The multifunctional nucleolus

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Abstract | The nucleolus is a distinct subnuclear compartment that was first observed more than 200 years ago. Nucleoli assemble around the tandemly repeated ribosomal DNA gene clusters and 28S, 18S and 5.8S ribosomal RNAs (rRNAs) are transcribed as a single precursor, which is processed and assembled with the 5S rRNA into ribosome subunits. Although the nucleolus is primarily associated with ribosome biogenesis, several lines of evidence now show that it has additional functions. Some of these functions, such as regulation of mitosis, cell-cycle progression and proliferation, many forms of stress response and biogenesis of multiple ribonucleoprotein particles, will be discussed, as will the relation of the nucleolus to human diseases.

Acrocentric chromosome
A chromosome with a centromere that is located near one end of the chromosome. Humans have five pairs of acrocentric chromosomes.

Small nucleolar ribonucleoproteins
Nucleolar RNA–protein complexes that function in pre-ribosomal RNA processing.

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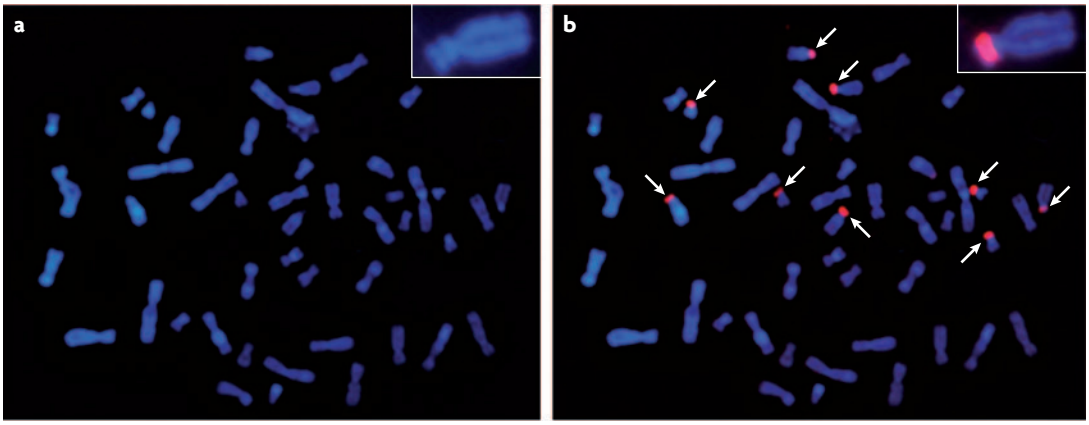
The primary function of the nucleolus is as the site of ribosome-subunit biogenesis in eukaryotic cells. Nucleoli form at the end of mitosis around the tandemly repeated clusters of ribosomal DNA (rDNA) genes and result in a subnuclear compartment that locally concentrates the transcription and processing machineries that are responsible for generating ribosome subunits. The process of assembling a ribosome subunit requires the initial transcription of rDNA genes by a specialized RNA polymerase — RNA polymerase I (RNA pol I). These genes are arranged in arrays of head-to-tail tandem repeats, termed nucleolar organizer regions (NORs), which are located on acrocentric chromosomes[1] (BOX 1). The initial 47S ribosomal RNA (rRNA) precursor transcript is subsequently cleaved to form the mature 28S, 18S and 5.8S rRNAs, post-transcriptionally modified through interaction with small nucleolar ribonucleoproteins (snoRNPs) and additional protein-processing factors and, finally, assembled with the many ribosomal proteins before interaction with the export machinery and transport to the cytoplasm (FIG. 1).

The complex processes that are involved in the formation of ribosome subunits occur in distinct subregions of the nucleolus, which can be distinguished by their morphology using electron microscopy (EM; FIG. 2d). Nucleoli are also clearly visible by light microscopy and can be specifically labelled for fluorescence microscopy (FIG. 2b), which has revealed that many nucleolar factors (depending on their functions) are localized to subregions of the nucleolus (FIG. 2b–f). These subregions are termed fibrillar centres (FCs), dense fibrillar components (DFCs) and granular components (GCs) (FIGS 1,2). Transcription of the rDNA repeats occurs largely at the border between the FC and DFC, with RNA pol I subunits being enriched in the FC region. The processing and modification of the pre-rRNA transcripts occurs largely in the DFC where the snoRNPs accumulate, whereas most proteins concentrate in the GC region where ribosome subunit assembly is completed (for a detailed review of the process of ribosome biogenesis, see REF. 2).

Until recently, our knowledge of the protein content of nucleoli has been limited. However, the ability to purify nucleoli on a large scale, combined with important advances in the identification and analysis of proteins using mass spectrometry, has now provided a wealth of information regarding the nucleolar proteome[3]. Several proteomic analyses have now been undertaken to characterize the nucleolar proteome in human and plant cells[3–6]. These studies have identified more than 200 plant proteins and over 700 human proteins that stably co-purify with isolated nucleoli. A comparison of nucleolar proteome data from humans and budding yeast showed that ~90% of human nucleolar proteins have clear yeast homologues[6]. This demonstrates that the nucleolar proteome is largely conserved through evolution.

Bibliographic and bioinformatic analyses of the proteomic data have allowed the classification of nucleolar proteins into functional groups and have suggested potential functions for ~150 previously uncharacterized human proteins[7–9] (FIG. 3). A classification of the molecular functions of these nucleolar proteins shows that approximately 30% have a function that is related to the production of ribosome subunits. However, the diverse identities and functions of many of the other nucleolar

Box 1 | **Nucleolar organizer regions**



Nucleoli are formed around nucleolar organizer regions (NORs), which are composed of clusters of ribosomal DNA (rDNA) repeat units. In humans, approximately 400 copies of 43-kb repeat units are distributed along all acrocentric chromosomes (chromosomes 13, 14, 15, 21 and 22) to form NORs. In many cell types, only a subset of NORs are transcriptionally active, although NORs are still assembled into nucleoli. Active NORs are associated with RNA polymerase I; these regions often stain positive for silver and are therefore called Ag-NORs. rDNA in these transcriptionally active NORs is ten times less condensed than surrounding chromosomal regions, and the active NORs form a feature called the secondary constriction. NORs that are active in one cell cycle are likely to become transcriptionally active again following mitosis. rDNA repeat units in inactive NORs are highly methylated and are not associated with the RNA polymerase I machinery. Fluorescence *in situ* hybridization (FISH) was carried out for rDNA repeats on the NORs of all acrocentric chromosomes in a metaphase spread of human lymphocytes from a male. Panel **a** shows that 4′,6-diamidino-2-phenylindole (DAPI) stains the DNA of all chromosomes (blue); the inset shows a DAPI-stained acrocentric chromosome. Panel **b** shows staining of the rDNA repeats on the acrocentric chromosomes by FISH using an 11.9-kb *Eco*RI fragment from the intergenic spacer of the rDNA repeat (red, indicated by arrows). An enlargement of the rDNA repeat units on an acrocentric chromosome is shown in the inset of panel **b**. For further information, see REFS 12,96,97.

proteins are consistent with additional processes that occur within the nucleolus. This includes many pre-mRNA processing factors and proteins that are involved in cell-cycle control as well as DNA replication and repair. A further dimension has been added to the analysis of the nucleolar proteome by recent studies that have characterized dynamic changes in the proteome of the nucleolus under different metabolic conditions, such as inhibition of transcription following treatment of cells with actinomycin D (REF. 6). The ability to analyse the parallel increases and decreases in the levels of many protein components quantitatively and in a high-throughput manner has highlighted just how dynamic the nucleolar proteome can be. It will be interesting in the future to see how the nucleolar proteome differs between cultured cell lines and primary cells.

Recent advances have uncovered a range of other cellular functions of the nucleolus in addition to its key role in ribosome-subunit biogenesis, and these are changing our understanding of this complex nuclear organelle. Here, we review the complex processes that take place within the nucleolus to coordinate ribosome synthesis and also discuss the importance of the nucleolus in relation to its role in mitosis, cell-cycle regulation and human diseases.

Nucleoli in mitosis

Consistent with the view that the nucleolus is a dynamic structure, the nucleolus is disassembled when cells enter mitosis and transcription shuts down (FIG. 4).

Prophase. At the onset of prophase, a rapid increase in the levels of cyclin B1–cyclin-dependent kinase-1 (CDK1) results in the phosphorylation of components of the rDNA-transcription machinery, including SL1 and transcription terminator factor-1 (TTF1)[10]. However, nascent transcripts are completed and remain associated with components from the DFC region during mitosis. So, hyperphosphorylation of components of the RNA pol I initiation complex might be the trigger for nucleolar disassembly at mitosis, which starts with the loss of RNA pol I subunit RPA39 from the FC region before breakdown of the nuclear envelope (NE)[11].

The RNA pol I transcription machinery remains associated with the rDNA repeats of the active NORs[12]. However, detailed live-cell fluorescence imaging of cells that were undergoing mitosis showed that some RNA pol I subunits, including RPA39, RPA16, RPA20 and RPA194, transiently dissociate from NORs during metaphase and reappear in anaphase, whereas other rRNA transcription factors, such as upstream binding factor (UBF) remain associated with the NORs throughout mitosis[11,13].

In contrast to the rDNA-transcription machinery, the rRNA-processing machinery does not remain associated with NORs during mitosis but, instead, is redistributed from NORs at prophase. Processing components leave the nucleolus, together with the partially processed pre-rRNAs that were associated with the DFC components after transcription was shut

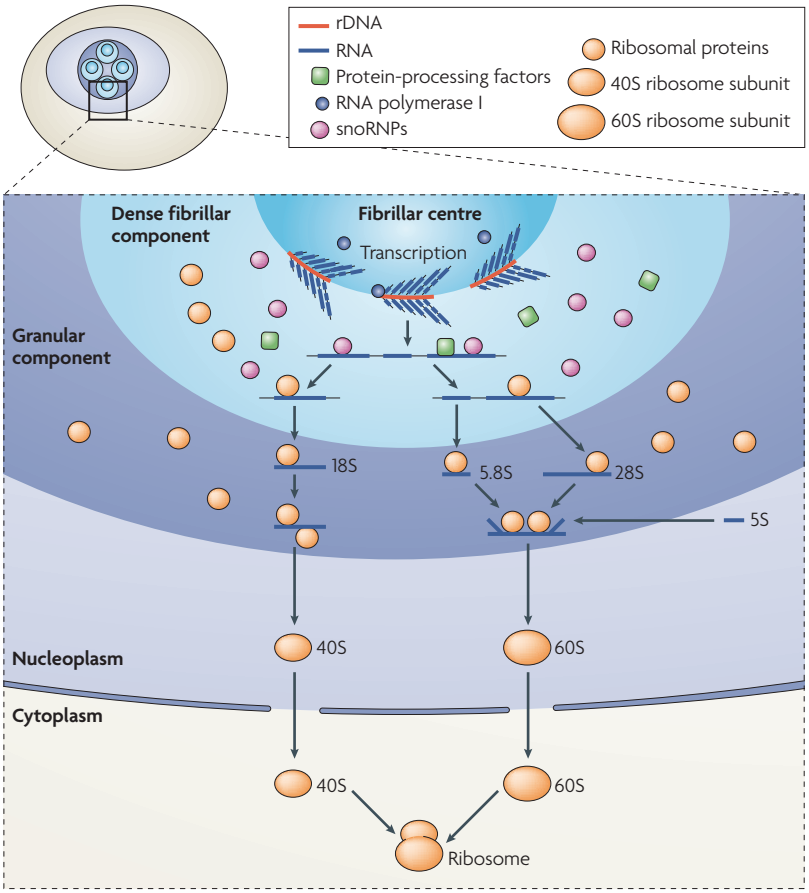


Figure 1 | **Model of ribosome biogenesis.** Transcription of ribosomal DNA (rDNA) by RNA polymerase I occurs either in the fibrillar centres (FCs) or at the boundary between the FC and the dense fibrillar component (DFC) region. The pre-ribosomal RNA transcripts are spliced and modified by small nucleolar ribonucleoproteins (snoRNPs) in the DFC. Final maturation of the pre-ribosomal ribonucleoprotein and assembly with ribosomal proteins occurs mostly in the granular component (GC) region. In the GC, the 5.8S and 28S ribosomal RNAs (rRNAs) assemble with the 5S rRNA transcript to form the 60S subunit, whereas the 18S rRNA alone assembles into the 40S ribosome subunit. The 40S and 60S ribosome subunits are both exported to the cytoplasm, where they bind to mRNA to form functional ribosomes.

down. Following the loss of some RNA pol I subunits from the FC, rRNA-processing components, such as fibrillarin and B23, simultaneously dissociate from the DFC and GC regions, respectively. Interestingly, there seems to be a correlation between the timing of the loss of fibrillarin from the DFC and the disintegration of the NE[11], which suggests that these events might be triggered by a similar mechanism.

Whereas many processing components are redistributed from the nucleolus to the cytoplasm at mitosis, others become attached to the surface of condensed chromosomes — the perichromosomal region (PR)[14]. The PR is a layer of irregular thickness that surrounds chromosomes, with the exception of the centromeres; this exclusion might be due to the high concentration of CENP proteins. The PR layer consists of homogeneously distributed rRNA-processing components, such as ribonucleoproteins (RNPs), small nucleolar RNA U3 (U3 snoRNA), pre-rRNA and fibrillarin, but also contains non-nucleolar proteins, such as phosphorylated nucleoplasmin[15]. The exact role of the PR is unknown but several functions have been proposed: first, the PR might function as an insulator that protects the chromosomes during mitosis by forming a barrier between chromosomes and the cytoplasm; second, the PR might function as a binding site for chromosomal passenger proteins; or third, the PR might help to ensure that there is an equal distribution of processing components between daughter cells because the PR-associated components will be dragged with the chromosomes to the respective daughter nuclei by the spindle apparatus.

Anaphase. In anaphase, most rRNA-processing components will either remain associated with chromosomes in the PR or become packaged in the cytoplasm into nucleolar-derived foci (NDF) in rapidly growing cells. NDF are highly mobile structures of 0.1–3 μm in diameter that number up to ~100 in each cell[16]. They contain early and late rRNA-processing proteins but also contain U3 snoRNAs and partially processed pre-rRNAs.

Late anaphase–early telophase. In late anaphase–early telophase, cyclin B1–CDK1 levels decrease, which results in the reactivation of rRNA transcription[17]. Subsequently, the NE starts to re-form and small particles dissociate from NDF in telophase and pass into the nucleus through the newly formed nuclear membrane. Consequently, the number of cytoplasmic NDF decreases until they completely disappear in early G1 phase[13]. Initially, most of the content of the former NDF is transferred to fibrogranular structures that form on the surface of chromosomes, known as pre-nucleolar bodies (PNBs)[18]. PNBs are found in both plant and animal cells, which indicates that they have a conserved and important function in the cell. The content of PNBs is similar to that of NDF as they both contain processing proteins, snoRNAs and partially processed pre-rRNAs. In parallel, the PR breaks down when the chromosomes start to decondense and most of its components are incorporated into the PNBs, whereas other processing components are initially distributed in the nucleoplasm (reviewed in REFS 15,19).

Originally, it was thought that PNBs moved to nucleoli to deliver their contents. However, Savino *et al.* showed that PNBs are immobile and that they instead release their processing components, which then move to the nucleolus[18]. However, as chromosomes start to decondense, PNBs can be brought into the vicinity of other PNBs or can be moved closer to nucleoli. When PNBs are in close proximity to each other and to nucleoli, they can deliver processing factors via 'bridges' that are formed by thin threads that can be visualized by electron microscopy[13,18]. Therefore, PNBs can function as transit sites for rRNA-processing factors and can locally concentrate proteins such as B23 and the nucleolar protein NOP52 that are involved in late rRNA-processing steps. These proteins seem to accumulate in the PNBs before they are recruited to nucleoli[20].

CENP proteins
Proteins that associate with the centromere, the region of a chromosome that is attached to the spindle during nuclear division.

Chromosomal passenger protein
A protein that shares a characteristic pattern of association with chromatin in prophase, centromeres in metaphase and early anaphase, and the midzone and midbody in late anaphase and telophase, respectively.

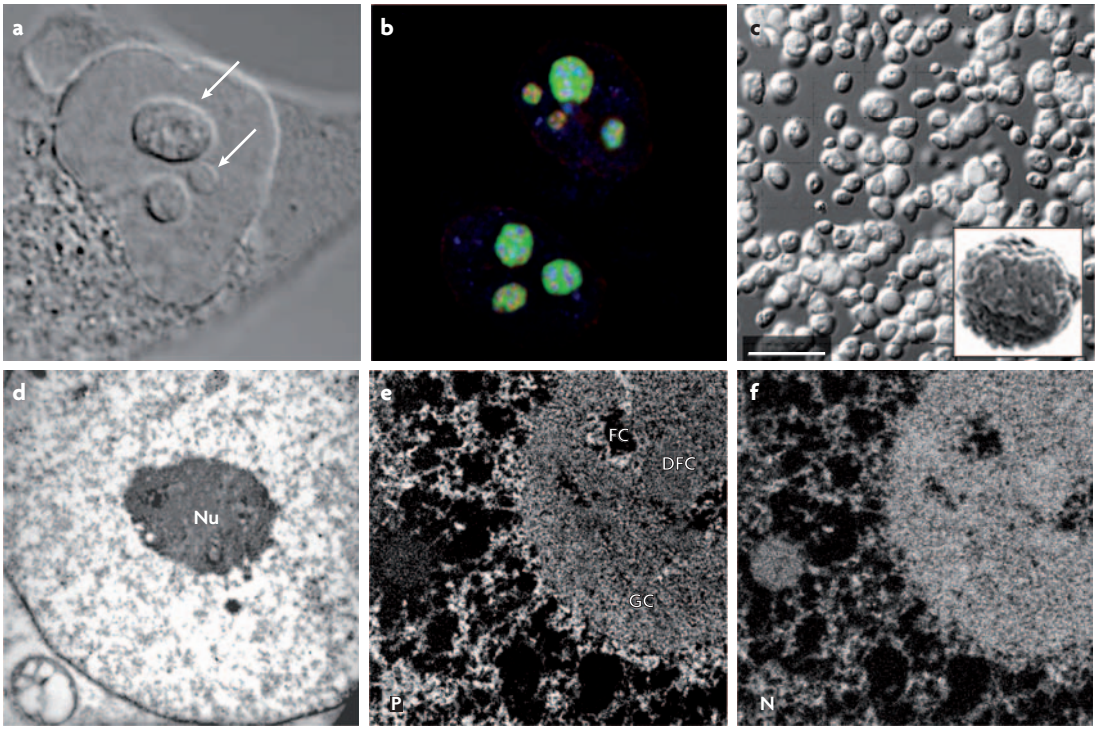


Figure 2 | **Visualization of the nucleolus.** Different imaging techniques can be used to identify distinct aspects of nucleolar morphology and composition. The fibrillar centre (FC), dense fibrillar component (DFC) and granular component (GC) regions can be individually visualized using transmission electron microscopy (EM) and specifically labelled by fluorescence microscopy using reporter proteins fused to fluorescent protein tags. The dense shell of heterochromatin that surrounds nucleoli can be identified by scanning EM of either intact cells or isolated nucleoli, or labelled by 4′,6-diamidino-2-phenylindole (DAPI) in the fluorescence microscope. **a** | Differential interference contrast (DIC) image of a HeLa cell showing prominent nucleoli within the nucleus (indicated by arrows). **b** | Immunofluorescence labelling of a HeLa cell with antibodies that are specific for proteins enriched in the GC (B23; shown in green), the DFC (fibrillarin; shown in red) or the FC (RNA polymerase I subunit RPA39; shown in blue). **c** | DIC image of nucleoli purified from HeLa cells. The inset shows a scanning EM image of a purified HeLa nucleolus. **d** | Uranyl-acetate-stained cell section showing a characteristic image of a nucleus with a nucleolus (Nu) imaged by transmission EM. **e**,**f** | Ultrastructural analysis of nucleoli by electron spectroscopic imaging (ESI). A nuclear region of interest that contains the nucleolus with phosphorus (**e**) and nitrogen (**f**) enriched images that reveal nucleic-acid-based and protein-based components, respectively.

Reassembly during G1 phase. Despite previous reports about different types of PNB with distinct lifetimes, Angelier *et al.* demonstrated that both early and late processing proteins pass through the same PNB before entering the nucleolus[20]. Processing components are subsequently released in a defined order. First, components such as fibrillarin, which have early roles in the pre-rRNA-processing pathway, are recruited to nucleoli, after which the DFC progressively expands in early G1 phase. The accumulation of fibrillarin closely correlates with the re-establishment of a functional NE, which supports the idea that these events might share a mechanistic link[11]. Proteins that are involved in later stages of the pre-rRNA-processing pathway are subsequently released from PNBs and move to the newly assembling nucleolus to form the GC[11,21]. The release of these proteins is thought to be regulated by an as-yet-uncharacterized cyclin-dependent kinase activity. Finally, with both the rRNA-transcription machinery and the rRNA-processing machinery reassembled on the rDNA, the NORs move together in the nucleoplasm and fusion of the new nucleoli results in the typical functional nucleoli that are seen during interphase.

In conclusion, nucleoli undergo cycles of disassembly and reassembly each time cells go through mitosis. This involves a complex and highly regulated series of events and stepwise pathways that are still not fully understood. It seems that rDNA-transcription and rRNA-processing events might be regulated independently during the cell cycle. For example, inhibition of cyclin B1–CDK1 activity *in vivo* results in a reinitiation of rDNA transcription but does not allow rRNA processing to occur[21]. It is possible that the events that surround the cycles of nucleolar disassembly and reassembly might be closely linked with parallel regulatory events that control the mitotic disassembly and reassembly of other nuclear structures, such as the nuclear lamina and NE, although the mechanisms that are involved remain to be established.

Nucleoli during interphase

Nucleoli respond to changes in cellular growth rate and metabolic activity, which indicates that they constantly receive and respond to signalling events. One important reason for the regulation of nucleolar activity is the crucial need for the cell to maintain a sufficient

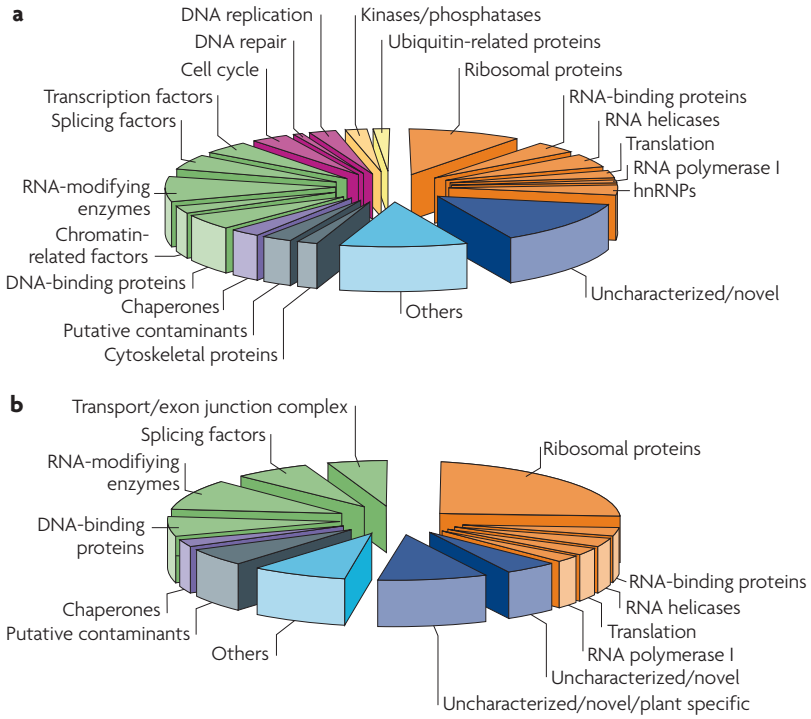


Figure 3 | **The nucleolar proteome.** Purification and mass-spectrometric identification of nucleolar proteins have led to the identification of 726 human nucleolar proteins (**a**) and 217 *Arabidopsis thaliana* nucleolar proteins (**b**). Proteins are clustered according to their molecular functions and colour-coded for similar and related functions. Orange represents proteins that are involved in the different aspects of ribosome biogenesis. Green represents proteins that function in RNA polymerase II transcription. Pink represents proteins that are involved in the cell cycle or DNA repair. Grey represents proteins that are either putative contaminants or known cytoskeletal proteins. Light blue represents other proteins that have not been reported as being nuclear or nucleolar. Dark blue represents previously uncharacterized proteins. hnRNP, heterogeneous nuclear ribonucleoprotein.

pool of ribosome subunits to support protein-synthesis levels during cell growth and division. Considering that the key role of the nucleolus is to ensure that the cell receives its essential supply of ribosomes, it is not surprising that its activity is tightly regulated. In addition, recent data indicate that nucleoli are also involved in coordinating and regulating cell-cycle-control events and stress responses[22].

Cell-cycle regulation. Although a fully functional nucleolus is reassembled during G1 phase, the structure and function of nucleoli remain dynamic throughout interphase. Various proteins have been shown to associate with the nucleolus specifically at different stages of the cell cycle, which suggests a role for nucleoli in regulating specific aspects of cell-cycle progression (FIG. 5).

Post-translational modifications are perhaps the most dynamic changes that are observed throughout the cell cycle and these modifications can regulate a plethora of activities. Some of these protein modifications, including sumoylation and phosphorylation, are regulated by the nucleolus. Conjugation of small protein chains, such as the small ubiquitin-like modifier (SUMO) protein[23], is a dynamic and reversible process, and several SUMO-specific proteases that remove SUMO chains from protein substrates have been described[24]. A new SUMO-specific protease, SENP5, was recently identified and found to be predominantly localized in the nucleolus[25]. Knockdown of *SENP5* by RNA interference causes defects in cell division and aberrant nuclear morphology[26]. This suggests a role for the nucleolus in regulating sumoylation of proteins that affect progression through cell division.

Reversible protein phosphorylation is the main post-translational regulatory mechanism that controls key events during the cell cycle. There is now evidence that links the nucleolus with the regulation of cell-cycle proteins by phosphorylation. In yeast, for example, Cdc14 is a protein phosphatase that is crucial for promoting exit from mitosis by dephosphorylating the activator of mitotic cyclin degradation Cdh1 (also known as Hct1), thereby activating mitotic cyclin-dependent kinases[27]. Cdc14 is sequestered in the nucleolus in an inactive state during interphase by the anchoring protein Net1 until the onset of anaphase, thus preventing an uncoordinated mitotic exit[28]. Upon entry into anaphase, the Cdc14 early anaphase release (FEAR) network initiates the release of active Cdc14 from the nucleolus through a mechanism that involves the phosphorylation of Net1 (REFS 29,30). Cdc14 controls the dissolution of cohesion-independent chromosome linkages at repeated DNA sequences and is necessary for the completion of chromosome segregation[30].

An example in humans involves protein phosphatase 1 (PP1), a ubiquitous serine–threonine phosphatase that regulates many cellular processes, including cell division[31]. The three PP1 isoforms, PP1α, PP1β/δ and PP1γ, are active phosphatases that have distinct localization patterns[32,33]. A pool of the PP1γ isoform accumulates in nucleoli during interphase but, on entry into mitosis, it becomes diffusely localized in the cytoplasm, with some PP1γ concentrated also at kinetochores where it exchanges rapidly with the diffuse cytoplasmic pool[34]. Subsequently, PP1γ is rapidly relocalized from the diffuse cytoplasmic pool to chromosomes at the onset of anaphase, a process that is mediated by the PP1γ-specific binding subunit, Repo-Man. This relocalization is probably important for controlling chromosome condensation[35]. A large pool of PP1γ then remains associated with chromatin throughout the following interphase and accumulates again within nucleoli. The regulated distribution of PP1γ through the cell cycle implicates the nucleolus in possibly contributing to the regulation of chromosome segregation and cytokinesis[36]. These examples suggest that the nucleolus has an important role in regulating the state of phosphorylation of key factors that are responsible for cell-cycle progression.

The sequestration of specific proteins in the nucleolus is another mechanism used by this subcompartment to regulate specific activities during the cell cycle. For example, telomerase reverse transcriptase, the RNP enzyme that adds telomeric nucleotide-repeat sequences to the ends of chromosomes, remains sequestered in nucleoli until the telomeres are replicated at late stages of S phase[37]. The nucleolar sequestration of telomerase

Cdc14 early anaphase release (FEAR) network
A signalling network in which the role for the protein phosphatase Cdc14 is key in the coordination of the multiple events that occur during anaphase, such as partitioning of the DNA, regulation of spindle stability, activation of microtubule forces and initiation of mitotic exit.

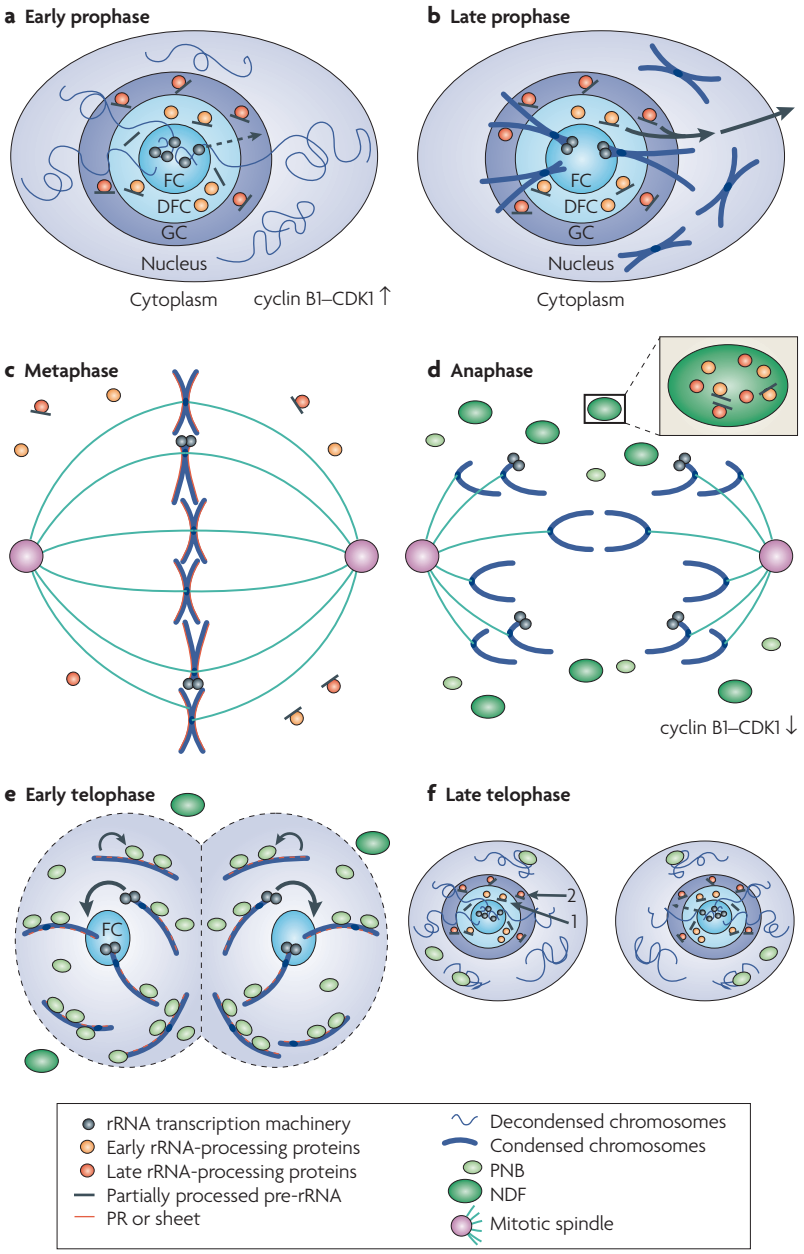


Figure 4 | **Nucleolar disassembly and reassembly during cell division. a** | During early prophase, cyclin B1–CDK1 levels increase and chromosomes start to decondense, whereupon they can be seen as thin threads in the nucleus. Although the transcription machinery usually remains attached to active nucleolar organizer regions (NORs) during mitosis, some RNA polymerase I subunits either leave the fibrillar centre (FC), or their concentration becomes too low for detection. **b** | In late prophase, when the chromosomes become more condensed, early and late processing factors and partially processed pre-ribosomal RNAs (pre-rRNAs) leave the nucleolus at the same time. **c** | In metaphase, the majority of processing components are associated with the surface of chromosomes as a perichromosomal region (PR). **d** | During anaphase, cytoplasmic processing components become packaged in nucleolar-derived foci (NDF), whereas the other components remain around the condensed chromosomes. In late anaphase, cyclin B1–CDK1 levels decrease. **e** | In early telophase, the number of NDF decreases and prenucleolar bodies (PNBs) are formed on the surface of each chromosome. The PR breaks down (indicated by an interrupted line) and processing components are taken up by PNBs. Nucleoli start to re-form around NORs of acrocentric chromosomes. **f** | Finally, in late telophase, the nuclear envelope is re-formed and early (1) and late (2) processing components relocate in an ordered manner to the dense fibrillar component (DFC) and granular component (GC), respectively. CDK1, cyclin-dependent kinase-1; rRNA, ribosomal RNA.

is reportedly mediated by its binding to the nucleolar protein nucleolin[38]. Therefore, the regulated release from this interaction might ensure the appropriate timing of telomerase activity during DNA replication. Importantly, the cell-cycle-dependent nucleolar localization of telomerase is not detected in either transformed cells or in cells that have experienced DNA damage, which implies that the loss of telomerase sequestration in the nucleolus might be an important diagnostic indicator of abnormal cells[37].

Stress response. An important, recently described example of the role of the nucleolus in regulating aspects of stress responses and cell-cycle arrest concerns the tumour-suppressor protein p53 (FIG. 6). Under normal conditions, p53 is a short-lived protein that is present in cells at a barely detectable level. On exposure of cells to various forms of exogenous stress (such as DNA damage, heat shock, hypoxia and so on) p53 becomes stabilized and is then responsible for an ensuing cascade of events, which results in either cell-cycle arrest or apoptosis. The accumulation of p53 in the cell induces the p21-mediated inhibition of cyclin D1–CDK4 and cyclin E1–CDK2, which results in cell-cycle arrest in G1 phase. The stability of the p53 protein in mammals is primarily regulated in non-transformed cells by the interplay of two proteins, HDM2 and p14ARF in humans (MDM2 and p19ARF in mice, respectively)[39]. HDM2 functions as a specific E3 ubiquitin ligase for p53, which results in a low level of p53 due to proteasome-mediated degradation of ubiquitin-conjugated p53 in the cytoplasm. Various stimuli, including stress pathways and oncogenic signals, increase the expression of p14ARF, which then associates with HDM2 to inhibit the ubiquitylation, nuclear export and subsequent degradation of p53. It has been proposed that p14ARF physically sequesters HDM2 in nucleoli, thereby relieving nucleoplasmic p53 from HDM2-mediated degradation[40]. p14ARF is predominantly a nucleolar protein and might also regulate ribosome biogenesis by retarding the processing of early 47S–45S and 32S rRNA precursors, perhaps through interaction with B23 (REF. 41). Exposure of cells to stress such as DNA damage, heat shock and aberrant ribosome biogenesis results in an increase in p53 and cell-cycle arrest. So, the nucleolus acts as a sensor for cellular stress signals through p53 stabilization.

There is also evidence that the nucleolus might have other roles as a coordinator of cellular stress responses[42]. For example, the regulation of ribosome-subunit biogenesis through the control of rRNA production is also an important aspect of the cellular response to many stimuli. Downregulation of rDNA transcription following stress has recently been shown to be regulated by the transcription factor TIF-IA[43]. Following exposure of cells to stress, TIF-IA is phosphorylated by c-Jun N-terminal kinase-2 (JNK2), which prevents the interaction of TIF-IA with RNA pol I, thus inhibiting transcription of rDNA. TIF-IA was also found to relocalize from the nucleolus to the nucleoplasm in response to stress. These findings suggest a mechanism whereby cells react to different forms of stress by inhibiting rDNA transcription.

The nucleolus and human diseases

The nucleolus has been linked to multiple forms of human disease, which are likely to involve a range of different mechanisms. For example, multiple genetic disorders have been mapped to human genes that encode nuclear proteins that are known to associate with nucleoli under specific conditions, including Werner syndrome and Bloom syndrome[44,45] but also Treacher Collins syndrome[46], dyskeratosis congenita syndrome[47] and, more recently, Rothmund–Thomson syndrome (RTS)[48]. In other cases, forms of cancer and viral infections seem to affect nucleolar structure or the biogenesis of ribosomes.

Cancer predisposition and genomic instability. Werner syndrome, Bloom syndrome and RTS result from mutations in a gene that encodes a specific member of the RECQ class of DNA helicases; that is, the WRN, BLM and RECQL4 DNA helicases, respectively[49]. The expression levels and nuclear localization of *WRN* and *BLM* vary during interphase. Interestingly, these proteins colocalize with promyelocytic leukaemia protein (PML) nuclear bodies in the nucleoplasm. However, specifically during S phase, both proteins relocalize and accumulate in the nucleolus[44,50]. These observations support the hypothesis that the WRN and BLM helicases are involved in temporally regulated, DNA-related events during the cell cycle[49].

The wild-type version of the RECQL4 protein has also been found to accumulate in the nucleolus, but specifically after oxidative stress[48]. In fibroblasts isolated from patients with RTS, the RECQL4 protein is absent and the cells show sensitivity to oxidants[51]. Proteomic studies have shown that many helicase family proteins can localize in nucleoli, some of which accumulate at certain cell-cycle stages or in response to specific stimuli[3]. It is interesting that multiple helicases that are found in the nucleolus give rise to human genetic disorders when mutated. Although other non-nucleolar functions are likely to be affected, the nucleolar localization of these helicases suggests that specific functions of DNA or RNA processing that occur in nucleoli are important for normal cell growth. Predisposition to forms of cancer and chromosome instability seem to be common features of these diseases, which suggests that these helicases might have a protective role in maintaining genomic integrity or in resisting external stress.

Another protein of medical importance that is linked with the nucleolus is parathyroid-hormone-related protein (PTHrP). Humoral hypercalcaemia of malignancy is a common complication of lung and certain other cancers. PTHrP was first discovered as the hypercalcaemia factor that is produced by solid tumours[52]. Its N terminus is homologous to that of parathyroid hormone (PTH), which enables PTHrP to share the same receptor and carry out similar biological functions to PTH. Recent findings have shown that PTHrP localizes to the nucleus and the nucleolus in certain tissues, such as skin, cartilage and bone, and that this localization is cell-cycle regulated and involves the nuclear import receptor importin-β1 (REFS 53,54). Levels of *PTHrP* mRNA increase in response to mitogenic factors specifically when cells are in G1 phase and this corresponds to the G1-specific accumulation of PTHrP in the nucleolus. This accumulation was shown to be negatively regulated by the cyclin-dependent kinases CDC2 and CDK2 (REF. 55).

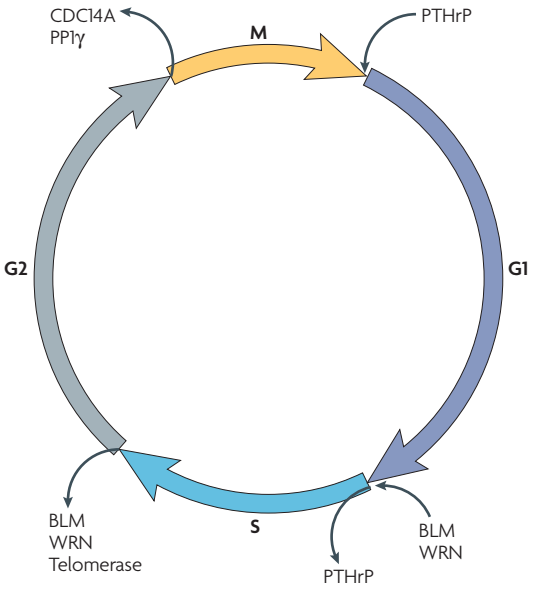


Figure 5 | **Roles of nucleoli in the cell cycle.** Several proteins have been shown to accumulate in the nucleolus at specific times during cell growth and division. The diagram shows proteins that are enriched in the nucleolus at different stages during interphase. As cells enter G1 phase, levels of parathyroid-hormone-related protein (PTHrP) mRNA increase in response to mitogenic factors specifically and this corresponds to the G1-phase-specific accumulation of PTHrP in the nucleolus. Many proteins have been found to be specifically associated with the nucleolus during DNA replication. Telomerase reverse transcriptase, the ribonucleoprotein (RNP) enzyme that adds telomeric nucleotide repeat sequences to the ends of chromosomes, remains sequestered in nucleoli until the telomeres are replicated at late stages of S phase. Several DNA helicases such as BLM (Bloom syndrome) and WRN (Werner syndrome) relocalize and accumulate in the nucleolus specifically during S phase. A pool of the protein phosphatase-1γ (PP1γ) isoform accumulates in nucleoli during interphase but, on entry into mitosis, it becomes diffusely localized in the cytoplasm with some PP1γ that is concentrated at kinetochores, where it exchanges rapidly with the diffuse cytoplasmic pool. Also during mitosis, upon entry into anaphase, the Cdc14 early anaphase release (FEAR) network initiates the release of active CDC14A from the nucleolus through a mechanism that involves the phosphorylation of NET1.

Diseases linked with aberrant ribosome biogenesis. Mutations in genes other than those that encode DNA helicases also link the nucleolus to disease pathogenesis, as shown by Diamond–Blackfan anaemia, which results from mutations in the gene that encodes ribosomal protein RPS19. This seems to cause defects that affect 18S rRNA maturation and 40S-ribosome-subunit production[56], which indicates that impairment of functions in the nucleolus is responsible for the disease phenotype.

Werner syndrome
A rare autosomal recessive disorder, characterized by the early development of various age-related diseases. The gene that is responsible for Werner syndrome (*WRN*) encodes a DNA helicase that is homologous to *Escherichia coli* RecQ.

Bloom syndrome
An autosomal recessive disorder that is characterized by growth deficiency, unusual facial features, sun sensitivity, telangiectatic erythema, immunodeficiency and a predisposition to cancer. *BLM*, the gene that is mutated in Bloom syndrome, encodes a DNA helicase of the RECQ family.

Rothmund–Thomson syndrome
(RTS). Patients exhibit chromosome fragility, skin and skeletal defects, cataracts and an increased predisposition to osteosarcoma. Some cases of RTS are caused by mutations in the DNA helicase gene *RECQL4*.

Promyelocytic leukaemia nuclear body
A round nuclear structure that contains several proteins, including the promyelocytic leukaemia protein (PML). It is thought to be the site of recruitment of various proteins and might also have a role in gene transcription.

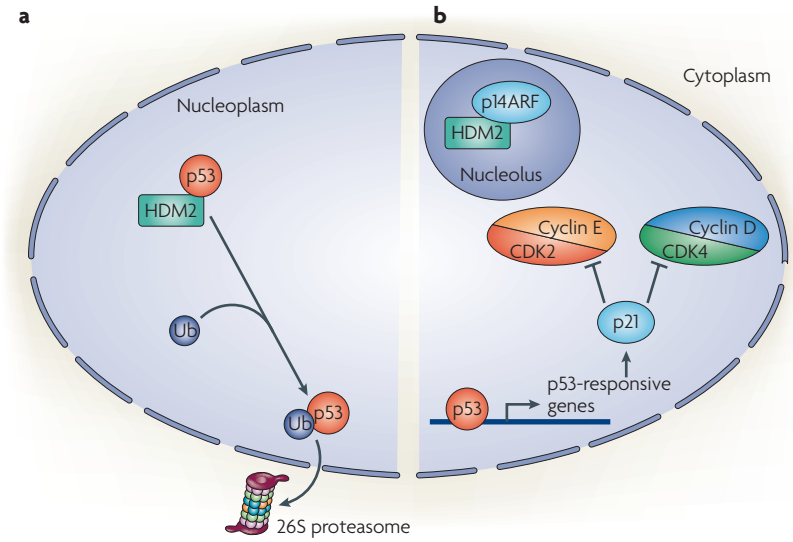


Figure 6 | **p53 in the nucleolus. a** | Under normal conditions, p53 is a short-lived protein that barely reaches a detectable level as a result of rapid degradation. HDM2 functions as an E3 ubiquitin ligase, resulting in rapid cytoplasmic export and degradation of p53 by the 26S proteasome. **b** | The p14ARF tumour suppressor induces the p53 pathway in response to oncogene activation or DNA damage. p14ARF is predominantly nucleolar and engages in several interactions with nucleolar proteins, whereas p53 is nucleoplasmic. Following upregulation by oncogenic signals, p14ARF associates with HDM2 and sequesters it within the nucleolus. This segregation of p53 from HDM2 prevents the ubiquitylation, nuclear export and subsequent degradation of p53. CDK, cyclin-dependent kinase.

The disease is associated with various malformations and is characterized by a severe reduction in erythroid precursors. It is surprising that a mutation in a ubiquitously expressed nucleolar protein such as RPS19 results in a cell-type-specific defect. Nonetheless, similar examples of this phenomenon have been described for other nucleolar proteins such as dyskerin, which is encoded by the gene that is mutated in dyskeratosis congenita. Patients suffering from dyskeratosis congenita are characterized by reticulated skin hyperpigmentation, nail dystrophy and oral leukoplakia.

Dyskerin has two separate functions in the nucleolus: pseudouridylation of rRNA[57,58] and stabilization of the telomerase RNA component, which is necessary for telomerase activity[59]. In both normal and transformed cells, dyskerin protein is predominantly nucleolar. However, in the cases of specific tumours that arise in patients with dyskeratosis congenita, levels of dyskerin expression are reduced and this correlates with abnormally low levels of rRNA pseudouridylation[60]. This suggests that rRNA modification catalysed in the nucleolus is crucial for its function and, therefore, is a cause of disease when it is disrupted or reduced. This view is further supported by data from a mouse model that indicate a role for dyskerin as a tumour suppressor[61]. In addition to dyskerin, several other proto-oncogenes and tumour-suppressor proteins affect the production of ribosomes[62]. For example, the c-Myc protein seems to drive cell growth and tumorigenesis[63] and results in enhanced rRNA synthesis[64]. By contrast, B23 has been reported to have both oncogenic and tumour-suppressive functions[41,65]. Mutation of *B23* is implicated in haematological cancers[66,67], whereas deletion of the chromosome region (5q) that contains the gene encoding B23 is a common aberration in *de novo* human myelodysplastic syndromes (MDS)[66].

Considering that a common feature of cancer cells is their high growth rates and consequent requirement for high levels of ribosome-subunit production to support the increased protein-synthesis levels, it is perhaps not surprising that various forms of oncogenic transformations result in changes in nucleolar structure and function. For example, in certain types of breast cancer, Cajal bodies (CBs) are found in nucleoli rather than in the nucleoplasm or at the nucleolar periphery[68]. The frequent linkage between cancer and nucleolar alterations has diagnostic utility. This is demonstrated by the use of nucleolar staining to detect prostate adenocarcinoma[69] and also seems to be useful in distinguishing benign from malignant salivary-gland tumours[70].

Viral infections. Many viruses, including plant and animal viruses, target nucleolar functions as part of their infectious strategy. Changes in the nucleolar morphology and proteome also occur as a result of viral infection[71]. Many virally encoded proteins are detected in the nucleolus of animal and plant cells[72–74]. This localization can be mediated either through nucleolar targeting signal (NOS) sequences on viral proteins[75], or through the interaction of the viral proteins with endogenous cellular nucleolar proteins, such as B23. A portion of the adeno-associated virus protein Rep is associated with B23 in the nucleolus[76]. The Rev protein of human immunodeficiency virus (HIV) and the Rex protein of human T-lymphotrophic virus (HTLV-I) also depend on an interaction with B23 for localization in the nucleolus[77]. The potential importance of such viral nucleolar localization interactions is exemplified by the recent discovery of the inhibition of HIV replication through the expression of a nucleolar localizing Rev element, U16RBE[78].

Neurodegenerative disorders. Some recent studies have established a link between the nucleolus and certain human neurodegenerative diseases. For example, in Alzheimer's disease, a change has been detected in the activity of NORs compared with healthy patients, thus suggesting a reduction in the expression levels of rRNA genes[79]. Aberrant forms of proteins are implicated in human neurodegenerative diseases such as Huntington's disease and spinocerebellar ataxias, which are typified by expansions of amino-acid triplet repeats that result in the formation of insoluble polyQ aggregates[80]. Aggregates typically form in the cytoplasm but are visible in the nucleus or in the nucleolus through interactions between polyQ tracts and nucleolin[81].

Biogenesis of RNPs other than rRNPs

Whereas the nucleolus is known to have a major role in coordinating the processing and maturation of rRNAs, there are now several lines of evidence that demonstrate that the nucleolus is also involved in the processing and maturation of other classes of cellular RNA. For example, the nucleolus has been suggested as a site of covalent

Cajal body
A round nuclear structure that contains several proteins, including coilin and survival of motor neuron (SMN1). It is thought to be the site of small nuclear ribonucleoprotein assembly and small nuclear RNA maturation.

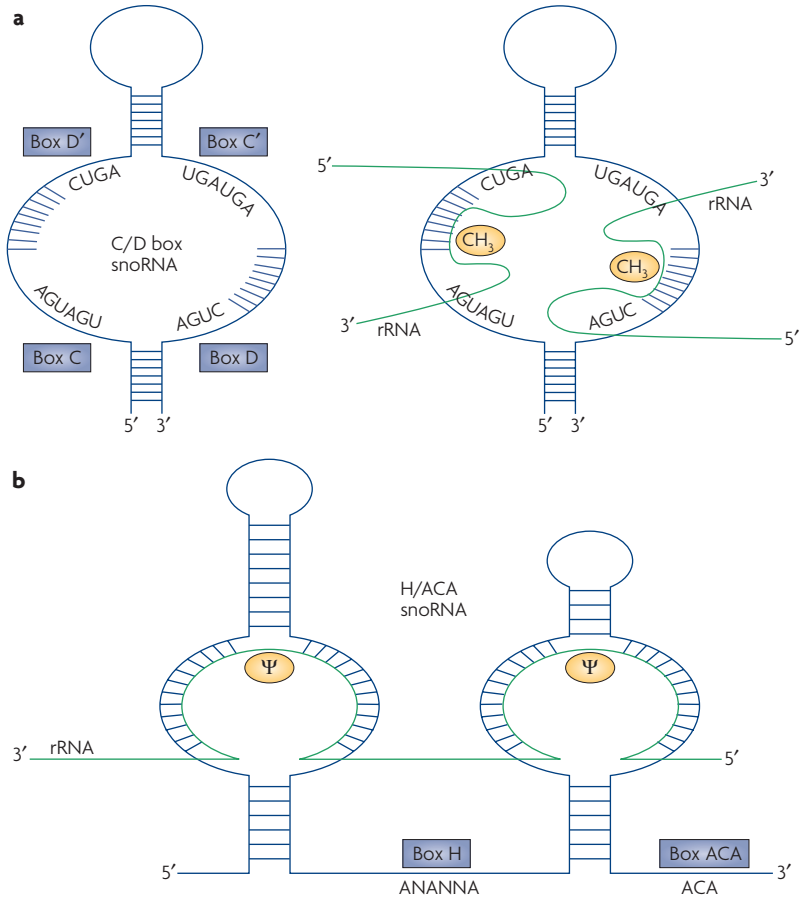


Figure 7 | **RNA modifications in the nucleolus.** In eukaryotes, the site-specific formation of the two prevalent types of modified ribosomal RNA (rRNA) nucleotides, 2′-*O*-ribose methylation and pseudouridylation, is directed by two large families of small nucleolar RNAs (snoRNAs). These are termed C/D box and H/ACA box snoRNAs, respectively, and exert their function through the formation of a canonical 'guide RNA' duplex at the modification site. These boxes are regions of complementary sequence to rRNA target sites that determine the sequence to be modified. **a** | 2′-*O*-ribose methylation is thought to be catalysed by C/D box small nucleolar ribonucleoproteins (snoRNPs). **b** | The site of rRNA pseudouridylation (Ψ) is determined by H and ACA box snoRNAs. The subsequent modification is carried out by the proteins that are part of the snoRNP complex.

RNA modifications and protein assembly for multiple RNP complexes, such as the spliceosomal small nuclear (sn)RNPs, telomerase and several other small RNAs that are transcribed by RNA pol III, such as 5S rRNA, some tRNAs, RNAse P RNA, the signal recognition particle (SRP) RNA and now also microRNAs (miRNAs)[82].

Covalent RNA modifications. The most common covalent modifications found in rRNA are 2′-*O*-ribose methylation and pseudouridylation. Both of these modifications are catalysed in the nucleolus by snoRNPs that act on the pre-rRNA substrate (reviewed in REF. 83). The snoRNAs function as 'guide RNAs'; that is, they have regions of complementary sequence to rRNA target sites that determine the sequence to be modified. The first type of post-transcriptional modification of rRNA is 2′-*O*-ribose methylation. It is produced by snoRNAs that have a conserved motif termed a 'C/D box', which contains the UGAUGA sequence (box C) and the CUGA sequence (box D) near the 5′ and 3′ ends, respectively. Some of those snoRNAs have additional conserved sequences, called boxes C′ and D′, in their centre. A complementary nucleotide sequence near box D targets the rRNA (FIG. 7a) and directs methylation five nucleotides upstream of the D/D′ box. Whereas the snoRNA sequence determines the site of modification, it is likely that the methyl-transferase activity is mediated by a snoRNP. A strong candidate for the methyltransferase is the highly conserved snoRNP protein fibrillarin because its crystal structure shows resemblance to an *S*-adenosyl-L-methionine binding domain, which is typically found in methyltransferases[84]. However, specific methyltransferase activity has yet to be confirmed for fibrillarin *in vitro*.

The pseudouridylation of rRNA is also catalysed by a distinct class of nucleolar snoRNPs and involves a 'guide RNA' targeting mechanism. In this case, the snoRNPs are characterized by a type of snoRNA that has a secondary structure defined by a 'hairpin–hinge–hairpin–tail' with two short conserved sequences called boxes H and ACA. One or both hairpins have an internal loop with two short sequences that are complementary to the rRNA substrate (FIG. 7b). The space between these sequences is called the pseudouridylation pocket. Whereas the snoRNA component dictates the site of pseudouridine formation, it is again a snoRNP that exhibits the pseudouridine synthase activity. Interestingly, the enzyme responsible is dyskerin, which is the same protein that, when mutated, causes the human genetic disorder dyskeratosis congenita[85,86].

Processing of other RNAs. The SRP complex consists of six proteins and an RNA of ~300 nucleotides[87]. Recent studies have shown that both the RNA and the proteins from the SRP transit through the nucleolus of mammalian cells before SRP export to the cytoplasm[88]. These results indicate a possible function for the nucleolus in the assembly and processing of the SRP complex and a potential association with newly formed ribosomes before their cytoplasmic export.

The RNase P RNA, which is a component of the pre-tRNA-processing enzyme RNase P, has been found in both the nucleolus and the nucleoplasm[89], which led the authors to suggest that some pre-tRNA processing occurs in the nucleolus. An alternative, although not mutually exclusive, possibility is that the nucleolus has a role in the assembly of RNase P. It was also shown that the nucleolus contains all the *trans*-acting factors that are responsible for the accurate and efficient synthesis of the eight 2′-*O*-methylated nucleotides and three pseudouridine residues that are carried by the mammalian U6 spliceosomal snRNA[90]. These results suggest a trafficking pathway in which the U6 spliceosomal RNA cycles through the nucleolus to undergo nucleolar RNA-directed processing. Interestingly, tRNA, RNase P RNA and U6 spliceosomal snRNA are all transcribed outside the nucleolus by RNA pol III and subsequently imported into the nucleolus, similar to 5S rRNA. This suggests that a possible common localization mechanism is shared by RNA pol III transcripts that are matured in the nucleolus.

Small nuclear RNPs
Nuclear RNA–protein complexes that combine with pre-mRNA and various proteins to form the spliceosomes.

Signal recognition particle
A ribonucleoprotein complex that is responsible for the recognition of the N-terminal signal-peptide sequence on nascent proteins and for the proper targeting of proteins onto a receptor on the cytoplasmic face of the endoplasmic reticulum.

ADAR1 and ADAR2 are editing enzymes that deaminate adenosine to inosine in long double-stranded RNA duplexes and specific pre-mRNA transcripts. Photobleaching experiments demonstrate that, in live cells, ADAR1 and ADAR2 are in constant flux in and out of the nucleolus[91]. Furthermore, it was shown that ADAR2-mediated, but not ADAR1-mediated, RNA editing occurs in the nucleolus, which indicates a role for the nucleolus in the regulation of RNA editing[92].

Recently, evidence has started to emerge concerning a role for the nucleolus in the small interfering RNA (siRNA) pathway. The finding that many of the proteins that are involved in siRNA processing — including RDR2, DCL3, AGO4 and NRPD1b (the largest subunit of RNA pol IVb) — colocalize with siRNAs in the nucleolus in plant cells suggests that processing of endogenous nuclear siRNAs, and possibly RNA-induced silencing complex (RISC) storage or sequestration, occurs within the nucleolus[93,94]. It was also reported that an miRNA (*miR-206*) colocalizes with 28S rRNA in the granular component of the nucleolus in mammalian cells, which implies that this miRNA associates with the ribosome subunits at an early stage[95]. It will be interesting to determine whether multiple forms of miRNAs arise in the nucleolus and whether they function in nucleolar processes or leave the nucleolus to regulate downstream cellular events such as protein translation.

Concluding remarks

Nucleoli are highly conserved features of eukaryotic cells that have a key role as the sites of ribosome-subunit production. However, multiple lines of investigation, including recent large-scale proteomic studies, have confirmed and characterized additional roles for nucleoli in important cellular processes beyond ribosome-subunit synthesis, including cell-cycle control, stress responses and coordination of the biogenesis of other classes of functional RNPs. Recent studies have also highlighted the dynamic nature of nucleoli and demonstrated that their composition can vary dramatically under different cellular conditions. These data underline the importance of studying the structure and function of subcellular organelles under a range of growth conditions and cell-cycle stages to evaluate their biological roles fully. Considering the large number of nucleolar proteins identified in proteomic analyses that are encoded by as-yet-uncharacterized open reading frames, it is also likely that further functions of nucleoli will be uncovered in the future.

Although the presence of a nucleolus might not be absolutely essential for the assembly of ribosome subunits, the fact that apparently all eukaryotic cells use a nucleolus to coordinate the complex events associated with transcribing rRNA, processing and modifying the rRNA transcript and assembling the large and small ribosome subunits from rRNAs and ribosomal proteins indicates that the nucleolus provides a major gain in efficiency that is of clear evolutionary advantage. At least in part, this advantage might arise from the nucleolar environment, which provides a high local concentration of related activities and also excludes other nuclear factors that could compete or interfere with these processes. It seems likely that the original impetus for evolution of the nucleolus stemmed from enhancing the efficiency of the crucial process of ribosome production. However, we suggest that once the nucleolus evolved, its presence was subsequently exploited by cells as a convenient site in which to incorporate additional functions, providing selective advantages by similarly enhancing their efficiency. For example, the presence in nucleoli of a complex machinery for RNA modification and RNP assembly would allow other forms of functional RNP, such as telomerase and splicing snRNPs, to exploit the nucleolus in their biogenesis pathways. It is possible that miRNAs and other types of RNP will also be found to make use of the nucleolar environment.

It is worth emphasizing that cell growth and proliferation are crucially dependent on an efficient supply of ribosomes to maintain protein-synthesis levels. Therefore, it should not be surprising that the nucleolus is emerging as a key centre, the activity of which is influenced by various signalling events that can modulate the efficiency of rRNA expression and ribosome-subunit assembly and transport. We anticipate that more information will emerge about the mechanisms that are involved in regulating nucleolar function and structure in response to processes during cell-cycle progression and proliferation. There are now several examples in which the disruption of nucleolar components and activities results in human disease, including inherited genetic disorders and predisposition to cancer. This directly reflects the severe impact on cell function of disrupting mechanisms that occur in the nucleolus, and we predict that further examples of molecular disease involving nucleolar components and functions will be documented. Therefore, despite more than 200 years of research on nucleoli, we look forward to discovering just how many more surprises the nucleolus holds in store.

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Competing interests statement
The authors declare no competing financial interests.

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