In the next steps, digoxigenin-labeled antisense probes are used for RNA hybridization and a HyPro (hybridization-proximity labeling) enzyme is added that biotinylates proteins and RNAs with an approximate labeling radius of 20 nm after providing a biotin substrate. HyPro is a recombinant < 50 kDa protein consisting of a high-affinity digoxigenin binding domain, and a codon-optimized version of APEX2, which requires biotin-phenol and H₂O₂ for *in situ* biotinylation. Biotinylated molecules are captured and subjected to mass spectrometry or RNA-seq analyses. RNA was analyzed after removal of mature rRNA by Illumina sequencing with 75 nt read lengths. Using this method, the Makeyev group has generated detailed 45S rRNA maps and profiled 45S rRNA neighboring lncRNAs in HeLa cells.

Several studies in which ncRNAs were functionally defined, and localization studies by *in situ* hybridization revealed their nucleolar localization have deepened our understanding of nucleolar ncRNAs. Examples include, nucleolar localization of the *Alu*-containing SRP (signal recognition particle) RNA in normal rat kidney (NRK) fibroblasts [32], the miRlet7d RNA as duplex with promoter-specific lncRNAs in MLE-12 mouse lung epithelial and MLg mouse lung fibroblast cells [33], the LETN RNA (lncRNA essential for tumor cell proliferation via NPM1) in Huh-7 cells [34], the Discn lncRNA (DNA damage-induced stem cell specific noncoding RNA) in mouse embryonic stem cells (ESCs) and MEF (mouse embryonic fibroblast) cells [35],

the PHACTR2-AS1 lncRNA (Phosphatase and actin regulator 2 – antisense 1) in Hs578T human breast carcinoma cells [36], and the box H/ACA small nucleolar RNA (snoRNA)-ended lncRNA that enhances pre-rRNA transcription (SLERT) in PA-1 human ovarian carcinoma and HeLa cells [37,38]. A significant observation is that SLERT, a snoRNA-ended lncRNA, can hint at a sequence-based prediction for nucleolar localization of similar ncRNAs. Sno-lncRNAs possess snoRNA sequences at both ends, while 5' SnoRNA capped and 3' PolyAdenylated lncRNAs (SPAs) have snoRNA sequences only on their 5' end. Their often high abundance suggests that sno-lncRNAs are functional RNAs and not simply snoRNA precursors [39–41]. Thus, the snoRNA parts might act as nucleolar or Cajal body targeting sequences, similar to what has been described and mechanistically uncovered for canonical snoRNAs [42,43]. Nucleolar localization has been demonstrated by RNA imaging and RT-qPCR analyses of cytoplasmic, nuclear and nucleolar RNA fractions also for LNC-SNO49AB in human leukemia cell lines [44].

Publicly available databases provide additional means to explore the localization of nucleolar RNAs and to select ncRNA candidates for further experimental validation of nucleolar localization and function. For example, the LncAtlas database (<https://lncatlas.org.eu>) allows rapid visualization of the relative levels of the most diverse class of ncRNAs, lncRNAs, in nucleolar, chromatin, nucleoplasmic, and cytosolic fractions of K562 cells analyzed in the GENCODE project [23]. LncBook, an integrative knowledge database of human lncRNAs (<https://ngdc.cncb.ac.cn/lncbook/>), also indicates the status of subcellular localization, if known [45]. The very comprehensive RNADisease database (<http://www.rnadisease.org>) [46], with 3,428,058 RNA disease entries to date, covering 18 RNA types, 117 species, and 4090 diseases, integrates subcellular localization information from RNALocate v2.0 [47] as well as drug and RNA interaction information, among other information. It also provides RNA disease prediction and disease enrichment tools and a common framework for ncRNA analysis in cancer RNA-seq data combined from The Cancer Genome Atlas (TCGA) [48] and International Cancer Genome Consortium (ICGC) data [49],

as well as data generated by the Therapeutically Applicable Research to Generate Effective Treatments (<https://www.cancer.gov/ccg/research/genome-sequencing/target>) initiative (TARGET). The Human microRNA Disease Database (<http://www.cuilab.cn/hmdd>) is a manually curated and regularly updated miRNA-specific database that collects information on experimentally validated associations of human miRNAs with any type of disease [50]. The current version 4.0 (HMDD v4.0) contains 53,553 miRNA-disease association entries for 1,817 human miRNA genes, 79 virus-derived miRNAs, and 2,379 diseases from 37,022 publications.

In sum, these concerted efforts in unraveling the mysteries of nucleolar RNAs underscore the importance of innovative methodologies and the vast potential that lies in the nucleolus for further genomic explorations. Given the diversity in cell types, cell and RNA fractionation protocols and sequence analysis platforms, the various techniques at our disposal offer a range

of alternative and complementary insights. However, our picture of nucleolar RNA content is far from complete. Techniques like subcellular spatial transcriptomics and highly multiplex RNA-FISH (MERFISH) [51,52]-based techniques present potential avenues for future exploratory studies. They could complement our methodological toolbox or serve as validation approaches combined with the methods already used to identify nucleolar ncRNAs. In addition, the novel CRISPR-Csm [53] method offers an exciting and promising tool for efficient depletion and imaging of nuclear RNA targets and, thus, paving the way for advanced functional analyses of nucleolar ncRNAs.

Types of RNA and their involvement in nucleolar biology

As illustrated in Figure 3, a variety of RNA types are synthesized in eukaryotic cells, and many of them are represented to some extent in the

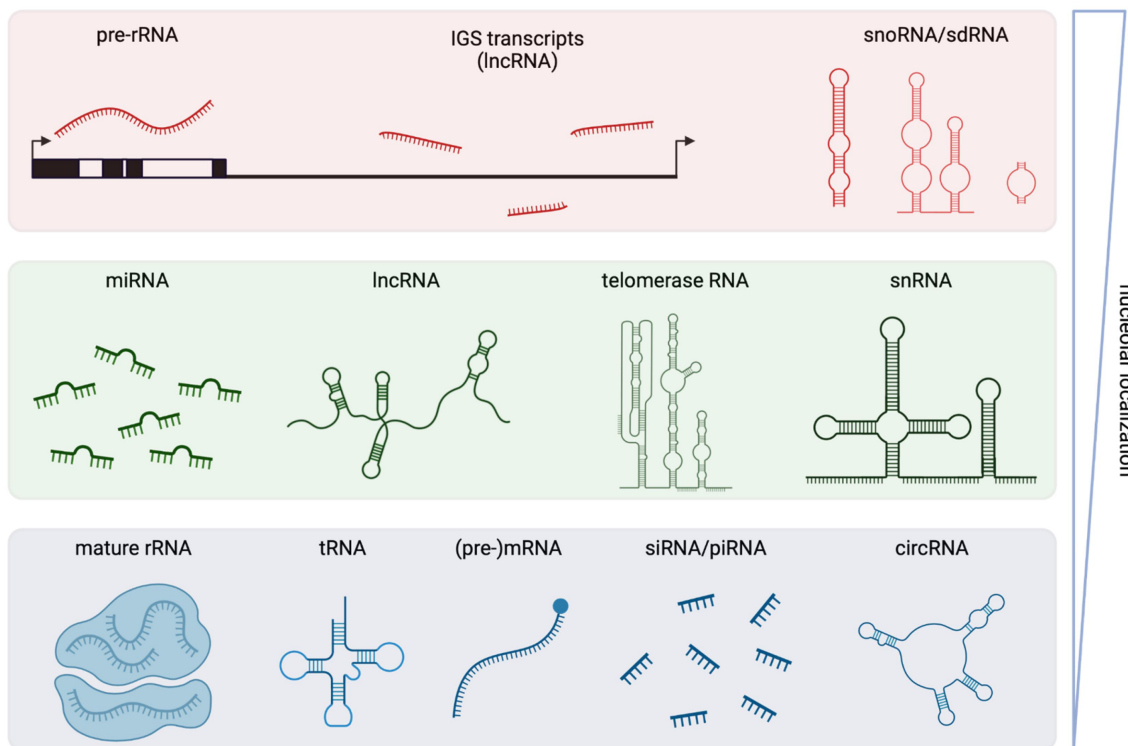


Figure 3. Representation of RNA types and their estimated abundances in the nucleolus. The main RNA types that have been so far identified and named, sometimes with yet not completely defined nomenclature [54,55], are rRNA, mRNA, tRNA, snRNA, snoRNA, sdRNA, siRNA, piRNA, circRNA, lncRNA, miRNA. Constitutive nucleolar RNAs include pre-rRNA, lncRNAs transcribed from the rDNA IGS and snoRNAs. Facultative nucleolar RNAs comprise various miRNAs, lncRNAs, telomerase RNA and snRNAs. Mature rRNAs, tRNAs, pre-mRNAs, mature mRNAs, siRNAs, piRNAs and circRNAs are not or very rarely localized in nucleoli. See text for details. Created with BioRender.com.

nucleolus. Specific steps of the biogenesis of several RNPs take place in the nucleolus (e.g., ribosome, miRNP, mRNP, SRP, U6 snRNP, RNase P, telomerase), which may explain their facultative nucleolar localization, even though the corresponding mature RNPs are not known to function in the nucleolus. In contrast, other RNPs (snoRNP, RNase MRP) or lncRNAs (e.g., synthesized from the rDNA IGS and the DJ) exert their function in the nucleolus [16,56].

Apart from pre-rRNA species synthesized from the rDNA coding region and processed partially co- and also post-transcriptionally in the nucleolus, small nucleolar RNAs (snoRNA) significantly contribute to the high RNA content of nucleoli. The current version of the human snoRNA database snoDB 2.0 contains 2123 unique entries [57]. SnoRNAs range from 60 to 300 nucleotides in length and most are derived from intron sequences of pre-mRNAs. The best-known function of snoRNAs is to guide the site-specific modification of rRNA in association with proteins as snoRNPs. The two major classes of snoRNAs are box C/D snoRNAs and box H/ACA snoRNAs, which drive sequence-specific 2'-O methylation and pseudouridylation by direct base pairing. However, it has also become apparent that snoRNAs or snoRNA-derived RNAs (sdrRNAs) play a role in processes other than ribosome biogenesis, such as modification of small nuclear RNAs (snRNAs) in Cajal bodies, alternative splicing, or miRNA-like regulation of mRNA levels [58–62]. Interestingly, in contrast to cellular, cytoplasmic, and nuclear small RNAs (sRNAs) enriched in miRNAs, the nucleolus predominantly harbors sdrRNAs that retain C or D motifs of box C/D snoRNAs. Particularly abundant sdrRNA species are 20 nt, 25 nt, and 120 nt RNAs derived from SNORD44, the latter of which binds fibrillarin and, thus, has a potential regulatory function [25]. Notably, dysregulation of specific snoRNAs has recently been linked to various cancer types, either correlated with or functionally associated with tumorigenesis [58]. The extent to which these processes involve nucleolar localization remains to be studied in detail. Interestingly, the RNA subunit of telomerase RNP, which is a reverse transcriptase complex that catalyzes the synthesis of telomeric DNA repeats, is structurally related to box H/ACA

snoRNAs [63]. This telomerase RNP, responsible for maintaining telomerase length, is reactivated in many cancer cells enabling them to bypass cellular senescence and apoptosis and achieving uncontrolled proliferation. It has been suggested that telomerase RNP biogenesis occurs in part in the nucleolus [56].

For snRNAs, the RNA subunit of the U6 snRNPs can be detected in the nucleolus because assembly of the ribonucleoprotein occurs partly there, even though the mature complexes operate in the nucleoplasm. Likewise, SRP RNPs localize transiently in the nucleolus during their maturation before translocating to their site of action in the cytoplasm [56]. In contrast, the RNase MRP complex with its RNA component, in addition to its mitochondrial function, also acts in the nucleolus, aiding in the processing of precursor rRNA [64,65]. Several other RNA types with nucleolar localization and function have been described. Examples include the circular RNA (circRNA) circANRIL, which binds to the pescadillo homologue 1 (PES1) protein, subunit of Pes1-Bop1-Wdr12 (PeBoW), a key pre-rRNA processing complex, thereby interfering with complex formation and ribosome biogenesis [66] (C/U)-rich sequence-containing inflammatory pre-mRNAs, which together with Rrp6 exosomes are directed to the nucleolus via nucleolin for degradation [67] nucleolar targeting of the mature Insulin-like growth factor 2 (IGF2) mRNA and perhaps other mRNAs together with their cognate miRNPs, possibly to regulate mRNA translation through sequestration [20]. Of the small repetitive DNA-encoded piRNAs, piR-49322 is an example for which nucleolar localization has been detected [68].

It has been long known that dysregulation of the core function of the nucleolus, ribosome synthesis, is linked to tumor biology making it a potential drug target [69,70]. This is further underscored by the numerous chemotherapeutic agents that target various steps of ribosome biogenesis [71]. Consequently, ncRNAs that regulate this key biological process are potential promoters or inhibitors of tumor cell growth and proliferation. Next, we will focus on specific lncRNAs and miRNAs with strong evidence of nucleolar

localization, which are not derived from rDNA and have a function beyond ribosome assembly in physiology and tumor biology.

Nucleolar lncRNAs, their functional roles and association with cancer

lncRNAs, typically defined as noncoding RNA transcripts longer than 200 nt, encompass a broad spectrum of transcripts with different functions. The 5.2 release of LNCipedia, a human lncRNA database, contains 127,802 transcripts from 56,946 genes, of which 107,039 and 49,372 are considered high confidence entries, respectively [72]. The more recent 2.0 version of LncBook, a curated knowledge base of human lncRNAs, counts even larger numbers, highlighting a high-quality collection of 323,950 lncRNA transcripts and 95,243 human lncRNA genes [45].

Various lncRNAs transcribed from the rDNA, such as promoter-derived pRNAs processed from the primary IGS-rRNA transcript [73,74], pyrimidine-rich noncoding transcript (PNCTR) [75], promoter and pre-rRNA antisense lncRNA (PAPAS)

[76], IGS16, IGS22, and IGS28 [77], exert their function in the nucleolus, as reviewed extensively by Feng and Manley [16]. Other nucleolar lncRNAs important for ribosome biogenesis include LoNA [28], SLERT [38], circANRIL [66], *alu*RNAs [27], Erythrocyte membrane protein band 4.1 like 4A antisense 1 (EPB41L4A-AS1) [78], and distal junction transcripts (DISNOR 187, DISNOR 238) [5]. They are discussed in detail by Baserga and colleagues [17]. HOXB Cluster Antisense RNA 3 (HOXB-AS3) is an additional lncRNA that has been described to regulate rDNA transcription in acute myeloid leukemia [79]. Here, we provide an overview of other notable lncRNAs including LNC-SNO49AB, SRP RNA, LETN, Discn, and PHACTR2-As1 with strong experimental evidence for nucleolar localization provided by e.g. RNA imaging. In addition, the lncRNAs for which quantitative 45S-HyPro-Seq analysis [31] yielded >3-fold enrichment and for which cancer-related information was available in the RNADisease database are also listed in Table 1.

While sno-lncRNAs and SPAs have both been linked to regulating mRNA splicing, and the lack

Table 1. Nucleolar lncRNAs beyond ribosome biogenesis and their association with tumor biology.

Name	Nucleolar localization	Function	Role in cancer
LNC-SNO49AB	RNA imaging by CRISPR-Cas13 system in NB4 cells and subcellular fraction analysis in MV4-11 and NB4 human leukemia cell lines [44]	enhances dimerization of ADAR1 and thus A-to-I RNA editing	upregulated in leukemia, oncogenic [44]
SRP RNA (75L RNA)	RNA <i>in situ</i> hybridization and microinjection in rat NRK cells [32,80]	involved in the subcellular targeting of nascent polypeptides as subunit of the signal recognition particle	upregulated in different tumors, potential oncogenic role by downregulating p53 level [81]
LETN	RNA <i>in situ</i> hybridization, live cell imaging of MS2-tagged RNA in Huh-7 cells and subcellular fraction analysis in HCC827 and Huh-7 cells [34]	facilitates pentamerization of NPM1, thereby supporting increased nucleolar activity and chromatin reorganization	upregulated in different tumors, potential oncogenic role [34]
Discn	RNA <i>in situ</i> hybridization in mESC and MEF cells [35]	expressed upon genotoxic stress, retains NCL in the nucleolus, thereby increasing the free RPA pool	unknown, putative role in chemoresistance and genome stability [35]
PHACTR	RNA <i>in situ</i> hybridization in Hs578T cells [36]	expression is (down)-regulated by EZH2; when expressed, silences rDNA by recruiting SUV39H1	lower PHACTR2-AS1 expression promotes breast cancer development and correlates with poor patient outcome [36]
MIR663AHG	45S-HyPro-Seq [31] (Log2FC=2,23)	supposed to bind to miR663a and its precursor pre-miR663a, and prevent the degradation of miR663a target mRNAs [82]	downregulated in colon cancer [82] according to the RNADisease database: upregulated in esophageal cancer [83,84], downregulated in thyroid cancer [85], downregulated in glioblastoma [86]
lnc-GCNT3-3	45S-HyPro-Seq [31] (Log2FC=2,10)	not determined	according to the RNADisease database: downregulated in glioblastoma [86]
lnc-LHFPL2-3	45S-HyPro-Seq [31] (Log2FC=1,91)	not determined	according to the RNADisease database: upregulated in breast cancer [87]

of five and two of them, respectively, is associated with the genetic disorder Prader-Willi syndrome [88], only sno-lncRNA LNC-SNO49AB has been definitely localized and functionally assigned to the nucleolus. LNC-SNO49AB binds to the ADAR1 RNA-editing enzyme in the nucleolus and facilitates its dimerization, thereby promoting A-to-I RNA-editing activity. Importantly, its upregulation has been linked to tumorigenesis. Leukemia cells exhibit higher expression of LNC-SNO49AB compared to normal hematopoietic cells, as shown by analysis of samples from leukemia patients. Consistent with this, knockdown of LNC-SNO49AB potently suppresses leukemia progression *in vitro* and *in vivo*, as demonstrated in preclinical models. Collectively, these observations connect RNA editing with leukemogenesis and the biology of the nucleolus [44].

SRP RNA, also known as 7SL RNA, is the non-coding RNA subunit of the signal recognition particle [89]. The SRP RNP complex is known to act in the cytoplasm, where it mediates the targeting of nascent polypeptides at the endoplasmic reticulum to secretory or membrane pathways. However, endogenous SRP RNA was detected in the nucleolus by *in situ* hybridization [32]. Intriguingly, imaging analyses of microinjected fluorescent SRP RNA in rat NRK cells by microscopy revealed nucleolar localization of its *Alu*-containing portion, suggesting *Alu* sequence-dependent nucleolar localization during RNP assembly [80]. This result is consistent with the nucleolar localization of *alu*RNAs [27] and the proposed evolutionary origin of *Alu* sequences as processed 7SL RNA genes [90]. Interestingly, a potential oncogenic function of SRP RNA was suggested, as it is strongly upregulated in various cancers such as liver, lung, breast, and stomach. Further detailed molecular analyses revealed that SRP RNA competes with the RNA-binding protein HuR at the 3'-untranslated region (UTR) of TP53 mRNA, thereby downregulating translation of the tumor suppressor protein p53 [81]. Whether and how the cancer-related function of SRP RNA hinges on its nucleolar localization needs to be investigated in future studies.

The renaming of the lncRNA RP11-196G18.22 gene to LETN for lncRNA essential for tumor cell proliferation via NPM1 already indicates the

association of this lncRNA with tumor biology. LETN has been detected in the nucleoli of Huh-7 cells by RNA-FISH, live cell imaging of MS2-tagged LETN via MS2-GFP, as well as cell fractionation and RT-qPCR in this and the HCC827 lung adenocarcinoma cell line. Analysis of The Cancer Genome Atlas (TCGA) database has highlighted the upregulation of lncRNA levels across multiple tumor types, frequently associated with gene amplification, in particular in hepatocellular carcinoma. Molecular analyses demonstrated that binding of LETN facilitates pentamerization of the multifunctional nucleolar protein nucleophosmin (NPM1), thereby controlling nucleolar function and promoting tumor cell proliferation [34].

The Discn lncRNA localizes to nucleoli of mouse ESCs when its level increases upon genotoxic stress, as demonstrated by RNA-FISH. Ectopically expressed Discn also localizes within nucleoli of MEFs but remains unaffected by genotoxic stress. Functional studies showed that the Discn lncRNA interacts with the nucleolin (NCL) protein and sequesters it in the nucleolus by hampering its subcellular shuttling. This prevents the interaction between NCL and replication protein A (RPA) in the nucleoplasm, increasing the pool of free RPA, which is thus available to respond to DNA replication stress and mediate DNA repair. Accordingly, the Discn lncRNA safeguards genome stability, and it can be potentially linked to chemotherapy resistance in tumor cells [35].

The lncRNA PHACTR2-AS1 was recently described as a mediator of EZH2-dependent activation of rDNA in breast cancer. Nucleolar localization of PHACTR2-AS1 was detected in Hs578T cells by RNA-FISH, and chromatin isolation by RNA purification indicated binding to the rDNA promoter in PHACTR2-AS1-overexpressing MDA-MB-231 breast cancer cells. Further mechanism of action studies revealed that PHACTR2-AS1 recruits the histone methyltransferase SUV39H1 to the rDNA locus. This promotes H3K9 methylation-dependent silencing of rDNA and inhibits ribosome biosynthesis. Consistent with the *in vitro* observations, overexpression of PHACTR2-AS1 suppresses breast cancer growth and metastasis in *in vivo* mouse models. In contrast, EZH2-mediated, H3K27 methylation-dependent

downregulation of PHACTR2-AS1 expression leads to increased ribosome synthesis through activation of rRNA transcription and to genomic instability. These processes correlate with increased proliferation and metastatic capacity of invasive breast cancer cells. Accordingly, high PHACTR2-AS1 RNA levels in patients correlate with better overall survival, while low levels, commonly observed in breast cancer, predict poor outcomes [36].

Notably, 45S-HyPro-Seq analysis revealed frequent association of lncRNAs encoded by satellite repeats that belong to the BSR/Beta, CER, GSATII and HSAT5 families [31]. The nucleolar localization of at least some of the satellite RNAs was predictable by taking into account the frequent association of centromeric and pericentromeric satellite DNA repeats with the nucleolus and the role of specific satellite RNAs in RNP assembly at the nucleolus and centromere [8,91]. In-depth analysis of satellite RNAs, including cellular imaging, could contribute to a better understanding of the relationship between the various satellite RNA species and nucleolar structure and function. Interestingly, pronounced overexpression of satellite RNAs has been shown to be common in pancreatic cancer and other epithelial cancers, but their function and localization in nucleoli or possible consequences on centromere-nucleolus associations have not been studied in this context [92–94]. Other lncRNAs identified by 45S-HyPro-Seq have also been associated with cancer. These include MIR663AHG, lnc-GCNT3–3 and lnc-LHFPL2–3, whose connection to tumor biology is outlined in Table 1. Despite the high ranking of these lncRNAs in 45S-HyPro-Seq analysis, their nucleolar localization needs to be further validated by *in situ* hybridization, and the link between their nucleolar localization and cancer-related function awaits detailed exploration in future research.

Nucleolar miRNAs and a brief overview of their function and association with cancer

MiRNAs are ~ 22 nt short ncRNAs that bind directly to target mRNAs and thus mediate post-transcriptional repression. They are transcribed as precursor RNAs that are processed by the endoribonucleases Droscha and Dicer and, after

processing, assemble as double-stranded RNA with Argonaute (Ago) family proteins to form the RNA-induced silencing complex (RISC). In the final maturation stage of RNP one strand of the duplex RNA is ejected [95,96]. The number of mature human miRNAs is currently estimated to be 2300 [97], with 567 recognized as authentic miRNAs according to the manually curated MirGeneDB database (<https://mirgenedb.org>) [98]. Canonical miRNAs are transcribed by RNA polymerase II from miRNA genes as part of longer primary miRNA transcripts. However, noncanonical origins of miRNAs, including rDNA origins, have also been proposed based on bioinformatic analyses and high-throughput sequencing of cellular small RNAs or their Ago-associated fraction [99–101]. Regardless of their subcellular localization, several miRNAs, such as members of the hsa-let7 family, hsa-miR-10a, hsa-miR-130a, hsa-miR-145, hsa-miR-146a, hsa-miR-24, hsa-miR-369-3p, hsa-miR-424-5p, hsa-miR-504, hsa-miR-595, and hsa-miR-7641 have been identified as regulators of various steps in ribosome biogenesis, as described in detail by Baserga and colleagues [17]. Experimental evidence for nucleolar localization is available for additional miRNAs, based mainly on work in the laboratories of Pederson [18,20], Lam [26], and Laiho [24]. The proposed functions behind their nucleolar localization include the formation of miRNA-mRNA pairings and thus pre-silencing prior to the export to the cytoplasm; nucleolar sequestration, which can be perturbed by exogenous genetic material (e.g., during viral infection) but is not sensitive to various forms of metabolic stress; and the possible nucleolar deactivation of miRNA precursors through ADAR-mediated A-to-I editing [17,102]. Nucleolar miRNAs without assigned regulatory role in ribosome biogenesis and whose localization was confirmed by *in situ* hybridization are listed in Table 2. Because of the wide variety of cancers with which each of these RNAs has already been associated (at least about 20 for each) and the diversity of targets through which these miRNAs act, details of specific settings are not discussed here. We recommend to survey the RNADisease and HMDD v4.0 databases, and in the case of miR-206, we also refer to a detailed recent review

Table 2. miRNAs beyond ribosome biogenesis and with clear evidence for nucleolar localization.

Name	Nucleolar localization
miR-1	RNA <i>in situ</i> hybridization in rat L6 myoblasts [18]
miR-206	RNA <i>in situ</i> hybridization in rat L6 myoblasts [18]
miR-494	RNA <i>in situ</i> hybridization in rat L6 myoblasts [18] top-ranked by NCode array in MCF cells [24]
miR-191	RNA <i>in situ</i> hybridization and miRNA RT-qPCR in HeLa cells [26] NCode array in MCF cells [24]
miR-484	RNA <i>in situ</i> hybridization and miRNA RT-qPCR in HeLa cells [26]
miR-193b	RNA <i>in situ</i> hybridization and miRNA RT-qPCR in HeLa cells [26]
miR-574-3p	RNA <i>in situ</i> hybridization and miRNA RT-qPCR in HeLa cells [26]

article [103]. Similar to some of the nucleolar lncRNAs mentioned above, the relationship between the nucleolar localization of these miRNAs and their multiple functions in tumor biology remains to be elucidated.

Concluding remarks

The nucleolus stands as the paramount hub for non-coding RNA metabolism in the eukaryotic cell. Characterized by its elevated local concentrations of rRNA precursors and associated snoRNAs, RNA binding proteins and RNA enzymes, the nucleolus serves as the epicenter for ribosome biogenesis. However, this unique and dynamic nucleolar micro-environment is permissive and ideal to exert multiple cellular functions related to RNA metabolism that extend beyond ribosome biogenesis. By leveraging common factors, the nucleolus exemplifies the principles of evolutionary tinkering [104]. Co-localization and physical interactions enable co-dependencies and regulatory relationships between the cell's most energy-demanding process, ribosome biogenesis, and other vital RNA metabolic processes. At the same time, the intricate nucleolar microenvironment is hijacked under disease conditions such as tumor development, inflammation, or viral infection catering to pathologic needs, further highlighting the important role of the nucleolus in RNA homeostasis.

Nucleolar noncoding RNAs can connect nuclear organization and nucleolus biology in different ways. They can modulate nucleolar architecture and function either by regulating ribosome synthesis or by shaping perinucleolar heterochromatin formation. Alternatively, their altered distribution in the nucleus, driven by sequestration in the nucleolus can influence nuclear organization.

While the past years have brought remarkable findings, a deeper dive into the nucleolar non-ribosomal RNA is crucial for a more comprehensive understanding of the structural and functional organization of nucleolar RNA metabolism that extends beyond ribosome biogenesis. For cancer research the nucleolus offers an enticing and, yet largely unexplored, avenue for exploration. The aberrant expression and function of nucleolar noncoding RNAs in various malignancies suggest their potential as biomarkers or therapeutic targets. Understanding the molecular interplay within the nucleolus could unravel innovative strategies for diagnostic and therapeutic interventions in oncology. In addition to the critical role of ribosome biogenesis in cancer cell proliferation and survival, targeting the nucleolar RNA landscape may present new avenues for precision oncology. The emerging advances in sub-nuclear transcriptomics hold great promises to identify and quantify RNAs across varying conditions and to estimate localization dynamics. Harnessing these innovations, in tandem with other complementary techniques, will undeniably shed more light on the multifaceted role of the nucleolus beyond ribosome biogenesis as a specialized site of noncoding RNA homeostasis in health and disease and its implications in the ever-evolving landscape of cancer biology and therapy.

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Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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