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Studium mechanizmu regulace genové exprese na úrovni funkční organizace chromatinových domén

Dynamika FC/DFC jednotek buněčného jadérka

Study of the mechanism of gene expression regulation at the level of functional organization of chromatin domains

Dynamics of FC/DFC units of cell nucleolus

Disertační práce

Ph.D. thesis

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ABSTRAKT

Jadérka (nucleoli) se vytvářejí na základě aktivity genů ribozomální DNA (rDNA), nazývaných Nucleolus Organizer Regions (NOR). Základní komponenty jadérek, fibrilární centra (FC) a denzní fibrilární komponenty (DFC) společně tvoří takzvané FC/DFC jednotky. Tyto jednotky jsou centry transkripce rDNA pomocí RNA polymerázy I (pol I), stejně jako raného procesingu, ve kterém hraje podstatnou roli protein fibrilarin. Každá FC/DFC jednotka pravděpodobně odpovídá jedinému transkripčně aktivnímu genu. V naší práci jsme studovali morfologicko-funkční změny FC/DFC jednotek v průběhu buněčného cyklu. Analýza pomocí korelativní světelné a elektronové mikroskopie prokázala, že pozitivní signály pro polymerázu I a fibrilarin v nukleárních kuličkách odpovídají jednotlivým FC/DFC jednotkám. Pozorování *in vivo* prokázala, že v časné S fázi, když byly replikovány transkripčně aktivní ribosomální geny, se počet jednotek v každé buňce zvýšil o 60 až 80 %. Během tohoto období jednotky přechodně ztratily pol I ale ne fibrilarin. Poté se až do konce interfáze počet jednotek nezměnil a jejich duplikace se dokončila až po rozdělení buněk v polovině G1 fáze. Tento zvláštní způsob reprodukce naznačuje, že značná podskupina ribozomálních genů zůstává transkripčně tichá od interfáze až po mitózu, ale opět se aktivuje v dceřiných buňkách. Ve výzkumu jsme pokračovali studiem FC/DFC jednotek a zkoumali jsme kinetiku jejich nejdůležitějších proteinů, polymerázy I a fibrilarinu. Po změnách fluorescenčních signálů v FC/DFC jednotkách jsme objevili dva druhy kinetiky: rychlé kolísání s periodami 2-3 minutami a pomalé kolísání intenzit signálů s periodami od 10 do 60 min. Naše data naznačují, že komplexní pulzující aktivita transkripce stejně jako raný procesing je pro ribozomální geny běžná. V první teoretické práci jsme se zaměřili na jadérkovou DNA, která je úzce spjata s našimi experimentálními daty o FC/DFC jednotkách a diskutujeme v ní o vlastnostech ribozomální DNA. V druhé teoretické práci jsme se zaměřili na diskontinuální transkripci, která je přímo spojena s daty naší druhé experimentální studie, které naznačují, že ribozomální geny v savčích buňkách jsou přepisovány diskontinuálně. Obě teoretické práce jsou podkladem teoretického úvodu práce.

Klíčová slova: Jadérko, FC/DFC jednotka, rDNA, polymeráza I, fibrilarin, buněčný cyklus, transkripce, replikace, fluktuace.

ABSTRACT

Nucleoli are formed on the basis of genes of ribosomal DNA (rDNA) clusters called Nucleolus Organizer Regions (NORs). The essential structural components of the nucleoli, Fibrillar Centers (FC) and Dense Fibrillar Components (DFC), together compose FC/DFC units. These units are centers of rDNA transcription by RNA polymerase I (pol I), as well as the early processing events, in which an essential role belongs to fibrillarin. Each FC/DFC unit probably corresponds to a single transcriptionally active gene. In our work we study changes of FC/DFC units in the course of cell cycle. Correlative light and electron microscopy analysis showed that the pol I and fibrillarin positive nucleolar beads correspond to individual FC/DFC units. *In vivo* observations showed that at early S phase, when transcriptionally active ribosomal genes were replicated, the number of the units in each cell increased by 60 to 80 %. During that period the units transiently lost pol I, but not fibrillarin. Then, until the end of interphase, number of the units did not change, and their duplication was completed only after the cell division, by mid G1 phase. This peculiar mode of reproduction suggests that a considerable subset of ribosomal genes remain transcriptionally silent from mid S phase to mitosis but become again active in the postmitotic daughter cells. In our research we continued the study of the FC/DFC units and examined kinetics of their most important proteins, polymerase I and fibrillarin. Following changes of the fluorescent signals in individual FC/DFC units, we found two kinds of kinetics: the rapid fluctuations with periods of 2-3 minutes and slow fluctuations with periods of 10 to 60 min. Our data indicate that a complex pulsing activity of transcription as well as early processing are common for ribosomal genes. In first theoretical work focused on the nucleolar DNA, which is closely linked to our experimental data about FC/DFC units we discuss the characteristics of ribosomal DNA. In second theoretical work we focused on the discontinuous transcription, and thus directly connected to the data of our second experimental study, which indicate that ribosomal genes in mammalian cells are also transcribed discontinuously. Both theoretical works are the basis of theoretical introduction of thesis.

Key words: Nucleolus, FC/DFC unit, rDNA, polymerase I, fibrillarin, cell cycle, transcription, replication, fluctuation.

ABBREVIATIONS

3C analysis - Chromosome conformation capture techniques

ActD - Actinomycin D

ARBP - Attachment region binding protein

A.U. - Arbitrary unit

AzdC - 5-aza-2'-deoxycytidine, Decitabine, deoxy derivative of Azacitidine (5-AzaC)

BLM - Bloom syndrome protein

BrdU - Bromodeoxyuridine

BSA - Bovine serum albumin

Cdc2 - Cell cycle controller

Chip-seq - Chromatin immunoprecipitation with DNA sequencing

CLEM - Corellative light and electron microscopy

C-Myc - Regulatory gene and proto-oncogene

CPBF - Cysteine protease binding protein family

CSB - Cockayne Syndrome group B protein

CTCF - Transcriptional repressor, CCCTC-binding factor (11-zinc finger protein)

CTCFL - Transcriptional repressor CTCFL

Cy3 - Cyanine dye (550 nm excitation, 570 nm emission)

Cy5 - Cyanine dye (650 nm excitation, 670 nm emission)

DAPI - 4',6-diamidino-2-phenylindole

DFC - Dense fibrillar component

DIC - Differential interference contrast

DKC1 - H/ACA ribonucleoprotein complex subunit DKC1

DMEM - Dulbecco modified Eagle's medium

dUTP - Deoxyuridine triphosphate

EdU - 5-ethynyl-2′-deoxyuridine

EM - Electron microscopy

EMCCD - Electron-multiplying charge-coupled device

EMS - Electron Microscopy Sciences (company)

FC - Fibrillar center

FC/DFC - Fibrillar center and Dense fibrillar component subunit of nucleolus

Fib, fib, FBL - Fibrillarin

FRAP - Fluorescence recovery after photobleaching

FU - 5-fluorouridine

GFP - Green fluorescent protein

hTAF(I)110 - Human TATA box-binding protein-associated factor RNA polymerase I subunit C

HDAC1 - Histone deacetylase 1

HeLa cell line - Human cells derived from cervical cancer

Hep 2 cell line - HeLa derivative human carcinoma cell line

HT-1080 cell line - Human fibrosarcoma cell line

HMG box - High mobility group box

IGS - Intergenic spacer

ITS1, ITS2 - Internal transcribed spacer (1,2)

Kb, kb - Kilobase

LEP cell line - Human embryonic fibroblast

LM - Light microscopy

lncRNA - Long non-coding RNA

Mb - Megabase

MBD - Methyl-CpG-binding domain

MCM - Minichromosome maintenance protein complex

N.A. - Numerical aperture

NAD - Nicotinamide adenine dinucleotide

NaOH - Sodium hydroxide

NCL - Nucleolin

Nopp 140 - Pre-rRNA transcription-processing linking factor

NOR - Nucleolus organization region

NoRC - Nucleolar chromatin-remodelling complex

NPM1 - Gene coding Nucleophosmin (NPM)

NuRD - Nucleosome remodeling deacetylase

PBS - Phosphate-buffered saline

PCNA - Proliferating cell nuclear antigen

PIC - Pre-initiation complex at ribosomal RNA gene promoters

Pol I, pol I - RNA polymerase I

pre-rRNA - Preribosomal RNA

pRNA - Promoter rRNA

R_{Δ} - Pearsons coefficient

rDNA - Ribosomal DNA

RFB - Replication fork barrier

RFP - Red fluorescent protein

RNAi - RNA interference

RNAP - RNA polymerase

RPA43 - Subunit of polymerase I

RPS - S ribosomal proteins

RPL - L ribosomal proteins

rRNA - Ribosomal RNA

RRN3 - RNA polymerase I-specific transcription initiation factor

S - Svedberg unit

SL1 - Selectivity factor 1

smFISH - Single molecule fluorescent *in situ* hybridization

snRNA - Small nuclear RNA

snoRNA - Small nucleolar RNA

snoRNP - Small nucleolar ribonucleoprotein

SNF2h, hSNF2H - Transcription factor, SMARCA5, (human) SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5

SSU processome - Small subunit processome

TAF - TATA binding protein associated factor

TAM - Translocation and assembly module protein complex

TBP - TATA-binding protein

TFIIH - Transcription factor II Human

TIP5 - Transcription termination factor I-interacting protein

tRNA - Transfer Ribonucleic Acid

TSA - Trichostatin A

TTFI - Transcription termination factor 1

t-UTP - Nan1p-containing subcomplex of 90S preribosome

U3 snoRNP - U3 small nucleolar RNP

UBF - Upstream binding factor

UCE (UPE) - Ubiquitin-conjugating enzyme E2

WRN - Werner protein

Xi - Inactive chromosome X

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1. INTRODUCTION

This thesis is based on four publications from one research field of study and published in international scientific journals with impact factor. Publications are attached in original formats as supplements of the thesis.

In first publication, which is theoretical, we discuss new information about the nucleolar DNA. The second publication presents our findings about reproduction of FC/DFC units in the nucleolus. Using correlative light and electron microscopy, we studied reproduction of the FC/DFC units in the course of the cell cycle. The third publication is the most important part of my thesis. In this work we examined the kinetics of pol I and fibrillarin in FC/DFC units. We discovered short-term and long-term fluctuations of these enzymes inside of nucleoli, and our data indicate that individual ribosomal genes are expressing discontinuously. In the last fourth publication, which is theoretical, we discuss various aspects of the phenomenon of discontinuous transcription.

The discussion contains a critical evaluation of the data presented in these publications and in the conclusion are our most significant findings.

2. THEORETICAL BACKGROUND

2.1. General introduction

Nucleolus is a non-membrane nuclear organelle found in all eukaryotes (Lam and Trinkle-Mulcahy, 2015). Nucleolus was formally described by Wagner (1835) and Valentin (1836). In the 1950s, the nucleoli were first isolated (Pederson, 2011). In the 1960s, the nucleolus was described as the site of ribosome synthesis. The average mammal cell nucleolus can produce up to 10,000 ribosomes per minute (Pederson, 2011). Nucleolus is the most prominent and distinctly differentiated nuclear body (Berger et al, 2008). Transcription of ribosomal genes, processing of the transcripts and early stages of assembling of pre-ribosomal precursor particles (figure 2.1) take place in the nucleolus (Huang, 2002).

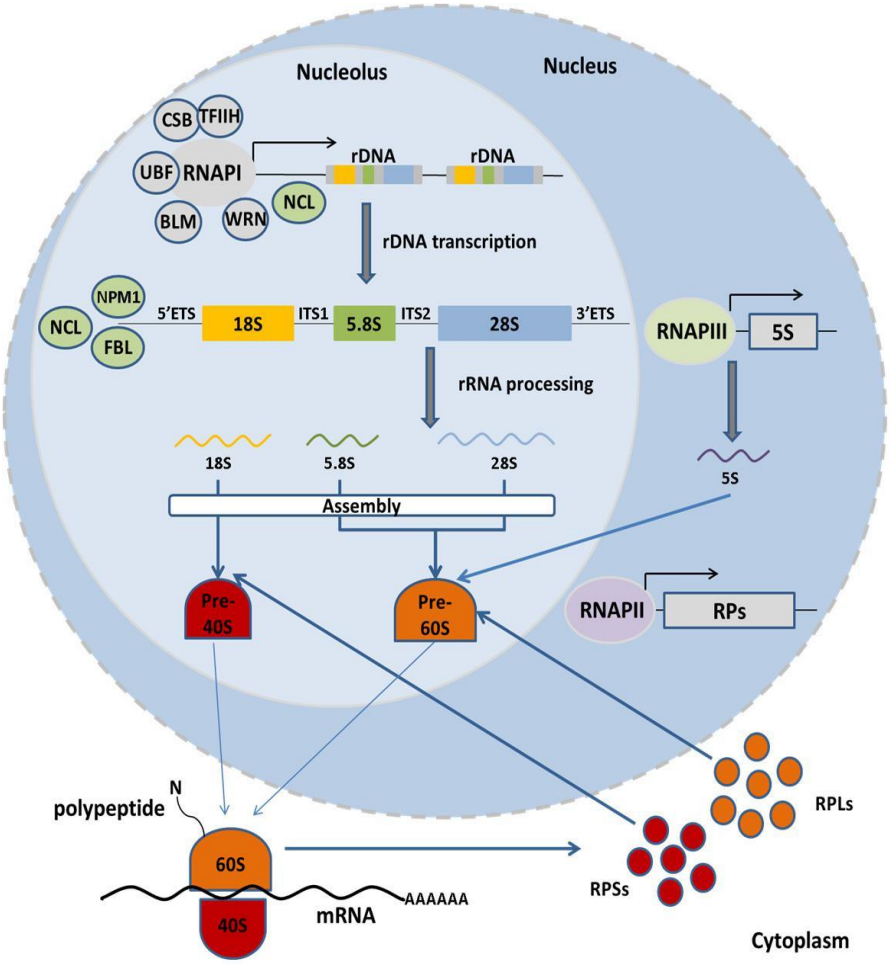

Figure 2.1. The function of nucleolus. In normal state, the RNA Pol I machinery transcribes a polycistronic pre-rRNA molecule, which is subsequently processed to mature rRNAs (18S, 5.8S, and

28S) of the small and large ribosomal subunit. Outside the nucleolus, RNA Pol III transcribes the fourth rRNA (5S), while RNA Pol II transcribes the ribosomal protein genes to be translated in the cytoplasm. Ribosomal proteins of the large (RPLs) and the small (RPSs) subunit enter the nucleolus to associate with rRNAs and assemble the ribosomal subunits, which are then exported to the cytoplasm (Tsekrekou et al., 2017).

Initially the nascent pre-rRNA is assembled into an 80–90S nucleolar particle. Structural rearrangement and nucleotide modifications occur as the ribosomal proteins are incorporated, followed with cleavages which ultimately give rise to the mature ribosomal subunits. In addition, 80 or more ribosomal proteins, a surprisingly large number (more than 150) of accessory proteins and dozens of snoRNAs are involved (Nazar, 2004). Nucleoli in mammals have typically tripartite organization: fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC). Nucleolar structure forms around clusters of ribosomal gene at the end of the mitosis (figure 2.2 and figure 2.3), persists in the interphase, and then decays when cells enter mitosis (Prieto and McStay, 2005).

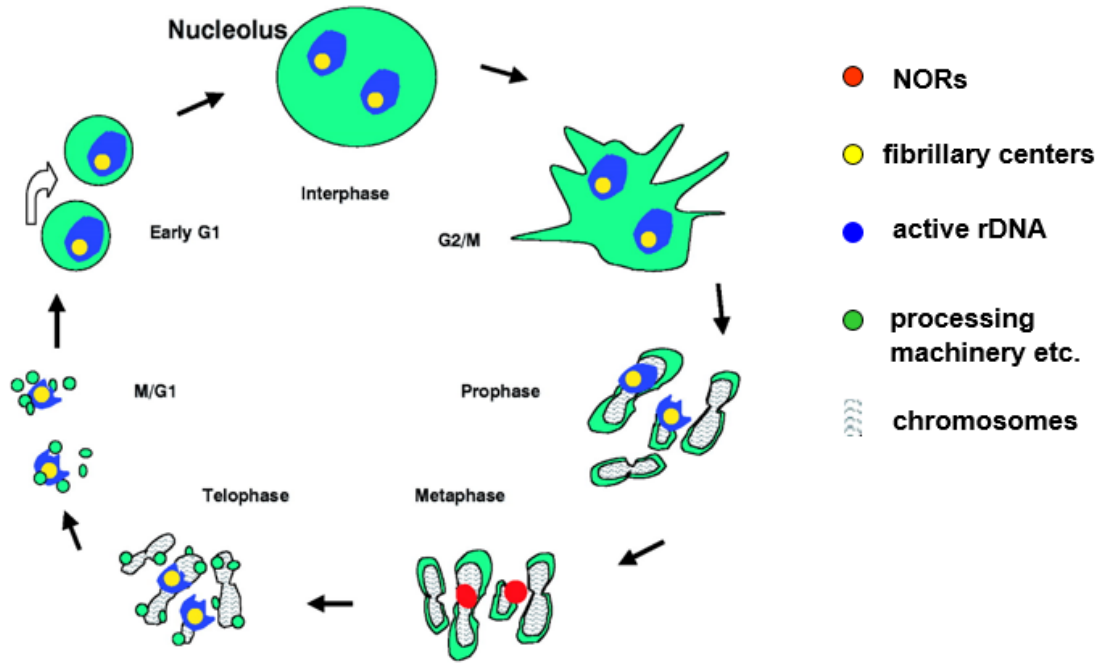

Figure 2.2. Nucleolus is formed by Nucleolus Organizer Regions (NORs). (Hernandez-Verdun et al., 2002).

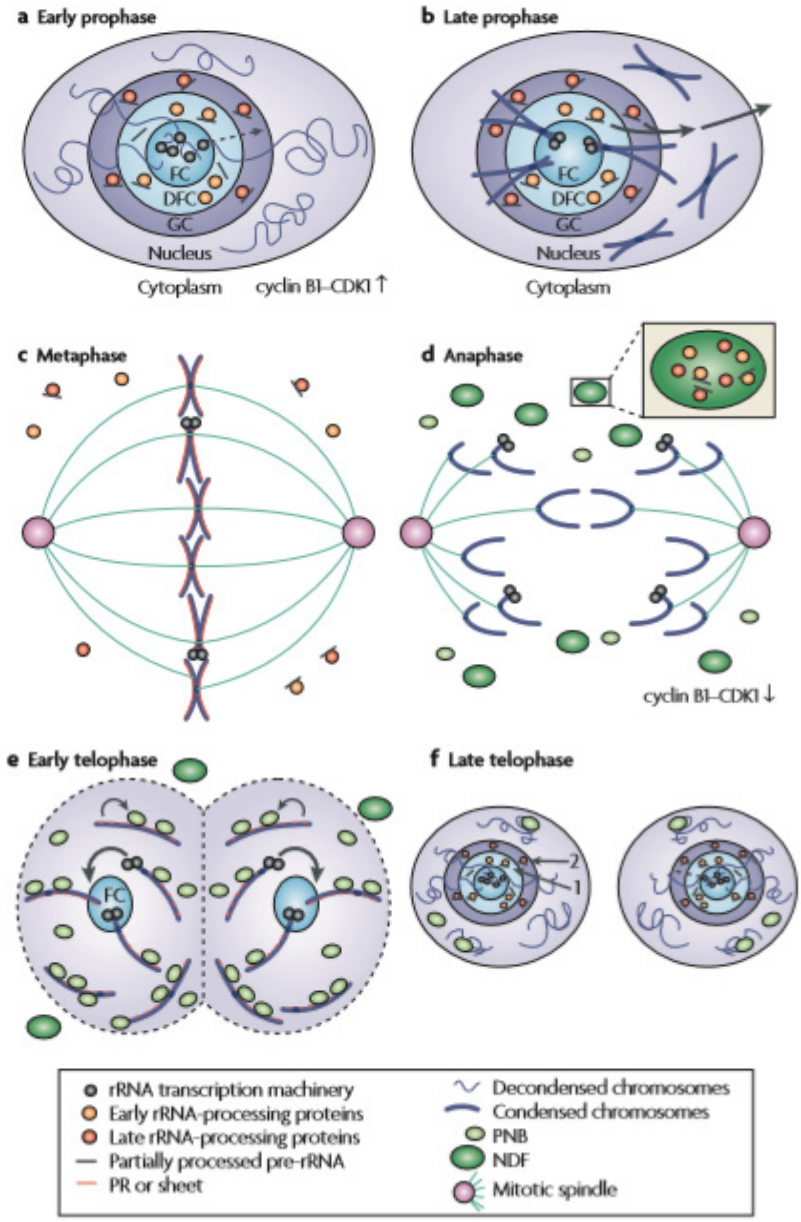

Figure 2.3. Nucleolar disassembly and reassembly during cell division. a.) During early prophase, cyclin B1–CDK1 levels increase and chromosomes start to decondense, whereupon they can be seen as thin threads in the nucleus. Although the transcription machinery usually remains attached to active nucleolar 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 reformed 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 (Boisvert et al., 2007).

Nucleoli are formed on the basis of clusters of ribosomal DNA (rDNA) called Nucleolus Organizer Regions (NORs) coding for RNAs of ribosomal particles (figure 2.4). The nucleoli also include a great variety of other DNA regions (Smirnov et al., 2016a).

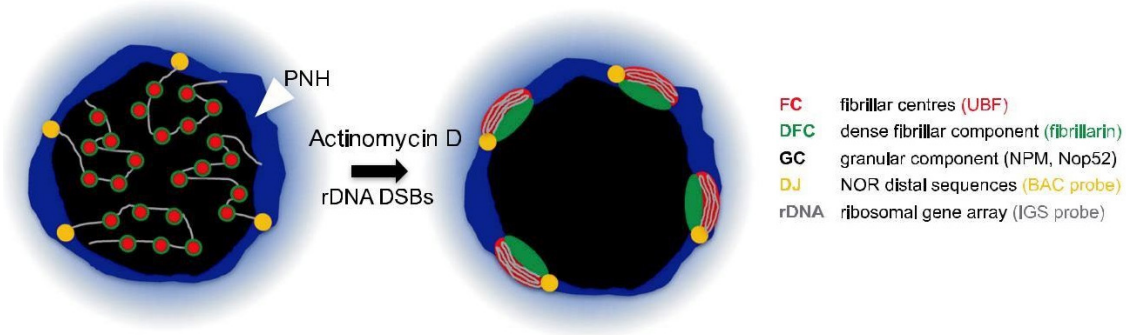

Figure 2.4. Organization of human NORs. Organization of NORs and DJ (NOR distal) sequences (gray line and yellow circle, respectively) in a normal mature nucleolus and in a nucleolus undergoing stress by actinomycin D treatment or the introduction of rDNA DSBs (double-strand breaks). FC, DFC, and GC components are colored as indicated and the perinucleolar heterochromatin (PNH) is indicated by a white arrowhead (Mangan et al., 2017).

The initial formation of nucleoli around the transcriptionally competent NORs are followed by fusion of the NORs into a smaller number of nucleoli. Thus one functional domain is produced by cooperative interaction of the regions belonging to different chromosomes (Savino et al., 2001). At the end of interphase, shortly before the nuclear envelope breakdown, nucleolar disassembly starts with the loss of RNA polymerase I subunits from the fibrillar centers. Dissociation of proteins from the other subcompartments occurs later (Leung et al., 2004; Jimenez-Garcia et al., 1994). When

cells enter mitosis, the synthesis of RNA stops, but the transcriptional apparatus of RNA polymerase I remains bound to the NORs. Only positive NORs become active at telophase and the level of transcription activity is related to the amount of rDNA transcription enzymes present in these NORs (Weisenberger and Scheer, 1995; Gebrane-Younes et al., 1997; Roussel and Hernandez-Verdun, 1994; Roussel et al., 1996). Nucleolar proteins and RNAs involved in rRNA processing and nucleolar RNAs are associated with chromosomes, creating a perichromosomal compartment which surrounds the chromosomes and prevents their collapse into a single mass (Booth et al., 2014; Dousset et al., 2000).

Being a locus of the ribosome biogenesis, nucleolus also has non-ribosomal functions (Raska et al., 2006b; Sirri et al., 2008; Jacob et al., 2013). Nucleoli are closely linked to cellular homeostasis and human health (Hernandez-Verdun et al., 2010). Recent biochemical and proteomic analyzes have revealed a broad functional complexity of the nucleoli including their role in role in cell cycle regulation, repair of DNA damage, pre-mRNA processing, telomere metabolism, processing of noncoding RNAs and coordination of cellular response (Lam and Trinkle-Mulcahy, 2015). The perinucleolar chromatin region which is related to the transcription of mRNA and tRNA is regarded as the fourth functional compartment (Pederson, 1998; Smetana, 2011).

The nucleoli are classified based on the size and distribution of the components (Smetana, 2011)

Large nucleolus with a relatively uniform RNA is usually present in proliferating cells. Such nucleoli are very active in respect of RNA transcription and contain small multiple fibrillar centers (Smetana, 2011).

Compact nucleolus has a relatively uniform distribution of ribonucleoprotein components. They can differ in size. Large compact nucleoli without segregation of nucleolar components are very active in transcription and maturation of ribosomal RNA (Smetana, 2011).

Nucleolus with distinct nucleolonemas is characterized by the presence of coarse nucleoprotein vesicular structures - nucleolonemas. It is the commonest type of

nucleolus in cells and is characterized by the active synthesis of ribosomal RNA (Smetana, 2011).

Ring shaped nucleolus with RNA is present only in the nucleolar peripheral part. It is characteristic for resting cells, which, however, may return to the proliferation. Ring shaped nucleoli are characterized by a reversible decrease of the RNA transcription (Smetana, 2011).

Micronucleoli, the size of which is smaller than 1 μm, are often found in the terminally differentiated or pre-apoptotic cells (Smetana, 2011).

Pronucleolus (prenucleolar body) is at the end of the mitotic division. Pronucleoli are mostly in the proximity or at mitotic chromosomes and, in contrary to micronucleoli, they are characterized by the onset of the RNA transcription (Smetana, 2011).

2.2. Structural organisation of the nucleoli

2.2.1. DNA and chromatin structure in the nucleoli

Eukaryotic genomes associated with histones, other proteins and other non-protein constituents form chromatin (Rickards et al., 2007). In the nucleoli, chromatin exhibits three different levels of organization: compact clusters, fibers with the diameter ranging from 11 to 30 nm, and free agglomerates of loose DNA fibers. Both clusters and chromatin fibers exhibit a nucleosomal organization which is absent in the free agglomerates of loose DNA fibers. These fibers are 2-3 nm thick, just like the double helix DNA molecule. Free agglomerates of DNA filaments are located in fibrillar centers (FC) and apparently correspond to ribosomal DNA (Derenzini et al., 2006). The architecture of the nucleoli during interphase reflects the distribution of the rDNA that is characterized by alternation of clustered and extended genes (Junera et al., 1995). Electron microscopic analysis has located nuclear transcription, replication and fibrillarin signals in the DFC and at its borders with FC (Pliss et al., 2005). Presence of the transcribed rDNA is necessary to overcome the highly stochastic nucleation step in the formation of nucleoli. In the absence of rDNA, some nucleolar proteins may form aggregates, but these aggregates are very variable in number, location, and time at which they form (Falahati et al., 2016).

Each human cell contains hundreds of copies of rDNA in the form of tandem repeats distributed among the chromosomes. A subset of the ribosomal genes remain permanently silent. Transcription of rDNA generates 45S pre-rRNA, which is then cleaved and processed into 28S, 18S and 5.8S rRNAs of ribosomal particles. The clusters of ribosomal genes are called Nucleolus Organizer Regions (NOR). In human cells, they are located on the short arms of acrocentric chromosomes (#13, 14, 15, 21 and 22) and appear as secondary constriction of metaphase chromosomes. Only some NORs, called competents, are transcriptionally active during the interphase and can be identified by the presence of RNA polymerase I and its transcription factors (Kalmarova et al., 2007; Kalmarova et al., 2008). Specific epigenetic modifications such as histone acetylation and methylation and methylation of DNA regulate the transcription of the rDNA (Cremer et al., 2004; Bartova et al., 2010).

Ribosomal RNA genes also occur in the form of extrachromosomal DNA. During oogenesis of some amphibians, fish, and insects, rDNA is amplified so that many extrachromosomal copies occur (Long and Dawid, 1980).

Ribosomal RNA genes in human cells are predominantly located in dense fibrillar component (DFC), and in the border region between FC and DFC (Hozak et al., 1993). But opinions on the exact localization of active ribosomal genes - in each of the two nuclear subcomponents FC and DFC, still differ (Raska, 2003). Some authors believe that transcription of rDNA takes place in fibrillar centers (FC), and the nascent rRNAs quickly enter the surrounding DFC (Cheutin et al., 2002). Data of the electron microscopy identify DFC (figure 2.5) as a nuclear subcomponent in which rRNA synthesis takes place (Koberna et al., 2002; Melcak et al., 1996). In metabolically active mammalian nuclei, fibrillar centers (FC) and dense fibrillar components (DFC) compose one functional domain for the transcription of rRNA genes, and the nascent transcripts form DFC (Raska et al., 1995; Raska, 2003). The tendency of the NOR bearing chromosomes to associate with the nucleoli correlates with the transcription competence of the NORs. But nucleoli may contain incompetent NORs as well; in such cases the rDNA seems to be connected to the chromosomal territory by a long protrusion (Kalmarova et al., 2007; Kalmarova et al., 2008).

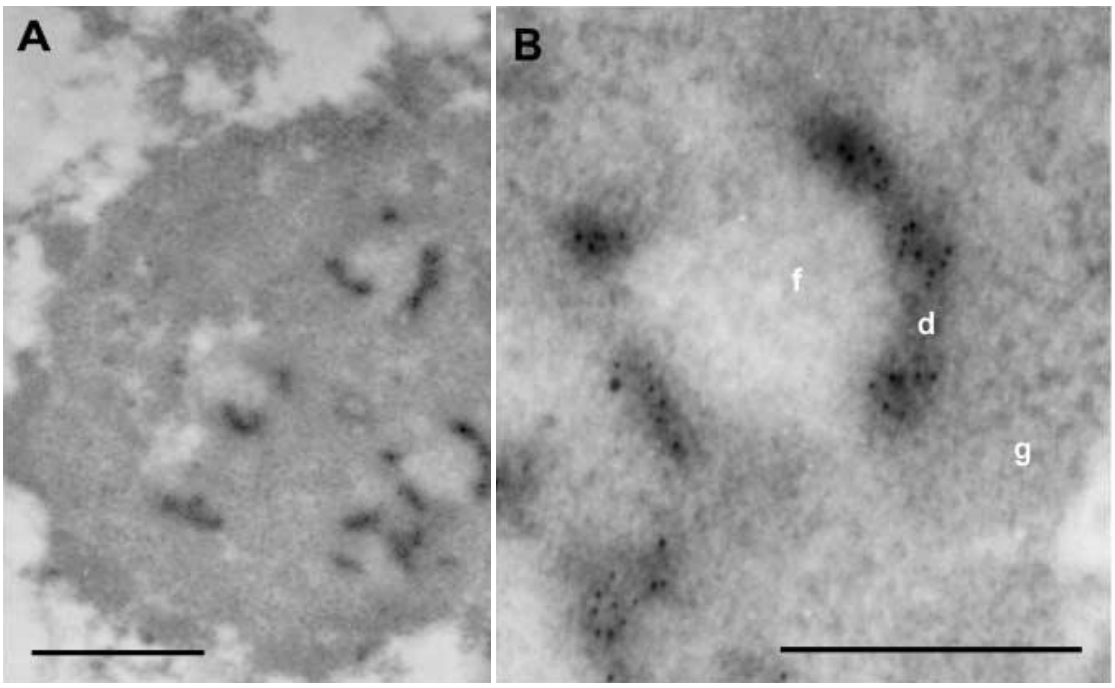

Figure 2.5. Transcription signal in FC-DFC units. Mapping of transcription signal (black dots), using electron microscopy in a thin section of permeabilized cell. A: FC-DFC units inside nucleolus. B: Detail of one FC-DFC unit; f - Fibrillar Center (FC), d - Dense Fibrillar Component (DFC), g - Granular Component (GC). Bars: (A) 0.5 μm; (B) 0.2 μm (Koberna et al., 2002).

A gene amplification system which maintains large clusters of tandemly repeated copies in the chromosome, with each species having a specific number of copies, preserves the stability of heavily transcribed ribosomal genes (Ide et al., 2010; McKeown and Shaw, 2009). Mutations occur randomly throughout the ribosomal DNA (rDNA) sequence. Molecular drive (unequal crossing-over, gene conversion, transposition) spreads these variations through the multiple copies of rDNA (Gerbi, 1986). Recombination and gene conversion stabilize human rDNA. Studies with randomly cloned genomic DNA fragments reveal homogenity among regulatory and coding regions of rDNA on all chromosomes, a homogenity among adjacent distal non-rDNA sequences, and the existence of one to three very divergent intergenic spacer classes within each array (Gonzalez et al., 2001).

One rDNA repeat consists of a transcribed and non-transcribed parts (figure 2.6). The non-transcribed parts, or intergenic spacers, proved to be biologically significant, complex and plurifunctional transcriptional units that appear central to proper cellular functioning. Through the timely induction of various ribosomal IGS

noncoding RNA (IGS RNA) transcripts, the cell is capable of both regulating rRNA synthesis and sequestering large numbers of proteins, thereby modulating essential molecular networks (Jacob et al., 2012). The sequence of the complete IGS reveals a collection of sequence motifs that can be correlated with functions known or expected to reside in the rDNA repeat: modulation of transcription, recombination, initiation of DNA replication, and chromosomal organization (Gonzalez and Sylvester, 1995).

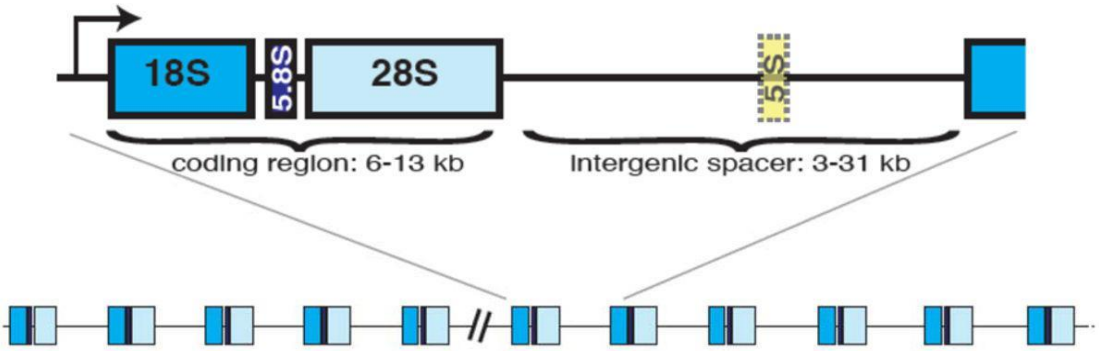

Figure 2.6. Structure of the eukaryotic rDNA repeat. The structure of a typical eukaryotic rDNA repeat unit is shown in the upper part of the figure (not to scale), with the regions encoding the three major rRNA species (18S, 5.8S and 28S) represented as blue boxes. The inclusion of the 5S rRNA gene (hatched box) within the rDNA repeat unit is observed only in some kinds of organisms. The direction of RNA pol-I transcription is indicated. Individual rDNA repeats are usually arranged into tandem arrays as illustrated (O'Sullivan et al., 2013).

Induction of IGS lncRNA by environmental signals operates as a molecular switch that regulates the structure and function of the nucleolus maintaining its tripartite organization as well as the transcriptional activity (Jacob et al., 2013). IGS transcripts are needed to create and maintain a specific heterochromatic configuration on the rDNA series subset promoter. The IGS transcripts influence nuclear localization of NOR, DNA methylation and transcription of rDNA (Mayer et al., 2006). The human IGS has the length of 30kb with a limited length heterogeneity localized in a specific region downstream of the 3' end of the transcribed region (La Volpe et al., 1985). Non-transcribed spacers of the ribosomal genes of a number of organisms contain repetitive sequences (Mroczka et al., 1984). Each repeating rDNA unit of Drosophila melanogaster consists of a region of a gene encoding a transcript comprising the 18S and 28S rRNA sequences and the non-transcribed spacer (Wellauer

and Dawid, 1977). These spacer regions in the *Xenopus laevis* ribosomal DNA vary in length, even within a single nuclear organizer (Reeder et al., 1976).

Although the coding sequences of the ribosomal DNA are rather conservative, the whole system of rDNA in the cell seems to be a mosaic of canonical and palindromic units that can be altered by factors linked to genomic instability and pathology (Caburet et al., 2005). The length of the rDNA clone shows marked variability between and within human individuals, ranging from 50 kb to more than 6 Mb, which may be revealed as electrophoretic patterns. Analysis of these rDNA fingerprints in multigenerational human families demonstrates that the rDNA clusters are subject to meiotic rearrangement at a frequency more than 10% per cluster, per meiosis. With this high intrinsic recombinational instability, the rDNA clusters may serve as a unique paradigm of potential human genomic plasticity (Stults et al., 2008; Kupriyanova et al., 2015).

A partial survey for sites of variation between clones of human rDNA has revealed a single point of variation among 18S rRNA gene sequences (a T/C variation at position 140), several sites of length variation in the regions of the transcribed spacers closely flanking the 18S rRNA genes, and some sites of length variation among 28S rRNA genes. Most of these sites of variation were associated with simple sequence tracts (Maden et al., 1987). The 5025 base sequence of the human 28S rRNA gene has great variability. This region is one of three large subunit rRNA regions that show extreme sequence and size variation among species. The interspecies differences suggest species-specific functions for these sections, while the intraspecies heterogeneity indicates differences among ribosomes (Gonzalez et al., 1985).

Sequences distal and proximal to ribosomal gene arrays are conserved among the acrocentric chromosomes, suggesting that they are sites of frequent recombination. These two flanking regions share a complex genomic architecture similar to other euchromatic regions of the genome, but have distinct genomic characteristics. Proximal sequences are similar to the regions bordering centromeres. In contrast, the distal sequence is predominantly unique to the acrocentric short arms and is dominated by a very large inverted repeat. The distal element is localized to the periphery of the nucleolus, where it appears to anchor the ribosomal gene repeats, and seems to be involved in nucleolar organization. (Floutsakou et al., 2013; Grob et al., 2014).

Other, non-ribosomal, DNAs may be also associated with nucleoli. Areas of the genome frequently associated with the nucleolus are called the Nucleolar Associated Domains (NADs). These domains are enriched with repeating elements, regions of inactive chromosome X (Xi) and certain genes transcribed by RNA polymerase III. NADs are often characterized by chromatin modifications characteristic of heterochromatin, including H3K27me3, H3K9me3 and H4K20me3. It has been supposed that NAD localization to the nucleolar periphery contributes to the establishment and/or maintenance of heterochromatic silencing (Matheson and Kaufman, 2017; van Koningsbruggen et al., 2010). NAD genes take part in specific biological processes, such as the response to other organisms, odor perception, and tissue development (Nemeth et al., 2010).

2.2.2. Nucleolar RNAs

Ribosomal RNAs are the most abundant and universal noncoding RNAs in living organisms. In eukaryotes, three of the four nucleolar ribosomal RNAs forming the 40S and 60S subunits are derived from the pre-ribosomal RNA. A complex sequence of processing steps is required to gradually release the mature RNAs from this precursor, concomitant with the assembly of the 79 ribosomal proteins (Henras et al., 2015). Eukaryotic ribosomal RNAs contain numerous modified nucleotides, in vertebrates about 115 methyl groups and some 95 pseudouridines. All but about ten of the methyl groups are methylations of ribose. The remaining ten are on heterocyclic bases. The ribose methylations occur very rapidly upon the primary rRNA transcript in the nucleolus, probably on nascent chains, and they appear to play an important role in ribosome maturation, at least in vertebrates. All of the methyl groups occur in the conserved core of rRNA. Experimental evidence indicates that structural motifs within the snoRNA species do indeed pinpoint the precise nucleotides to be methylated by the putative 2′-O-methyl transferase(s) (Maden and Hughes, 1997).

Eukaryotic cells contain about 150 different species of small nucleolar RNAs (snoRNAs). This surprisingly large number of snoRNAs can be divided into two major classes, designated the box C/D snoRNAs and the box H/ACA snoRNAs, on the basis of conserved sequence elements and conserved predicted secondary structures (Lafontaine and Tollervey, 1998; Balakin et al., 1996; Ganot et al., 1997; Samarsky et

al., 1998; Bachellerie et al., 2002). The ACA snoRNAs fold into two hairpin structures connected by a single-stranded hinge region and followed by a short 3' tail. The hinge region carries an evolutionarily conserved sequence motif, called box H. The H box, probably in concert with the flanking helix structures and the ACA box characterized previously, plays an essential role in the accumulation of human U64 intronic snoRNA (Ganot et al., 1997)

A group of lncRNAs coded by the loci upstream of the transcribed rDNA is known as promoter rRNA (pRNA). These RNAs interact with the target site of the transcription factor TTF-I to produce a triplex DNA:RNA that is specifically recognized by DNA methyltransferase DNMT3b. This pathway of RNA-dependent methylation of DNA is an important component of transcription silencing (Schmitz et al., 2010).

Under stress conditions lncRNAs transcribed from rDNA spacer regions can bind certain nuclear enzymes and temporarily inactivate them within the nucleoli; this phenomenon is known as “nucleolar detention” (Audas et al., 2012b; Jacob et al, 2013). Proteins subject to this detention are recognized by a discrete peptide code, referred to as Nucleoar Detention Sequence (NoDS). Interrupting the NoDS/lncRNA interaction allows proteins to escape from the nucleoli and restore their function. After dislocation of the intergenic lncRNA, the proteins may be immobilized outside the nucleolus, indicating that these types of ncRNAs can work independently of nucleolar architecture (Audas et al., 2012b). Notably, some RNAs produced in the nucleoplasm are then processed in the nucleolus (Pederson, 1998).

2.2.3. Nucleolar proteins

Hundreds of proteins have been discovered in the nucleoli (figure 2.7). Especially numerous are the proteins involved in expression of the ribosomal genes and production of ribosomes.

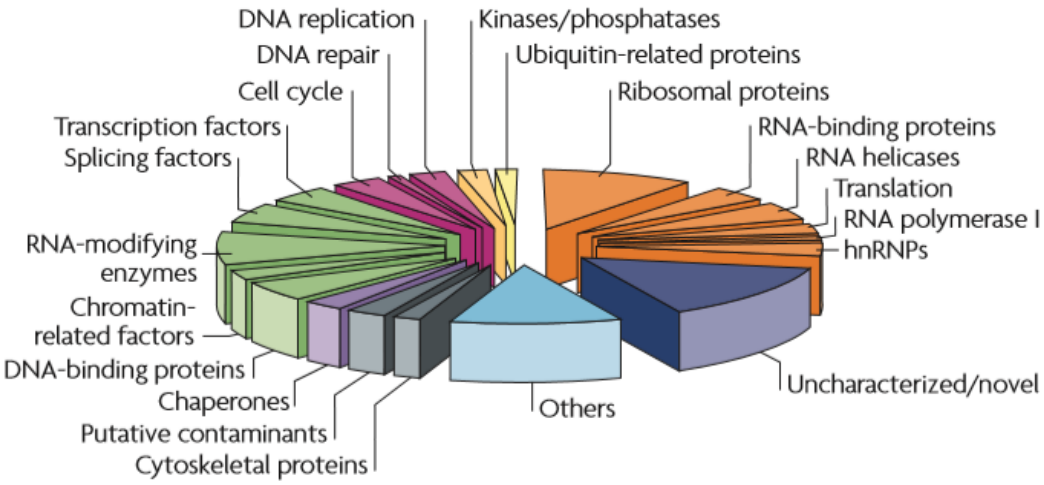

Figure 2.7. The nucleolar proteome. Purification and mass-spectrometric identification of nucleolar proteins have led to the identification of 726 human nucleolar proteins. Proteins are clustered according to their molecular functions and colour-coded for similar and related functions. Orange represents 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 (Boisvert et al., 2007).

Selected important proteins involved in transcription rDNA and early processing of rRNA:

RNA polymerase I (Pol I, pol I), (figure 2.8) produces ribosomal RNA (rRNA) (Albert et al., 2011). Transcription of ribosomal RNA by RNA polymerase (Pol) I initiates ribosome biogenesis and regulates eukaryotic cell growth (Engel et al., 2013).

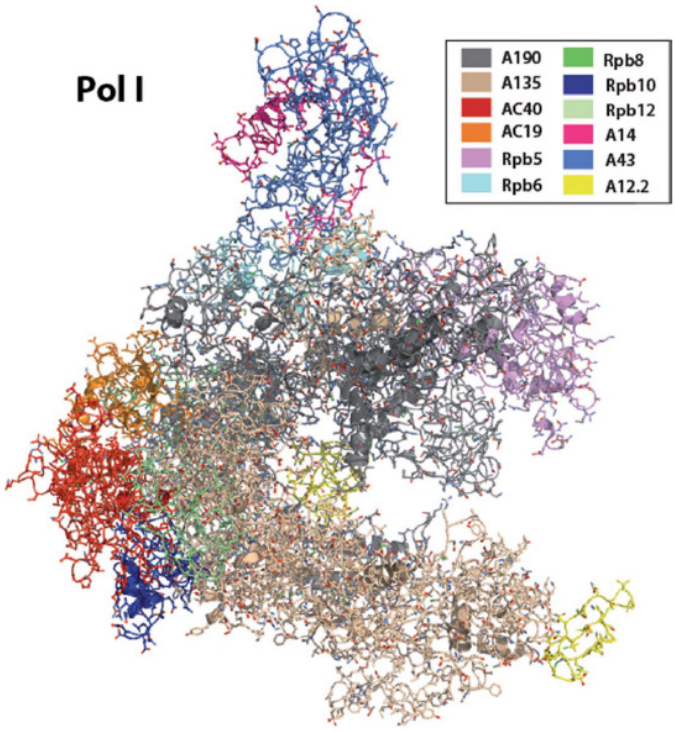

Figure 2.8. Model of yeast RNA Polymerase I. Initial 12-subunit model of Pol I obtained by molecular replacement followed by homology modelling. Subunit colours are as shown in the inset (Moreno-Morcillo et al., 2014).

An 'expander' element of RNA polymerase I occupies the DNA template site and stabilizes an expanded active centre cleft with an unwound bridge helix. A 'connector' element invades the cleft of an adjacent polymerase and stabilizes an inactive polymerase dimer. The connector and expander must detach during Pol I activation to enable transcription initiation (figure 2.9) and cleft contraction by convergent movement of the polymerase 'core' and 'shelf' modules. Conversion between an inactive expanded and an active contracted polymerase state may generally underlie transcription. Regulatory factors can modulate the core–shelf interface that includes a 'composite' active site for RNA chain initiation (figure 2.10 and table 2.1), elongation, proofreading and termination (Engel et al., 2013). The Rpa49 and Rpa34 subunits of the complex are functionally conserved and do not have counterparts in the Pol II and Pol III complexes. Loss of RPA49 leads to the disappearance of the nucleolar structure (Albert et al., 2011).

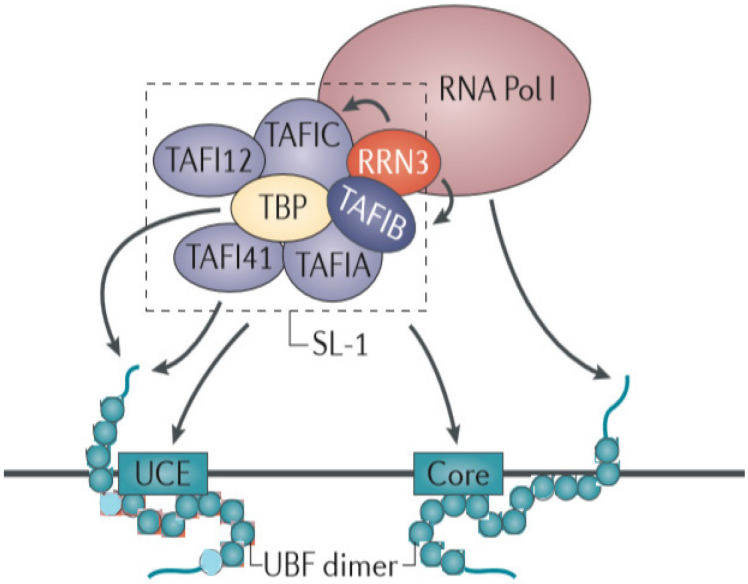

Figure 2.9. Basal transcription machinery and promoter structure of the eukaryotic DNA-dependent RNA polymerase I. Assembly of the RNA polymerase (Pol I) pre-initiation complex (PIC) at ribosomal RNA gene (rDNA) promoters begins with the binding of upstream binding factor (UBF) to the upstream control elements (UCEs) and core element of the rDNA promoter, leading to the recruitment of the SL-1 initiation factor, which contains TATA-box-binding protein (TBP) and at least five TATA-box-associated factors (TAFs). The resultant stable UBF–SL-1 complex recruits an initiation-competent form of RNA Pol I, which contains RRN3 that mediates interactions between RNA Pol I and SL-1 (Russell and Zomerdijk, 2006; Bywater et al., 2013).

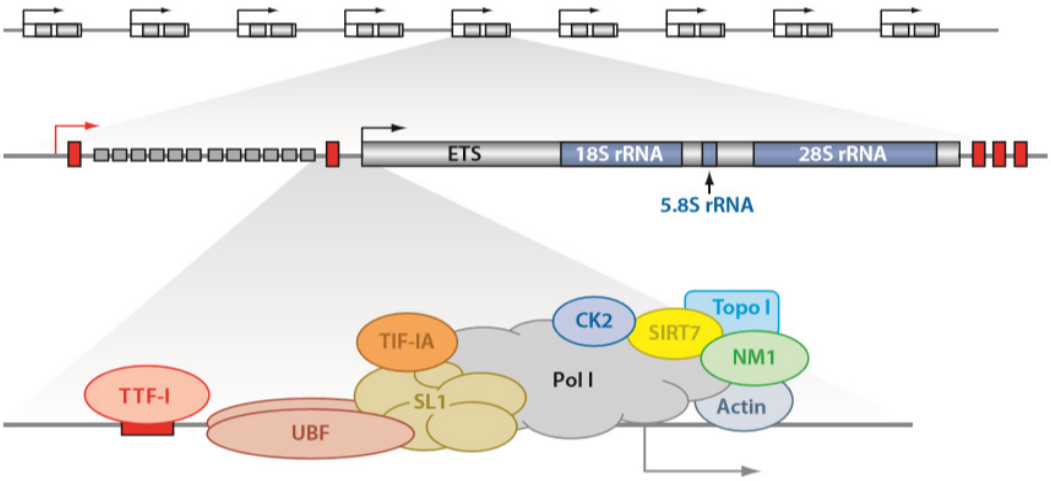

Figure 2.10. The structural organization of mammalian rDNA repeats and the basal factors required for transcription initiation. The diagram shows the arrangement of tandemly repeated rRNA genes. The site of transcription initiation of 47S pre-rRNA (black arrow) and intergenic transcripts from

the spacer promoter (red arrow) are indicated. Terminator elements are located downstream of the transcription unit (T1-10), downstream of the spacer promoter (TSP), and upstream of the gene promoter (To) (red boxes). Repetitive enhancer elements (gray boxes) are located between the spacer promoter and major gene promoter. The ellipsoids show the factors that are associated with the rDNA promoter and Pol I, respectively. TTF-I is associated with the upstream terminator To. Synergistic binding of UBF and TIF-IB/SL1 to the rDNA promoter is required for the recruitment of RNA polymerase I (Pol I) together with multiple Pol I-associated factors to the transcription start site to initiate pre-rRNA synthesis (Drygin et al., 2010).

Table 2.1. RNA polymerase I - dependent transcription factors in Human cells. (Adapted from Jacob, 1995).

Transcription factor	Molecular mass (kDa)	rDNA binding element(s)	Probable function(s)
UBF I and II	97 and 94	Core promoter, UCE (UPE), enhancer element (repetitive)	Enhances stable initiation complex formation and activates transcription. UBF 1 is the functional species. Also involved in the growth-dependent regulation of rRNA gene transcription.
TBP-TAFs complex SL I	100, 68 and 48 in Human	Core promoter, Human SL I binding to the core promoter requires UBF	Required for basal transcription and species-specific formation of transcription initiation complex.
CPBF	44	Core promoter	Involved in transcription initiation complex formation and activates basal transcription
TTF I	100, 90, 80 and 65	Terminator element GGGTCGACCAG	Termination of Pol I mediated transcription by binding to terminator elements.

Upstream binding factor (UBF) is a transcription factor for RNA polymerase I which contains several DNA binding motifs. These include a short basic region adjacent to a dimer motif plus five high-mobility-group (HMG) boxes. HMG box 1, in particular, requires association of two double helices before it will bind and, either by itself or in the context of the intact protein, will loop DNA and organize it into higher-order structures (Hu et al., 1994). UBF is important for activation of ribosomal RNA transcription and differs from other HMG-box proteins by binding both RNA (tRNA) and DNA (Copenhaver et al., 1994). UBF can also bind in vitro to a variety of

sequences found across the intergenic spacer in *Xenopus* and mammalian ribosomal DNA (rDNA) repeats. The high abundance of UBF, its colocalization with rDNA *in vivo*, and its DNA binding characteristics, suggest that it plays a more generalized structural role over the rDNA repeat. Indeed, it was found that UBF binding *in vivo* is not restricted to known regulatory sequences but extends across the entire intergenic spacer and transcribed region of *Xenopus*, human, and mouse rDNA repeats (O'Sullivan et al., 2002; Mais et al., 2005; Zentner et al., 2014). UBF is extensively bound to rDNA throughout the cell cycle, resulting in the transcriptionally competent form of chromatin. Pseudo-NORs (figure 2.11), the artificial arrays integrated into the ectopic chromosomes, being transcriptionally silent, recruit UBF, as well as other components of pol I transcription machinery, and mimic the chromatin structure of the competent NORs (Prieto and McStay, 2007). DNA-binding properties of UBF are conserved in vertebrates (Pikaard et al., 1990). UBF is the first common interaction partner of CTCF and CTCFL, indicating the role of these proteins in the organization of rDNA chromatin (van de Nobelen et al., 2010).

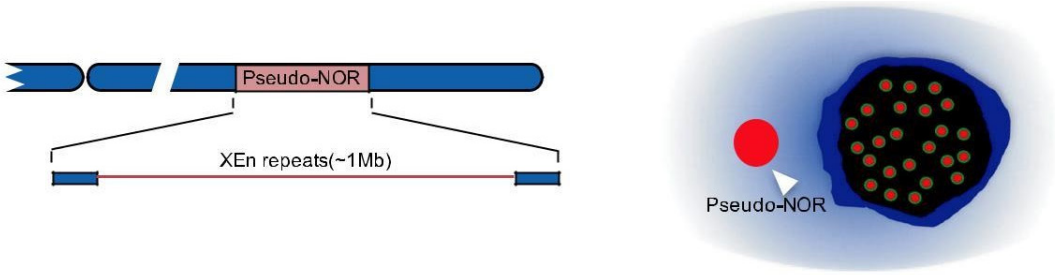

Figure 2.11. Organization of human pseudo-NORs. Pseudo-NORs are arrays of an optimal UBF-binding site (XEn) integrated into nonacrocentric chromosomes. An illustration of the pseudo-NOR as it appears during interphase is shown. Note that the pseudo-NOR (white arrowhead) is comprised solely of FC (red) (Mangan et al., 2017).

Selectivity factor 1 (SL1) is an important component of the pol I transcription machinery. This promoter-binding complex consists of TATA binding protein (TBP) and several TBP associated factors. SL1 is inactivated by Cdc2/cyclin B-directed phosphorylation and reactivated by dephosphorylation. Transcriptional inactivation in vitro is accompanied by phosphorylation of two subunits, TBP and hTAF(I)110. Mitotic phosphorylation inhibits the interaction of SL1 with UBF. SL1

phosphorylation may prevent complex formation before initiation and suspend the transcription of rDNA during mitosis (Heix et al., 1998).

Fibrillarin (Fib, FBL), (figure 2.12 and figure 2.13) is a key small nucleolar protein in eukaryotes (table 2.2), which has an important role in pre-rRNA processing (figure 2.14) during ribosomal biogenesis and would play a critical role in the maintenance of nuclear shape and cellular growth (Amin et al., 2007). Fibrillarin is accumulated in DFC of the nucleoli. The methyltransferase activity of fibrillarin is the primary source of methylation for more than 100 methylated sites during the first steps of pre-rRNA preocessing (Rodriguez Corona et al., 2015).

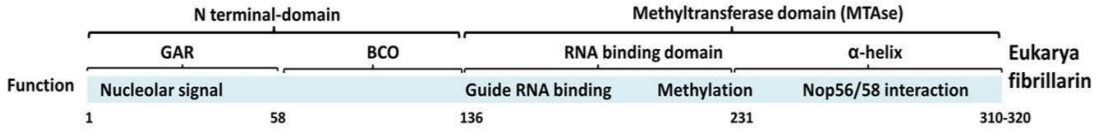

Figure 2.12. Structural alignment of eukaryotic fibrillarin. Representation of the primary structure of the Eukarya fibrillarin. The fibrillarin sequence is divided in four regions: The GAR domain is a sequence rich in glycine and arginine. BCO: a sequence with undefined activity. The methyltransferase domain contains the enzymatic activity as well as a conserved RNA binding sequence. This domain can be subdivided into RNA binding domain and the α-helix region that interacts with Nop56/58. The average amino acid position of each domain is given below the bar (Rodriguez-Corona et al., 2015).

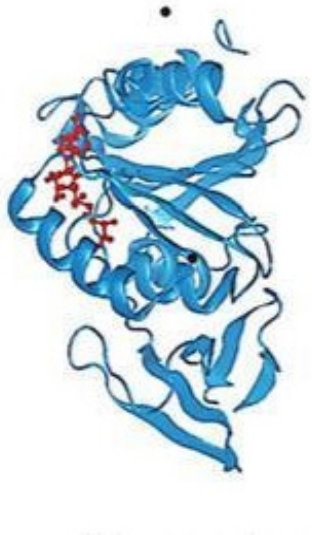

Figure 2.13. Crystal structure of fibrillarin from *Homo sapiens*. Protein data base ID: 2IPX. Black dots are Calcium ions. Red structure in left is *S*-Adenosyl methionine (Rodriguez-Corona et al., 2015).

Table 2.2. Two families of modification guide snoRNAs. Sets of associated proteins and categories of cellular RNA targets identified so far. Archaeal homologs of the two sets of snoRNP proteins are indicated (Bachellerie et al., 2002).

	C/D snoRNPs		H/ACA snoRNPs	
	Eukarya	**Archaea**	**Eukarya**	**Archaea**
Core proteins	Nop1p (Fibrillarin)	Fibrillarin	Cbf5p (Dyskerin)	Cbf5
	Nop56p (p62)	Nop56 /Nop58	Gar1p	Gar1
	Nop58p		Nop10p	Nop10
	snu13p (15.5 kDa)	rpL7Ae	Nhp2p	rpL7Ae
Transiently-associated proteins	Srp40p (Nopp140)		Srp40p (Nopp140p)	
	SMN		SMN	
	p50 (rvb2)	p50/p55		
	p55 (rvb1)			
RNA targets	rRNAs, snRNAs	rRNAs	rRNA, snRNAs	
	mRNAs (?)	tRNAs	SL RNA (trypanosome)	rRNAs
			other RNAs ?	

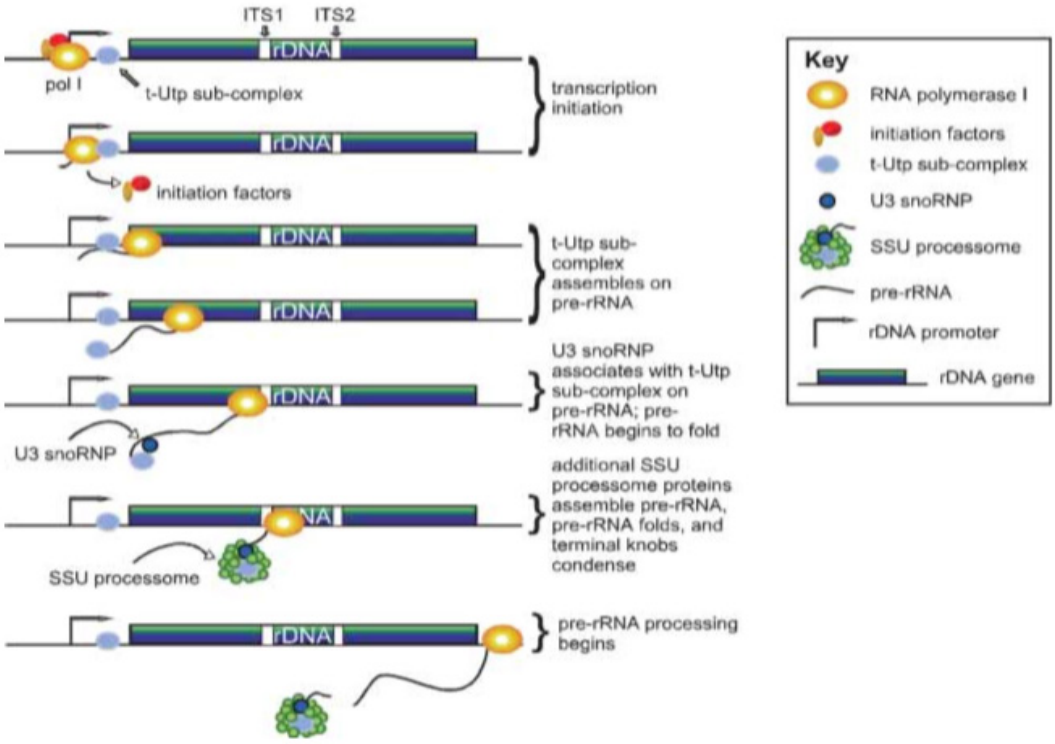

Figure 2.14. Model linking rRNA transcription and pre-rRNA processing via components of the SSU processome. Association of the t-Utps with the r-chromatin is required for efficient transcription, and allows their assembly onto the nascent rRNAtranscript.The U3 snoRNP then associates with the rRNA via base-pairing interactions, additional proteins join, and the SSU processome is formed. As a result, the pre-18S rRNA becomes accurately folded and ready for rRNA processing (Gallagher et al., 2004).

Nucleolin is involved in several steps of ribosome biogenesis, including regulation of rDNA transcription, rRNA processing and structural arrangement, but it is not well understood how nucleolin modulates the transcription (Roger et al., 2002; Cong et al., 2012). Depletion of nucleolin results in an increase in the heterochromatin mark H3K9me2 and a decrease in H4K12Ac and H3K4me3 euchromatin histone marks in rRNA genes. ChIP-seq experiments identified an enrichment of nucleolin in the coding and promoter region of rDNA. Nucleolin is preferentially associated with unmethylated rRNA genes and its depletion leads to the accumulation of RNAPI at the beginning of the transcription unit and a decrease in UBF along the coding and promoter regions. Nucleolin is able to affect the binding of transcription termination factor-1 on the promoter-proximal terminator T0, thus inhibiting the recruitment of TIP5 and HDAC1 and the establishment of a repressive heterochromatin state. Nucleolin is of great importance for the maintenance of chromatin status and the elongation of rDNA transcription (Cong et al., 2012). Nucleolin participates in the co-transcriptional packaging of the pre-rRNA, and the quality of this packaging will determine whether the 40S precursor undergoes maturation or is degraded. The interaction of nucleolin with nascent pre-rRNA could help the co-transcriptional assembly on pre-rRNA of factors necessary for the subsequent maturation of the pre-ribosomal particle containing the 40S pre-rRNA (Roger et al., 2003). Nucleolin allows RNA polymerase I transcription of chromatin templates *in vitro*. The results of chromatin immunoprecipitation experiments established that nucleolin is associated with chromatin containing rRNA genes transcribed by RNA polymerase I but not with genes transcribed by RNA polymerase II or III. Knockdown of nucleolin by RNA interference resulted in specific inhibition of RNA polymerase I transcription. (Rickards et al., 2007). Other studies suggest that nucleolin is involved in the regulation of rDNA transcription, rRNA processing, and ribosome assembly. Injection of a 2-4-fold excess of *Xenopus* or hamster nucleolin in stage VI *Xenopus* oocytes reduces the accumulation of 40 S pre-rRNA 3-fold, whereas transcription by RNA polymerase II and III is not affected. It was supposed that nucleolin affects RNA pol I transcription by acting directly on the transcription machinery or on the rDNA promoter sequences and not, as previously thought, through interaction with the nascent pre-rRNA (Roger et al., 2003).

Cockayne syndrome group B protein (CSB) plays a role in both transcription-coupled DNA repair and transcriptional regulation of all three classes of nuclear RNA polymerases. The active rRNA genes bind a complex composed of CSB, pol I and histone methyltransferase G9a. G9a methylates histone H3 on lysine 9 (H3K9me2) in pre-rRNA coding regions and facilitates the association of heterochromatin protein 1g (HP1g) with rDNA. H3K9 and HP1g methylation require ongoing transcription. CSB knockdown prevents association of Pol I with rDNA, disrupts the interaction of G9a with Pol I, and inhibits pre-rRNA synthesis. Likewise, knockdown of G9a leads to decreased levels of H3K9me2 in the transcribed region and downregulation of pre-rRNA synthesis (Yuan et al., 2007).

Nopp140 is pre-rRNA transcription-processing linking factor and is believed to shuttle between nucleolus and cytoplasm. Nopp140 and pre-rRNA processing factors such as DKC1 and fibrillarin (FBL) associate with r-chromatin during interphase; in mitosis Nopp140, DKC1, and FBL are released from r-chromatin (Ueshima et al., 2014).

C-Myc is transcription factor and protooncogen. C-Myc plays a crucial role in cell proliferation and apoptosis (Li and Hann, 2013). C-Myc levels are modulated by ubiquitin/proteasome-mediated decomposition. Proteasome inhibition leads to accumulation of c-Myc in the nucleolus. The c-Myc and Max proteins interact in the nucleolus and are associated with ribosomal DNA. C-Myc is required to activate rDNA transcription in response to mitogenic signals. C-Myc can activate Pol I transcription in the absence of Pol II transcription. C-Myc coordinates the activity of all three nuclear RNA polymerases and therefore plays a key role in the regulation of ribosome biogenesis and cell growth (Arabi et al., 2005). Human c-Myc also directly increases transcription of ribosomal RNA by pol I. Synthesis and rRNA accumulation rapidly follows activation of the Myc-ER allele encoding the Myc-estrogen-receptor fusion protein; the effect is resistant to inhibition of Pol II transcription and is significantly reduced by c-Myc RNAi. C-Myc binds to specific consensus elements located in human rDNA and associates with the selectivity factor SL1. The presence of c-Myc at specific sites on rDNA correlates with binding of SL1 to the rDNA promoter and increased histone acetylation of that region. Stimulation of c-Myc rRNA synthesis can be a key way for both cell growth and tumorigenesis (Grandori et al., 2005). The ability of c-Myc to induce the expression of all ribosomal components may explain its

strong effect on the cell growth, which depends on the accumulation of ribosomes (Gomez-Roman et al., 2006; Grewal et al., 2005). Nucleophosmin plays a significant role in c-Myc nucleolar localization and c-Myc mediated transcription of rDNA (Li and Hann, 2013). Activation of c-Myc induces association of TTF-1 with rDNA and c-Myc physically associates with induced genes from the rDNA gene (Shiue et al., 2009). It was found that active ribosomal genes form distinct chromatin loops by tethering to nucleolar matrix via the non-transcribed inter-genic spacer region, and that this process is dependent on the activity of the c-Myc protein (Shiue et al., 2014).

Transcription termination factor 1 (TTF-1) binds to Sal boxes at the 3'ends of the ribosomal genes and causes termination of the rDNA transcription. On the other hand, TTF-1 is necessary for the efficient transcription initiation. The spacer and the promoter proximal TTF-I-binding sites demarcate the enhancer. 3C analysis revealed an interaction between promoter and terminator regions, which brings the beginning and end of active rRNA genes into close contact (Nemeth et al., 2008). The binding of TTF-I is accompanied by changes in chromatin architecture, suggesting that TTF-I has a remodeling function upon the promoter (Strohner et al., 2001). During mitosis TTF-1 is specifically phosphorylated in a manner dependent on the Cdc2/cyclin B kinase pathway and on an okadaic acid-sensitive phosphatase. The chromatin-binding affinity of TTF-1 appears to be different in mitotic chromosomes compared to the interphase nucleolus (Sirri et al., 1999).

CCCTC-binding factor (CTCF) is a multifunctional factor involved in many cellular processes, such as gene regulation, chromatin isolation and genomic organization. CTCF through epigenetic mechanisms contributes to the regulation of ribosomal gene expression (Hernandez-Hernandez et al., 2012). CTCF is a highly conserved zinc finger protein that participates in local histone modifications, and RNA polymerase II mediated transcription. CTCF being an insulator binds to the spacer promoter of rDNA, suggesting that transcriptional insulation plays a role in regulating rRNA transcription (Zentner et al., 2011). The insulators can prevent activator of one gene from interacting with the promoter of a neighbouring gene. Almost all described insulators of vertebrates require for their activity binding of the CTCF (Yusufzai et al., 2004). CTCF bound to a site upstream of the rDNA spacer promoter and preferred non-methylated over methylated rDNA. The DNA binding of CTCF stimulates the binding of UBF (van de Nobelen et al., 2010). Analysis of CTCF subnuclear localization has

shown that it is distributed homogeneously in both DFC and GC subunits of nucleolus but is not associated with fibrillar centers (Torrano et al., 2006). CTCF can bind UBF to rDNA, forming part of the pathway that maintains active rDNA chromatin (van de Nobelen et al., 2010). CTCF also regulates the course of rDNA replication. Condensin competes with CTCF in relation to a specific rDNA locus and negatively regulates the CTCF-mediated transcription of rRNA gene (Huang et al., 2013). Transcription by pol I and protein synthesis are needed to maintain nuclear localization of CTCF (Torrano et al. 2006).

2.3. Transcription of ribosomal genes

2.3.1. General characterization

The activities of pol I and pol III dominate cellular transcription, combining to exceed 80% of total RNA synthesis in growing cells (Paule and White, 2000). Different types of cells of the same organism exhibit different numbers of transcriptional units reflecting their differential metabolic activity (Haaf et al., 1991). The number of the active repeats may affect the overall level of rRNA synthesis. (Birch and Zomerdijk, 2008). But nucleolar transcriptional activity does not always correlate with the total number of rRNA genes (Haaf et al., 1991)

Assembly of the RNA polymerase (Pol I) pre-initiation complex (PIC) at ribosomal RNA gene promoters begins with the binding of UBF to the upstream control element (UCE) and core element (CE) of the rDNA promoter, leading to the recruitment of the selectivity factor SL-1. SL1 and other pol I specific factors guide Pol I to the rDNA promotor and contribute to multiple rounds of transcription initiation, promoter escape, elongation and termination. In addition, many accessory factors are now known to assist at each stage of this transcription cycle, some of which allow the integration of transcriptional activity with metabolic demands (Goodfellow and Zomerdijk, 2013). In the course of the transcriptional elongation ribosomal genes with attached nascent transcripts appear on the preparation of spread chromatin as so-called “Christmas trees” (Miller and Beatty, 1969; Figure 2.15).

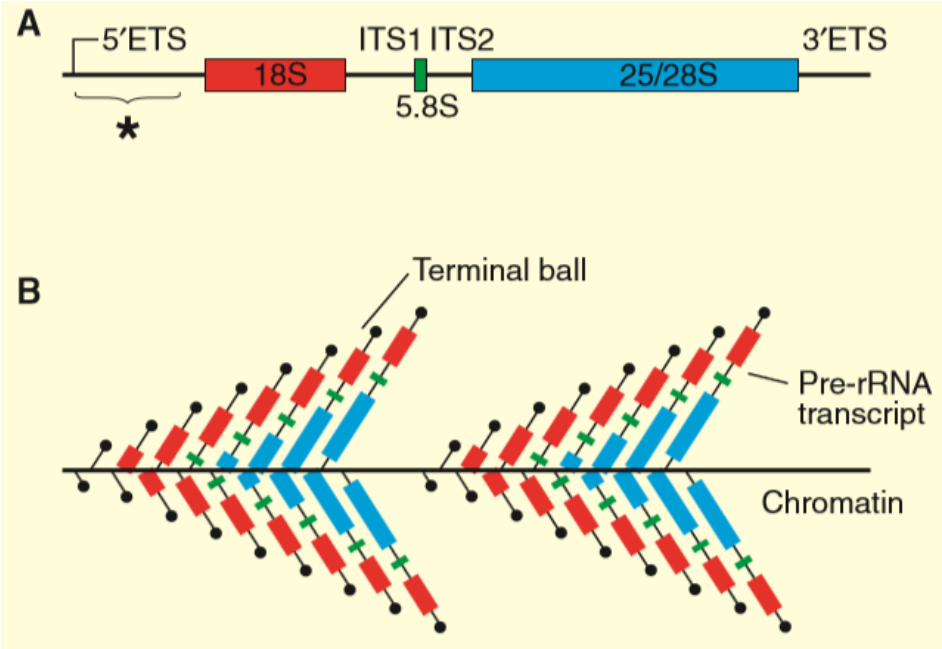

Figure 2.15. Ribosomal DNA transcription and beginning of the rRNA processing. (A) A schematic of pre-RNA, indicating the mature 18S (red), 5.8S (green) and 25/28S (blue) rRNAs which must be removed from the precursor. An ordered set of cleavages remove the 5′ and 3′ external transcribed spacers (ETS) and the internal transcribed spacers, ITS1 and ITS2. The bracket and asterisk indicate the region required for terminal ball formation and where U3 binds the pre-rRNA. (B) A schematic of the 'Christmas tree' appearance of actively transcribed rDNA loci visualized by electron microscopy: the chromatin is the 'trunk', the closely packed rRNA transcripts constitute the 'branches' and at the tip of each transcript is a 'terminal ball', all of which are indicated. The elongating transcript encodes 18S, 5.8S and 25/28S rRNAs color-coded as in panel A (Peculis, 2002).

Transcription elongation plays perhaps a key role in regulating the intensity of rRNA gene transcription (Moss et al., 2006). It is not clear how the various components of the "Christmas trees" are accommodated within the nucleoli *in situ.* But it is believed that the promoter and terminator of each active rRNA gene are held together spatially throughout the cell cycle, forming a stable core around which the transcribed region is organized (Denissov et al., 2011).

Stereological analysis of the clustered transcription signal corresponding to the "Christmas trees" sampled on the surface of thin EM sections suggests that these Christmas trees may be contorted in space and exhibit a DNA compaction ratio on the order of 4-5.5 (Koberna et al., 2002).

The interaction of transcription factors with the target genes is highly dynamic. Whether the dynamic nature of these interactions is only the intrinsic property of transcription factors or plays a regulatory role is unknown. Transcriptional upregulation is accompanied by prolonged retention of RNA pol I components in the promoter, resulting in longer promoter dwell time and an increase in the steady-state population of assembling polymerase. As a consequence, polymerase assembly efficiency and, ultimately, the rate of entry into elongation are elevated. (Gorski et al., 2008). Termination of the rDNA transcription has specific features. The termination of mouse rDNA transcription requires the element AGGTCGACCAGATNTCCG (Sal1 box) which is repeated eight times at the 3 'end of the rRNA segment. This sequential motif interacts with a specific nuclear protein that controls the termination of transcription (Grummt et al., 1986; Kuhn et al., 1988). Two out of the five human Sal box elements are functionally inactive due to natural point mutations which damage factor binding. A similar sequence motif with a 10 of 11 base identity with the downstream terminators is located upstream of the human transcription initiation site. The upstream element interacts with the same factor(s) as the downstream terminators and is also capable to stop elongating human RNA polymerase I (Pfleiderer et al., 1990).

Generally, chromatin may be in three different states: suppressed or silenced, nontranscribed but potentially transcribable (potentiated or prepared for transcription) and transcriptionally active. Various conformations of rDNA are probably equivalent to these three chromatin stages (Shaw and McKeown, 2011; Murano et al., 2008). In active nucleolar rRNA genes, promoters are almost completely demethylated, in the mutated genes the promoters are almost completely methylated (Pontvianne et al., 2013; Xie et al., 2012). Transcription of rDNA is accompanied by acetylation and methylation of nucleosomes near promoters, which is a general feature of eukaryotic genes (Heintzman et al., 2007). Epigenetic control mechanisms silence about half of the ribosomal genes in metabolically active cells (Santoro et al., 2002). Interplay of DNA methylation, histone modification and chromatin-remodeling activities establishes silencing at the rDNA locus in higher eukaryotes as well as at the underdominant genes in hybrid cells. (Santoro, 2005). Epigenetic silencing of a fraction of rDNA requires association of the nucleolar chromatin-remodelling complex NoRC to 150-250 nucleotide RNAs (pRNA) that originate from an RNA polymerase I

promoter located in the intergenic spacer separating rDNA repeats. (Santoro et al., 2010; Mayer et al., 2008). Active and silenced chromatin domains are often in close juxtaposition to one another, and enhancer and silencer elements operate over large distances to regulate the genes in these domains (Valenzuela and Kamakaka, 2006; Zillner et al., 2015). The ATP-dependent chromatin remodeling complex (NoRC) silences part of the mammalian ribosomal RNA genes (rDNA) as well as other large repetitive regions, such as centromeres and telomeres, by creating heterochromatic structures on the rDNA promoter (Li et al., 2004). SNF2h containing chromatin-remodeling complex NoRC is responsible for silencing a fraction of mammalian rRNA genes (Zhou and Grummt, 2005; Strohner et al., 2001). NoRC is the molecular machine that shifts the promoter-bound nucleosome downstream of the transcription start site into a translational position that is unfavorable for transcription complex formation (Li et al., 2006). Some studies show that NoRC is also an important determinant of replication timing and that epigenetic markers are maintained through DNA replication (Li et al., 2004). NoRC mediates inhibition of rRNA synthesis by affecting the activity of DNA methyl transferase and histone deacetylase on the rDNA promoter (Santoro et al., 2002). Association of NoRC with chromatin requires the interaction of the TAM (TIP5/ARBP/MBD) domain of TIP5 with noncoding pRNA, which targets NoRC to specific genomic loci. (Anosova et al., 2015).

The nucleosome remodeling and deacetylation (NuRD) complex establishes a specific chromatin structure at rRNA genes that are poised for transcription activation. The promoter of poised rRNA genes is unmethylated, associated with components of the preinitiation complex, marked by bivalent histone modifications and covered by a nucleosome in the "off" position, which is refractory to transcription initiation. Repression of rDNA transcription in growth-arrested and differentiated cells correlates with elevated association of NuRD and increased levels of poised rRNA genes. Reactivation of transcription requires resetting the promoter-bound nucleosome into the "on" position by the DNA-dependent ATPase CSB (Cockayne syndrome protein B). (Xie et al., 2012).

2.3.2. Kinetics of rDNA transcription

Recent studies based on new single-cell and single-gene techniques indicate that genes transcribed by pol II are often, perhaps even for the most part, expressed discontinuously, i.e. in pulses alternated by the periods of inactivity (reviewed in Smirnov et al., 2018). It is not clear yet, whether the same applies to the ribosomal genes as well. The kinetics of RNA polymerase I, which transcribes ribosomal genes, is particularly difficult to reveal, since one cell typically contains multiple copies of these genes, and their expression is usually very intensive. But direct measurements of rRNA (ribosomal RNA) production in the entire nucleoli by the label-free confocal Raman microspectrometry showed a pulse-like pattern of the rDNA (ribosomal DNA) transcription. In our work (Hornacek et al., 2017) on tumour-derived cells expressing a GFP-RPA43 (a subunit of pol I) fusion protein, we measured the fluorescence signal upon the nucleolar beads, which are likely to represent individual transcriptionally active genes (Haaf and Schmid, 1991; Haaf and Ward, 1996; Cheutin et al., 2002; Denissov et al., 2011). Our data, complemented with the measurements of the incorporated fluorouridine signal, suggested that the ribosomal genes are also transcribed in pulse-like manner (Hornacek et al., 2017).

2.4. Replication in the nucleoli

Chromosomal DNA replication in eukaryotes initiates at many sites on each chromosome during the S phase of the cell cycle. Each origin of replication lies in a unique chromosomal environment and can be regulated in different cell types both at the level of utilization and the time of initiation during S phase (Diffley and Stillman, 1990). Replication of genomic material is a process that requires not only high fidelity in the duplication of DNA sequences but also inheritance of the chromatin states (Santoro and De Lucia, 2005).

Replication of the 400 copies of the 43 kb human ribosomal rDNA locus spans most of the S phase (Scott et al., 1997). Expressed rDNA genes are believed to replicate during the first half of S phase. Paradoxically, attempts to visualize replicating rDNA during early S phase have failed. In human (HeLa) cells, early replicating rDNA, which predominantly represents active rDNA units, is well detectable only at the nucleolar periphery. It was suggested that replication of these

units takes place on the periphery or even outside the nucleoli (Dimitrova, 2011). PCNA is used as a replication signal in cells (figure 2.16).

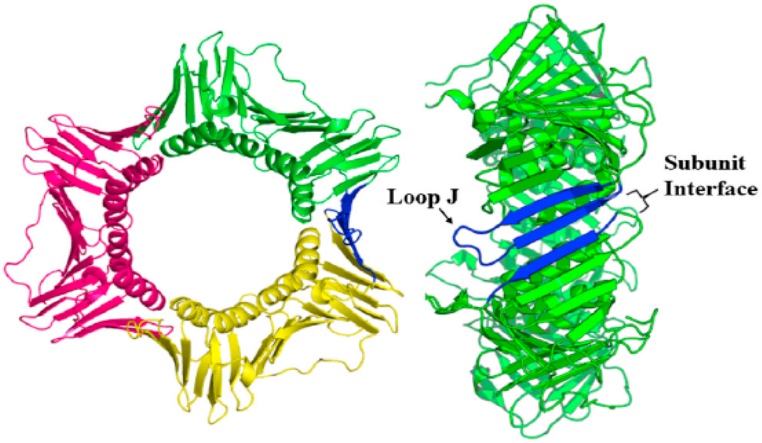

Figure 2.16. Structure of PCNA. The biological unit of PCNA is a trimeric ring with a central hole for binding double-strand DNA. Viewing PCNA from the side shows the subunit interface between two PCNA monomers (blue) (LuCore et al., 2015).

In *Saccharomyces cerevisiae* most ribosomal DNA is replicated unidirectionally by forks moving in the direction of transcription and most replicons are larger than the repeat unit (Liskens and Huberman, 1988). Replication forks (figure 2.17) move through most of the rDNA in the same direction in which RNA polymerase I transcribes the 35s rRNA precursor: the 3' end of this transcription unit acts as a barrier (replication fork barrier, RFB) to replication forks moving in the direction opposite to RNA polymerase I (Brewer and Fangman, 1988).

In mammalian rDNA genes replication may start at various points of the transcribed and non-transcribed units (Lebofsky and Bensimon, 2005). But there seem to be two preferable regions of initiation of DNA synthesis. The first one is located upstream of the transcription units and the second one is located at the 3′-end of the coding regions of the ribosomal DNA repeats (Gencheva et al., 1996; Little et al., 1993; Yoon et al., 1995). But because of low efficiency of the RFB some replication forks move in upstream direction (Lebofsky and Bensimon, 2005). Replication of the ribosomal genes is spatially separated from their transcription, and FC/DFC units of the nucleoli may provide a structural basis for that separation (Pliss et al., 2005; Smirnov et al., 2014).

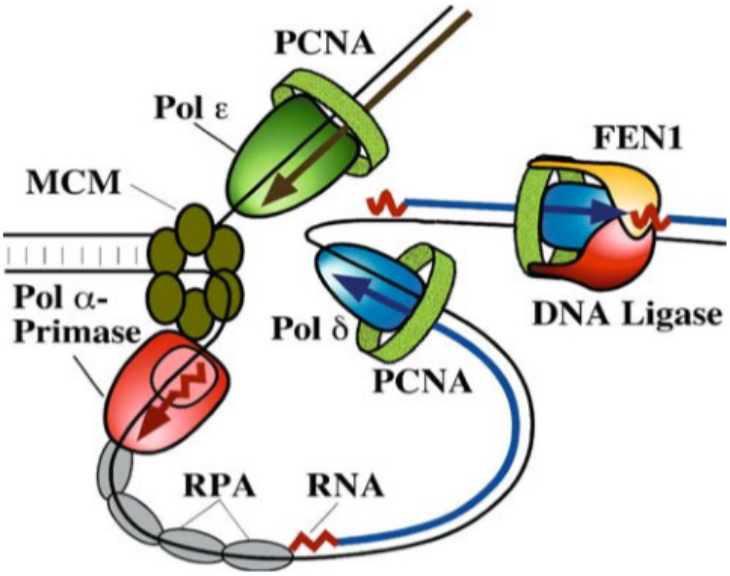

Figure 2.17. Eukaryotic DNA replication fork. The minimal set of proteins for fork propagation are indicated. PCNA is green ring. Abbreviations: RPA - Replication protein A; PCNA - Proliferating cell nuclear antigen; MCM - Minichromosome maintenance protein complex, DNA helicase; RNA - RNA primer; Pol α-Primase - DNA polymerase *α*; Pol ε - DNA polymerase ε; Pol δ - DNA polymerase δ; FEN1 - Flap endonuclease 1; (Garg and Burgers, 2005).

Alterations of the dynamics of DNA replication cause genome instability. These alterations known as “replication stress” have emerged as a major source of genomic instability in pre-neoplasic lesions, contributing to cancer development. The concept of replication stress covers a wide variety of events that distort the temporal and spatial DNA replication program (Magdalou et al., 2014). Thus recombination initiated at terminal single-strand hairpin loops can result in genetic exchange of ribosomal gene sequences and can lead to completion of 5’ nucleotide sequences at ends of newly replicated rDNA molecules (Bergold et al., 1983). Mutations in the terminator element, depletion of the transcriptional terminator factor TTF-I specific for RNA polymerase I or deletion of the termination domain of TTF-I eliminates RFB activity. Thus the same factor that blocks the pol I elongation prevents collision between the replication and transcription mechanisms (Gerber et al., 1997). Human rRNA genes represent a unique challenge to DNA replication since a large proportion of them exists as noncanonical palindromes in addition to canonical tandem repeats. (Lebofsky and Bensimon, 2005).

3. LIST OF PUBLICATIONS

This thesis is based on the following papers:

Hornáček M., Kováčik L., Mazel T., Cmarko D., Bártová E., Raška I. and Smirnov E. (2017). „Fluctuations of pol I and fibrillarin contents of the nucleoli.“ Nucleus 8(4):421-432. doi: 10.1080/19491034.2017.1306160. IF:3,148.

Smirnov E., **Hornáček M.**, Kováčik L., Mazel T., Schröfel A., Svidenská S., Skalníková M., Bártová E., Cmarko D. and Raška I. (2016). „Reproduction of the FC/DFC units in nucleoli.“ Nucleus 7(2):203-215. doi: 10.1080/19491034.2016.1157674. IF:3,148.

Smirnov E., **Hornáček M.**, Vacík T., Cmarko D. and Raška I. (2018). „Discontinuous transcription.” Nucleus 9(1):149-160. doi: 10.1080/19491034.2017.1419112. IF:3,148.

Smirnov E., Cmarko D., Mazel T., **Hornáček M.** and Raška I. (2016). „Nucleolar DNA: the host and the guests.“ Histochemistry and Cell Biology 145(4):359-372. doi: 10.1007/s00418-016-1407-x. IF:2,553.

Other author's publications:

Bártová E., Legartová S., Krejčí J., Řezníčková P., Kovaříková A., Suchánková J., Fedr R., Smirnov E., **Hornáček M.** and Raška I. (2018). „Depletion of A-type lamins and Lap2α reduces 53BP1 accumulation at UV-induced DNA lesions and Lap2α protein is responsible for compactness of irradiated chromatin.“ Journal of Cellular Biochemistry *In press*. doi: 10.1002/jcb.26770. IF:3,085.

Bártová E., Suchánková J., Legartová S., Malyšková B., **Hornáček M.**, Skalníková M., Mašata M., Raška I. and Kozubek S. (2017). „PCNA is recruited to irradiated chromatin in late S-phase and is most pronounced in G2 phase of the cell cycle.“ Protoplasma 254(5):2035-2043. doi: 10.1007/s00709-017-1076-1. IF:2,870.

Medel S., Syrová Z., Kováčik L., Hrdý J., **Hornáček M.**, Jäger E., Hrubý M., Lund R., Cmarko D., Štěpánek P., Raška I. and Nyström B. (2017). „Curcumin-bortezomib loaded polymeric nanoparticles for synergistic cancer therapy.“ European Polymer Journal 93:116-131. doi: 10.1016/j.eurpolymj.2017.05.036. IF:3,531.

Farkaš R., Pečeňová L., Mentelová L., Beňo M., Beňová-Liszeková D., Mahmoodová S., Tejnecký V., Raška O., Jůda P., Svidenská S., **Hornáček M.**, Chase B. A. and Raška I. (2016). „Massive excretion of calcium oxalate from late prepupal salivary glands of *Drosophila melanogaster* demonstrates active nephridial-like anion transport.“ Development Growth and Differentiation 58(6):562-574. doi: 10.1111/dgd.12300. IF:2,145.

4. THE AIMS OF THE WORK

- To follow cell cycle related changes of FC/DFC units in human sarcoma derived cell lines with stable expression of RFP-PCNA (the sliding clamp protein) and GFP-RPA43 (a subunit of RNA polymerase I, pol I) or GFP-fibrillarin.
- *In vivo* observations of nucleoli at early S phase of cell cycle, when transcriptionally active ribosomal genes are replicated.
- To correlate signals of polymerase I and fibrillarin (processing factor protein) in individual FC/DFC units.
- To correlate signals of polymerase I and fibrillarin (processing factor protein) in individual FC/DFC units using correlative light and electron microscopy analysis (CLEM).
- To follow changes of the fluorescent signals in individual FC/DFC units.
- To search types of kinetics of transcription signal intensity and intensity of early processing inside nucleolus.
- To discuss the characteristics of ribosomal DNA: the structure of the rDNA locus, complex organization and functions of the intergenic spacer, multiplicity of gene copies in one cell, selective silencing of genes and whole gene clusters, relation to components of nucleolar ultrastructure, specific problems associated with replication.
- To review current data on the role of non-ribosomal DNA in the organization and function of nucleoli.
- To discuss probable causes preventing efficient visualization of DNA in nucleoli.
- To discuss the occurrence of the transcriptional fluctuations, the techniques used for their detection, their putative causes, kinetic characteristics, and probable physiological significance.

5. MATERIAL AND METHODS

5.1. Cell cultures and cell lines

In our experiments we used these cell cultures:

- HeLa (human cell line derived from cervical cancer cells, Institute's own collection)
- HEp2 (human epithelial type 2 cells from laryngeal carcinoma, Institute's own collection)
- HT-1080 (human fibrosarcoma cells, Institute's own collection)
- Primary LEP (human embryonic fibroblasts, Sevapharm, Czech Republic).

Each cell lines were cultivated at 37 °C in Dulbecco´s modified Eagle's medium (DMEM, Sigma-Aldrich, #D5546) containing 10% fetal calf serum, 1% glutamine, 0.1% gentamicin, and 0.85 $g.l^{-1}$ $NaHCO_3$ in standard incubators.

We produced 2 cell lines stably expressing:

1.) GFP-RPA43 and RFP-PCNA (Smirnov et al, 2014),

2.) GFP-fibrillarin and RFP-PCNA (Smirnov et al, 2014).

5.2. Plasmids and Transfection

5.2.1. Plasmids

- The plasmid constructs for RFP-fibrillarin and RFP-PCNA were received from the Max Planck Institute for Molecular Cell Biology and Genetics, Dresden.
- GFP-RPA43 and GFP-fibrillarin vectors were received from Laboratory of Receptor Biology and Gene Expression, Bethesda, MD.

5.2.2. Transfection

- The constructs were transfected into HT-1080 cells using Fugene (Promega, #E2312) by standard protocol procedure of „FuGENE HD Transfection Reagent" (Promega): (3 µl of Fugene, 97 µl of medium without serum and 1 µg of plazmid DNA).
- Geneticin, aminoglycoside commonly known as G418 or G-418 (GIBCO, #11811031) was used for selection of stable clones with two-colored fluorescence.

5.2.3. Transfection protocol of mammalian cells (according to Promega)

1.) Dilute 3 µl of „FuGENE HD Transfection Reagent“ with 97 µl of serum-free medium without antibiotics or fungicides.
2.) After dilution vortex for one second or flick the tube to mix. Incubate for 5 minutes at room temperature.
3.) Add 1 µg DNA to solution of diluted FuGENE HD Transfection Reagent in serum-free medium. Mix and incubate the complex for a minimum of 15 minutes.
4.) After 15 minutes add complex to the Petri dish with cells and with 1 ml of complete DMEM growth medium (remove 1 ml from 2 ml of total volume of medium) and return the cells to the incubator for incubating for 18 to 72 hours.

5.3. Immunocytochemistry

Primary antibodies against human rRNA polymerase (pol I), human fibrillarin and Upstream Binding Factor (UBF) were kindly provided by Dr. U. Scheer (Biocenter of the University of Wurzburg). We also used polyclonal (rabbit) anti-RPA43 (Thermo Fischer Scientific, # PIPA525184). We visualized fibrillarin in nucleoli with antibodies against human fibrillarin or mouse monoclonal fibrillarin (clone 17C12), kindly donated by Kenneth M. Pollard (Scripps Research Institute, La Jolla, CA).

Secondary anti-human, anti-rabbit and anti-mouse antibodies were conjugated with Alexa 488 (Invitrogen, #H10120), Alexa 532 (Invitrogen, #A-11002), Cy3 (Jackson ImmunoResearch Laboratories, #109-165-088, #111-165-003, #115-025-062), or DyLight 488 (Jackson ImmunoResearch Laboratories, #109-005-044, #111-475-003, #115-475-003).

After incorporation of 5-fluorouridine (FU), the signal was visualized by a mouse monoclonal anti-BrdU antibody (Sigma-Aldrich, #B8434).

Coverslips with the cells were mounted in Mowiol (Sigma-Aldrich) containing DABCO (Sigma-Aldrich). One drop of mounting medium, approximately 7 µl per one coverslip.

5.3.1. Immunostaining protocol

1.) Cells overnight on coverslips.
2.) Fixation with 2% PFA (paraformaldehyde) in 1x PBS at room temperature.
3.) 3x3 minutes 1x PBS rinse.
4.) Permeabilization with Triton X100 (20 µl Triton, 5 ml 1x PBS).
5.) 3x3 minutes 1x PBS rinse.
6.) Blocking with 1% BSA in 1x PBS
7.) 3x3 minutes 1x PBS rinse.
8.) Primary antibody(ies): in blocking solution (1% BSA in 1x PBS), 1 hour at room temperature.
9.) 3x3 minutes 1x PBS rinse.
10.) Secondary antibody(ies): in blocking solution (1% BSA in 1x PBS), 45 minutes at room temperature in dark chamber.
11.) 5x3 minutes 1x PBS rinse.
12.) 3x1 minutes 1x H_2O rinse.
13.) Mount on slides using Mowiol (7 µl).

5.4. Incorporation of nucleotides

For labeling of replication and transcription sites, sub-confluent cells were incubated 5 min with 5-ethynyl-2´-deoxyuridine (EdU) (Invitrogen, #C10337) at a final concentration of 10 mM and 5-fluorouridine (FU) (Sigma-Aldrich, #F5130) at a concentration of 100 mM.

The replication signal was visualized using EdU Alexa Fluor 647 Imaging Kit (Invitrogen #C10337). Additionally, we used incorporation of Cy3-dUTP and Cy5-dUTP, which were introduced into the cells by means of the scratch procedure. Each antibodies were dissolved in 1% BSA-PBS.

5.4.1. 5-ethynyl-2´-deoxyuridine (EdU) incorporation protocol

1.) Cells overnight on coverslips in Petri dish with growth medium.
2.) Add 2 µl EdU (1:1000) to Petri dish and incubate for 5 minutes.
3.) Fixation with 2% paraformaldehyde (PFA) in 1x PBS (1g paraformaldehyde dissolve in 50 ml of heated solution 1x PBS. Cool

under a stream of water and finally, add 9 µl of 4M NaOH for pH adjustment) for 10 minutes in dark chamber.

4.) 3x3 minutes 1x PBS rinse.
5.) Permeabilization with Triton X100 (20 µl Triton, 5 ml 1x PBS) for 5 minutes.
6.) 3x1 minute 1x PBS rinse.
7.) Add EdU cocktail for 30 minutes.

Components (solutions) of commercially selling EdU cocktail (Invitrogen, #C10337):

a.) Component D – 45 µl
b.) Copper – 2 µl
c.) H_2O – 9 µl
d.) Component F – 1 µl
e.) Colour – 0,5 µl

Total volume of EdU cocktail: 50,5 µl

At first mix component D + copper. At second mix H_2O + component F. After that mix these two solutions to one volume and at last add colour and stir.

8.) 3x1 minute 1x PBS rinse.
9.) 2x1 minute in H_2O and mount on slides using Mowiol (7 µl).

5.4.2. 5-fluorouridine (FU) incorporation protocol

1.) Cells overnight on coverslips in Petri dish with growth medium.
2.) Add 10 µl FU (1:200) to Petri dish and incubate for 5 minutes.
3.) Fixation with methanol on ice for 30 minutes in dark chamber.
4.) 3x3 minutes rinse in PBS.
5.) Primary antibody (1:100) in blocking solution of 1% BSA in 1x PBS for one hour in humid dark chamber.
6.) 3x3 minutes rinse in 1x PBS.
7.) Secondary antibody, anti-mouse Cy3 (1:100) in blocking solution of 1% BSA in 1x PBS for 40 minutes in humid dark chamber.
8.) 3x1 minutes rinse in 1x PBS.
9.) 3x1 minutes rinse in H_2O and mount on slides using Mowiol (7 µl).

5.4.3. Scratching protocol

1.) Cells overnight on coverslips in Petri dish with growth medium.
2.) Mix 25 µl PBS + 0,5 µl Cy5 dUTP in Eppendorf tube, pack all tube to the aluminium foil and vortex.
3.) Replace all coverslips from Petri dish with growth medium to new clean Petri dish and dry them on filtering paper. All the following steps are done in the dark.
4.) Drop 25 µl of prepared solution of antibody from Eppendorf tube on the top of each coverslip with cells. After that scratch all surface of coverslip with needle 2 times from one side of the coverslip to the other side. After that turn the slide around 90 degrees and scratch it the same way.
5.) After that replace Petri dish with coverslips to incubator for 10 minutes.
6.) 2x1 minutes fast rinse in 1x PBS.
7.) Replace coverslips back to Petri dish with primary growth medium for 10 minutes.
8.) Fixation with 4% PFA in 1x PBS for 10 minutes.
9.) 3x3 minutes rinse in 1x PBS.
10.) 3x1 minutes rinse in H_2O and mount on slides using Mowiol (7 µl).

5.5. Confocal microscopy and FRAP

The basic principle of Fluorescence recovery after photobleaching (FRAP) is that fluorescent molecules are irreversibly photobleached in a small region of the cell with a high-power laser beam. The subsequent movement of surrounding nonbleached fluorescent molecules into the bleached region (i.e., recovery) is monitored with low-power laser imaging. The rate at which fluorescence in the bleached area recovers is a measure of mobility. Mobility is a combination of diffusion and binding interactions. Comparison of the recovery of a nonbinding control to that of the molecule of interest reveals a difference in mobility attributable to the molecule of interest. The degree of slowed mobility in the recovery of the protein of interest is a measure of binding strength (Hardy, 2012).

FRAP is a widely used tool for estimating mobility parameters of fluorescently tagged molecules in cells (Braga et al., 2004). Comparison of the diffusion times ($t_{1/2}$) from a FRAP experiment provides qualitative estimates of

diffusion rates. In comparison with other fluorescent markers, the green fluorescent proteins (GFP) possess characteristics that are ideal for use in such experiments (Pucadyil and Chattopadhyay, 2006).

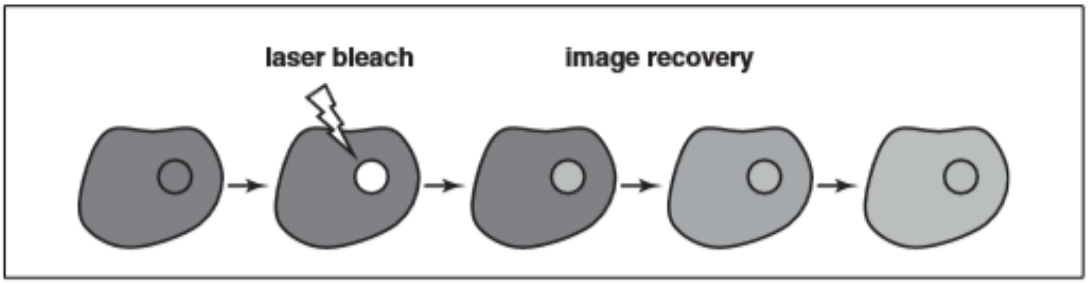

Figure 5.1. Schematic illustrating of Fluorescence recovery after photobleaching (FRAP). A cell expressing fluorescently tagged protein under goes high-power laser photobleaching in a defined region of interest (ROI) in the cell. Recovery from the laser bleach is monitored over time by low-power laser imaging. Due to imaging there is a reduction in whole-cell fluorescence concurrent with a recovery of fluorescence in the bleached ROI (Hardy, 2012).

After the bleaching, a low intensity laser light is used to follow the recovery of fluorescence caused by the concurrent inward diffusion of neighbouring (non-bleached) molecules into the bleached region and the outward diffusion of the bleached molecules. The analysis of the resulting fluorescence recovery versus time curve yields recovery times that are used to obtain diffusion coefficients of the diffusing species (Rayan et al., 2010).

The diffusion coefficient is equal to:

$$D = \frac{r^2}{4\tau}$$

where r is the radius of the circular beam, and τ is the time constant obtained from the fit of the curve (Rayan et al., 2010; Soumpasis, 1983).

The r value needs to be precisely known as any deviation in this value will lead to incorrect D values. The fluorescence recovery curve was fit via the following equation (Rayan et al., 2010; Soumpasis, 1983; Braeckmans et al., 2003):

$$\frac{F_{tot}(t)}{F_0} = 1 + \left\{ \sum_{n=1}^{+\infty} \left[\frac{(-K_0)}{n!} \frac{1}{\sqrt{1+n}} \right] \times \left(1 - e^{-2(\tau/t)} \left(I_0\left(2\frac{\tau}{t}\right) + I_1\left(2\frac{\tau}{t}\right) \right) \right) \right\}$$

where F_0 is the total fluorescence at time zero (i.e., before bleaching), K_0 is the bleaching parameter, and I_0 and I_1 are the modified Bessel functions of the 0th and 1st order respectively (Rayan et al., 2010; Braeckmans et al., 2003).

Two parameters can be deduced from FRAP: the mobile fraction of fluorescent molecules and the rate of mobility, which is related to the characteristic diffusion time, τ_D. An equation below shows a typical fluorescence recovery curve, allowing the determination of the two parameters. The mobile fraction can be determined by comparing the fluorescence in the bleached region after full recovery (F_∞) with the fluorescence before bleaching (F_i) and just after bleaching (F_0).The mobile fraction R is defined as:

$$R = (F_\infty - F_0)/(F_i - F_0)$$

The mobile fraction can change in different circumstances, for example when the fluorescent protein interacts with other molecules or membranes. The mobile fraction can also be affected by membrane barriers and microdomains in the membrane. These discontinuities can prevent, or temporarily restrict, the free diffusion of membrane molecules (Reits and Neefjes, 2001).

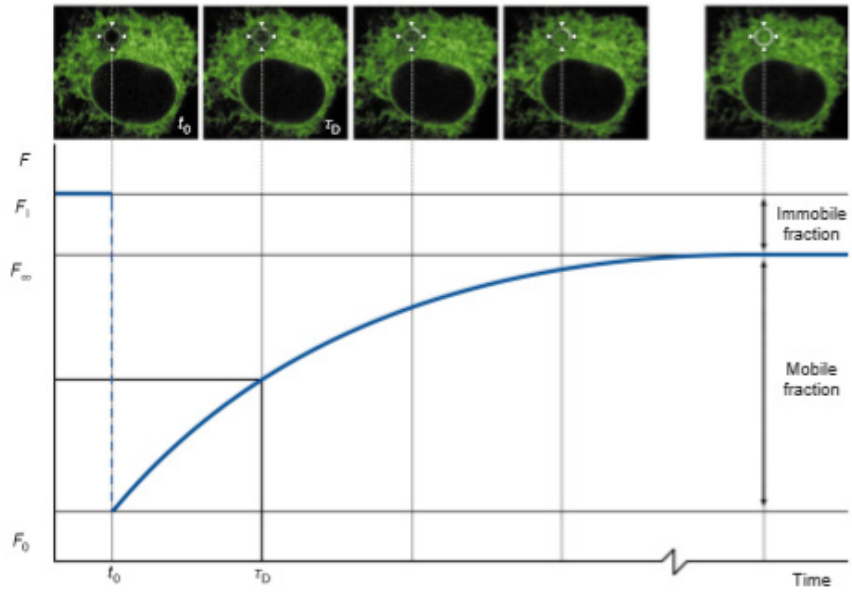

Figure 5.2. Time period diagram of FRAP. When a region in the fluorescent area (here the endoplasmic reticulum) is bleached at time t_0 the fluorescence decreases from the initial fluorescence F_i to F_0. The fluorescence recovers over time by diffusion until it has fully recovered (F_∞). The characteristic diffusion time τ_D indicates the time at which half of the fluorescence has recovered. The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery (F_∞) with that before bleaching (F_i) and just after bleaching (F_0) (Reits and Neefjes, 2001).

In our experiments were confocal images of fixed samples acquired using a confocal laser scanning microscope Leica SP5 equipped with a 63x/1.4 NA oil immersion objective.

5.5.1. *In vivo* observations and FRAP

For *in vivo* cell imaging we used a spinning disk confocal system based on Olympus IX81 microscope (Olympus, Tokio, Japan) equipped with Olympus UPlanSApo 100x/1.4 NA oil immersion objective with CSU-X spinning disk module (Yokogawa, Tokio, Japan) with speed 5000 rpm per minute, Ixon Ultra EM-CCD camera (Andor, Belfast, UK) and FRAPPA unit (Andor, Belfast, UK) for FRAP experiments. The live cells were maintained in glass bottom Petri dishes (MatTek) at 37 °C and 5% CO_2 using a microscope incubator (Okolab). For assessment of the technical error during the image acquisition, control measurements of signal intensity dynamics were performed on 170 nm carboxylate yellow-green microspheres (Invitrogen, #P7220).

5.6. Correlative light and electron microscopy (CLEM)

Fundamental to understanding how biomolecules regulate life is the ability to study them at the near-molecular level. Microscopic techniques are highly valuable to determine protein location in space and time. Ongoing development of genetically encoded fluorescent proteins, such as the green fluorescent protein (GFP), allows imaging in living cells. However it can still not provide information about the (ultrastructural) cellular context, as can be determined with electron microscopy (EM) (Sjollema et al., 2012). Correlative microscopy is a method when for the analysis of the very same cell or tissue area several different methods of light or electron microscopy are used. Usually these methods of analysis are separated by the period of additional preparation (Mironov and Beznoussenko, 2013).

CLEM is typically performed in one of two ways:

(A.) samples are analyzed by fluorescence imaging-for instance, timelapse studies of tagged with fluorescent proteins followed by fixation and further EM processing, acquisition and analysis (de Boer et al., 2015).

(B.) ultrathin sections prepared for EM still contain fluorescent label, or are fluorescently labeled, and are imaged with both LM and EM, for instance after immunolabeling. The latter approach also allows for analysis with integrated microscopes as discussed below. Probes to identify specific molecules, organelles or cells are either genetically encoded or affinity based (de Boer et al., 2015).

5.6.1. CLEM protocol

1.) Cells for experiment were grown in glass bottom Petri dishes with grid (MatTek) under standard conditions (37 °C, 5% CO_2) in incubator.
2.) DIC images and confocal z-stacks from the regions of interest were obtained by the confocal microscope Olympus with spinning disk Yokogawa. Number of rotations of spinning disc was 5000 per minute.
3.) After obtained images in light microscopy, we fixed cells in 2% glutaraldehyde solution (EMS, #16019) in Sörensen buffer with pH=7,3.
4.) After fixation, we post-fixed our samples in 2% OsO_4 solution (EMS, #19140).
5.) Dehydration was in series of ethanol solutions from 60 to 100% (60, 70, 80, 90, 95 and 2x 100%)
6.) After dehydration we embedded our samples in epoxy embedding mixture (EMS, #14130).
7.) The blocks contained cells were separated from the cover slips after brief submerging in liquid nitrogen.
8.) The region of interest was localized by the a-numeric imprint on the surface of the block.
9.) Thin sections made on Leica Ultracut UCT ultramicrotome were mounted on formwar/carbon-coated nickel grids.
10.) Grids were contrasted with lead citrate and uranyl acetate.
11.) Samples were contrasted and after that were examined on the transmission electron microscope Morgagni (FEI company) at voltage 80 kV.
12.) We compared the ultrastructural images with optical sections of the z-stacks and we looked for correspondence between GFP-positive nucleolar beads and FC/DFC units which appeared distinctly on the thin sections.

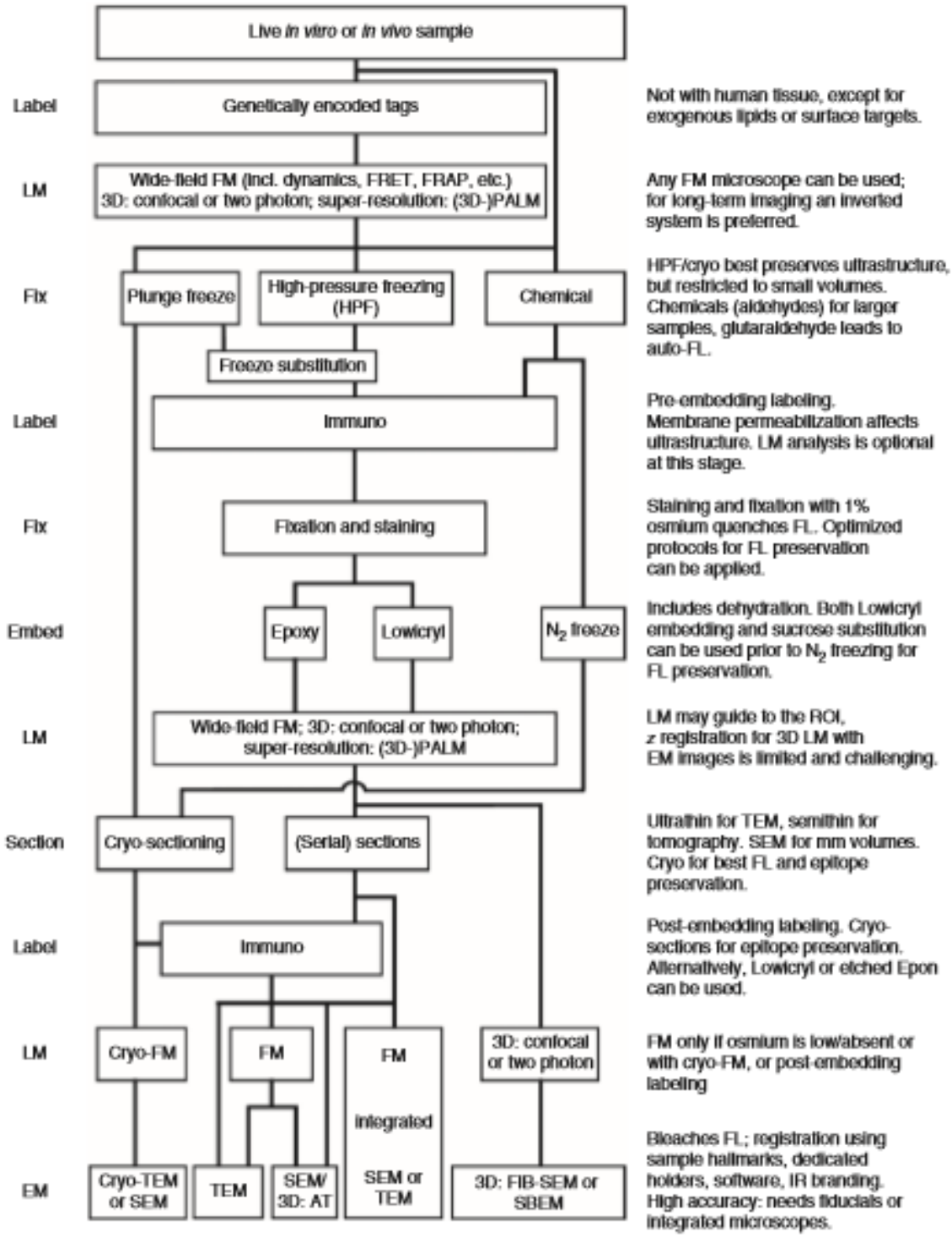

Figure 5.3. CLEM procedures and considerations. A single flowchart for the numerous diverse CLEM applications cannot be given. The general layout is depicted (left), emphasizing considerations on the sample under study, preparation and preservation, as well as reagents and microscopes available (right). FRAP - fluorescence recovery after photobleaching; AT - array tomography; FL - fluorescence; IR - infrared (de Boer et al., 2015).

5.7. Microscopic instrumentation

Electron microscope:

- **Morgagni** (FEI, Eindhoven, Holland) accelerating voltage 100 kV, tungsten cathode, 1K CCD camera Mega View III.

Light microscopes:

- **Olympus AX 70 Provis** (Olympus, Tokio, Japan) epifluorescence microscope, equipped with 10x, 20x and 40x dry lenses and 60x immersion objective and PlanApochromat 100x.
- **Leica SP5** confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63x PlanApochromat / 1.4 NA and 40x / 1.25 NA oil lens. Lasers: 405 nm diode laser, 488 nm argon laser, 561 nm DPSS laser and 633 nm HeNe laser. Leica incubator was used to provide a suitable cell environment.
- **Spinning disk microscope based on the Olympus IX 83 microscope** and equipped with 100x / 1.4NA immersion oil lens (Olympus, Tokyo, Japan), CSU-X spinning disk unit (Yokogawa, Tokyo, Japan), FRAPPA unit (Andor, Belfast, UK) and Ixon Ultra EM-CCD (Andor, Belfast, UK). Okolab incubator (Okolab, Naples, Italy) was used to provide a suitable environment for cells.

5.8. Software and data analysis

For measurement of FC/DFC units in 3D confocal images, we developed a MatLab based software.

The program identifies each unit by creating a maximum intensity projection of the confocal stack and blurring the projection with a Gaussian filter (SD 8-10 pixels), thresholding the blurred image with a value obtained by Otsu's method for automatic threshold selection. After that, the optical section whereupon the unit had maximum intensity was identified.

The final result contains 3D coordinates of each unit, its size (fullwidth half-maximum), the value of x2, and integral intensities in the spheres with radii 1.0, 1.5,

2.0, 2.5, 3.0, 3.5, and 4.0 pixels respectively. FC/DFC units were counted after deconvolution with Huygens software.

For measuring signals in the entire nucleoli we used a custom ImageJ plugin (Smirnov et al., 2014), available at https://github.com/vmodrosedem/segmentation-correlation. Based on the confocal stacks in two channels, the program identifies the regions occupied by nucleoli, measures their areas (in pixels), and the average intensities of both signals within these areas.

Additionally it calculates Pearson's correlation coefficient and Spearman's rank coefficient between the signals corresponding to the two channels within the volume of nucleoli.

MatLab software was used for Fourier analysis of the time series.

6. RESULTS

6.1. Reproduction of the FC/DFC units in the nucleoli

6.1.1. Nucleolar beads correspond to FC/DFC units

At the beginning of our research, which we published under the title "Reproduction of the FC/DFC units in the nucleoli", we were interested in whether there were significant changes in FC/DFC units during a cell cycle, and if so what kind of changes. Our experiments were performed on two stable cell lines based on human sarcoma cells. The first cell line stably expressed RFP-PCNA (sliding clamp protein) and GFP-RPA43 (subunit of RNA polymerase I, pol I) fusion proteins (figure 6.1).

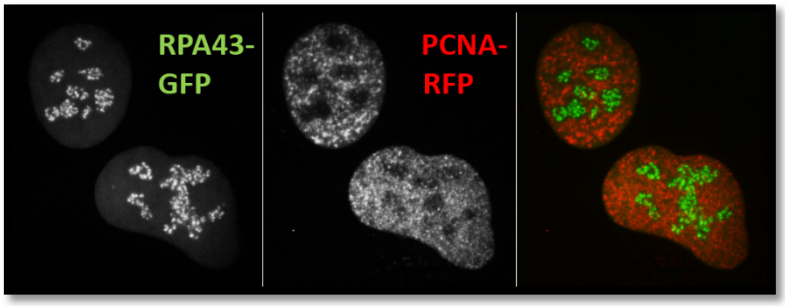

Figure 6.1. Stable cell lines based on HT1080: RFP-PCNA and GFP-RPA43. (Smirnov et al., 2016b; not published).

The second cell line stably expressed RFP-PCNA and GFP-fibrillarin (protein involved in early processing of rRNA transcripts) fusion proteins (figure 6.2.a).

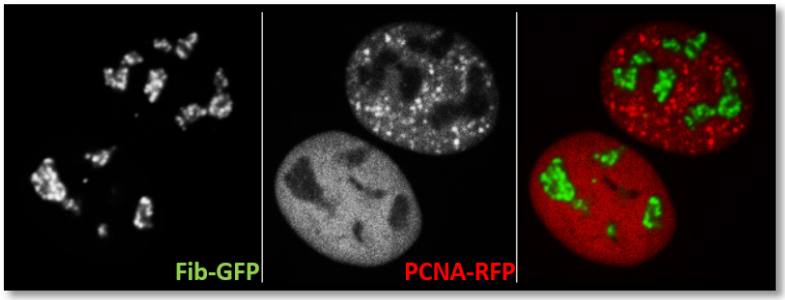

Figure 6.2A. Stable cell lines based on HT1080: PCNA-RFP and fibrillarin-GFP. (Smirnov et al., 2016b; not published).

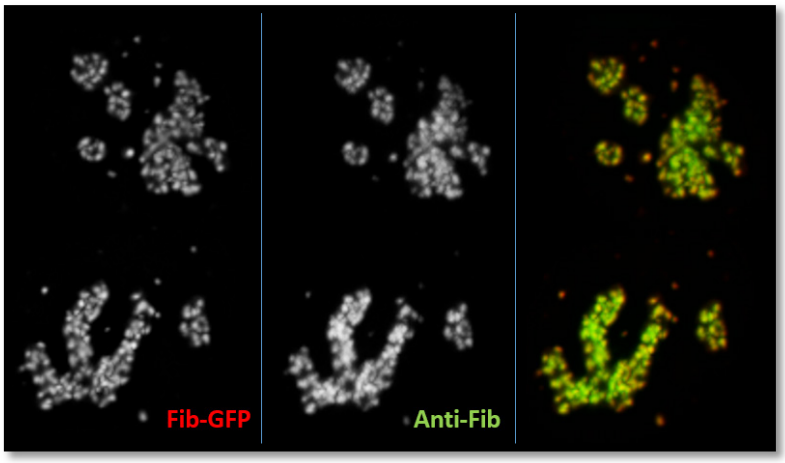

Figure 6.2B. Stable cell lines based on HT1080: Correlation of signals for fibrillarin-GFP and anti-fibrillarin antibody. (Smirnov et al., 2016b; not published).

The basic method used in our experiments was correlative light-electron microscopy (CLEM). Based on the CLEM observation results, we have been able to confirm that the FC/DFC units of mammalian cells fully correspond to signals of fibrillarin and pol I proteins (figure 6.3).

6.1.2. FC/DFC units may lose polymerase I signal but not fibrillarin signal in early S phase

Moreover, *in vivo* observations revealed that during early S phase when transcriptionally active ribosomal genes are replicated, the amount of individual FC/DFC units in each cell increases by 60-80%. Another interesting finding was that during this early S phase, the FC/DFC units temporarily lost signal for polymerase I but did not lose signal for fibrillarin. From middle S phase to the end of interphase, the number of units did not change and their duplication was completed only after the cell division in daughter cells halfway through the G1 phase. Our findings indicate that the reproduction of FC/DFC unit is two-step. From these findings, we come to a conclusion that a significant portion of the ribosomal genes remain transcriptionally inactive (silent) from the mid-S phase to mitosis but again become active in postmitotic daughter cells. FC/DFC units thus play an important role in cellular metabolism by regulating rRNA transcription and early transcription processing.

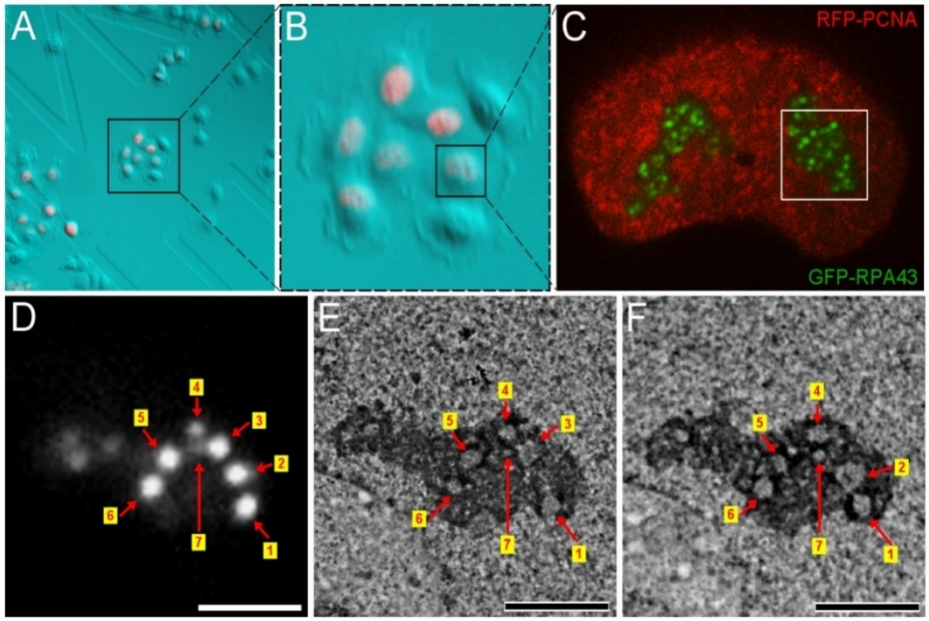

Figure 6.3. CLEM analysis of the nucleolar beads. A - selecting a suitable cell group, B - selecting a suitable cell, C - red points represent a replication signal, green beads represent a signal for pol I, D - white beads represent FC/DFC units, E, F - picture of ultrathin slices in TEM (transmission electron microscopy) - confirmation of the assumption that one white beads from observation in a light microscope corresponds to one FC/DFC subunit visible on an electron microscopic image (Smirnov et al., 2016b).

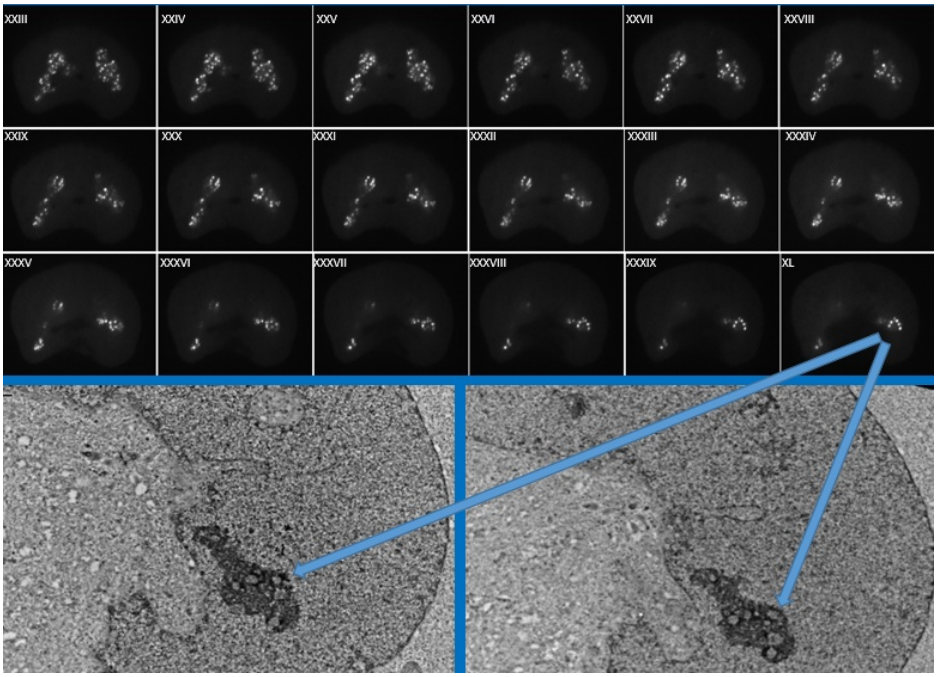

Figure 6.4. Optically sectioned images of a FC/DFC unit and two corresponding electron microscope images of thin sections. The top three series of images represent images of one FC/DFC unit from a confocal microscope. Two images at the bottom of the figure are images of two ultrathin

slices created by TEM corresponding to the same optical plane of FC/DFC unit captured in the upper row (Smirnov et al., 2016b; not published).

The FC/DFC units undergo fundamental changes during the two interphase intervals: early G1 phase and early S phase. At the beginning of the G1 phase, when the nucleoli is remixed after mitosis, NORs gradually unfold into nucleolar necklaces formed by nucleolar beads. Each NOR produces one necklace (figure 6.5).

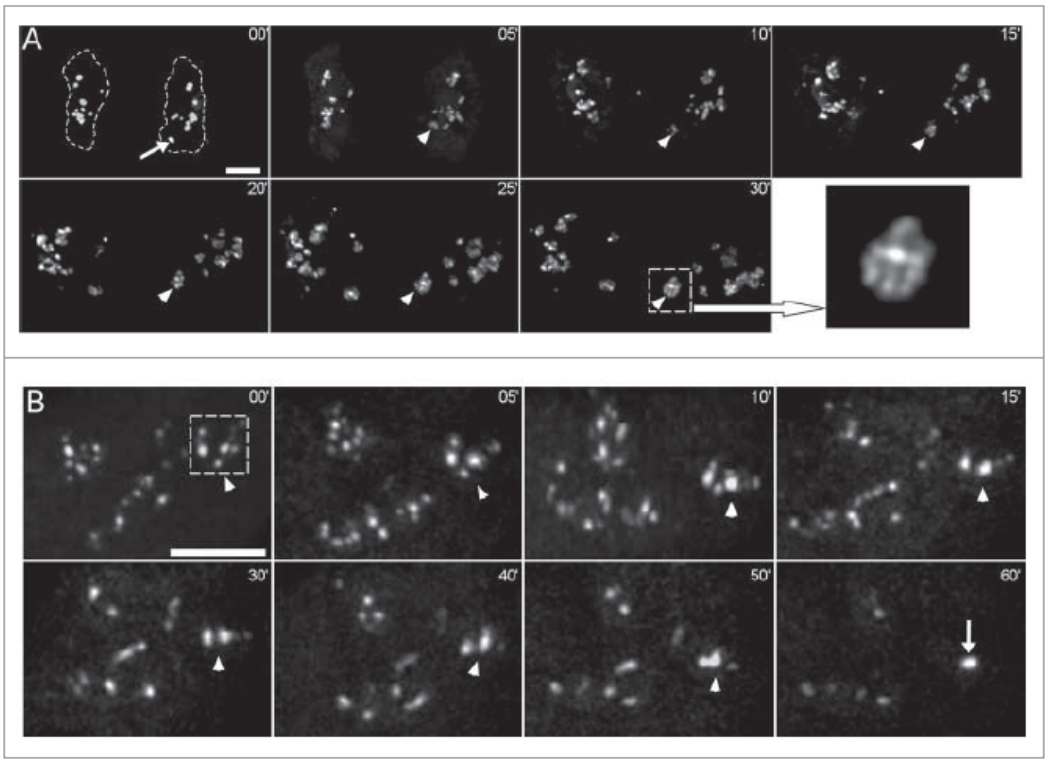

Figure 6.5. NORs and nucleolar necklaces in the cells expressing GFP-RPA43. Projections of confocal z-stacks. (A) After telophase, each mitotic NOR (arrow) gradually unfolds into a necklace (arrowheads). Nucleoli are formed from one or more necklaces. Emerging nuclei of the daughter cells are outlined in the first frame representing telophase. (B) Interphase cell treated with calyculin A for 1 h. A part of cell nucleus is shown. Nucleolar necklaces (arrowheads) gradually shrink into NORs (arrow). Scale bars: (A) 2 μm, (B) 3 μm (Smirnov et al., 2016b).

This process is stopped in the mid-G1 phase. Then, the number of beads or FC/DFC units will not change until the end of the mentioned G1 phase. Another significant changes occur in the early S phase when transcriptionally active

ribosomal genes are replicated. The FC/DFC units occasionally lose the pol I signal, but not the fibrillarin signal (figures 6.6. and 6.7.). We conclude that the disappearance of pol I, which is not observed in other parts of S phase, is directly related to the replication of ribosomal genes.

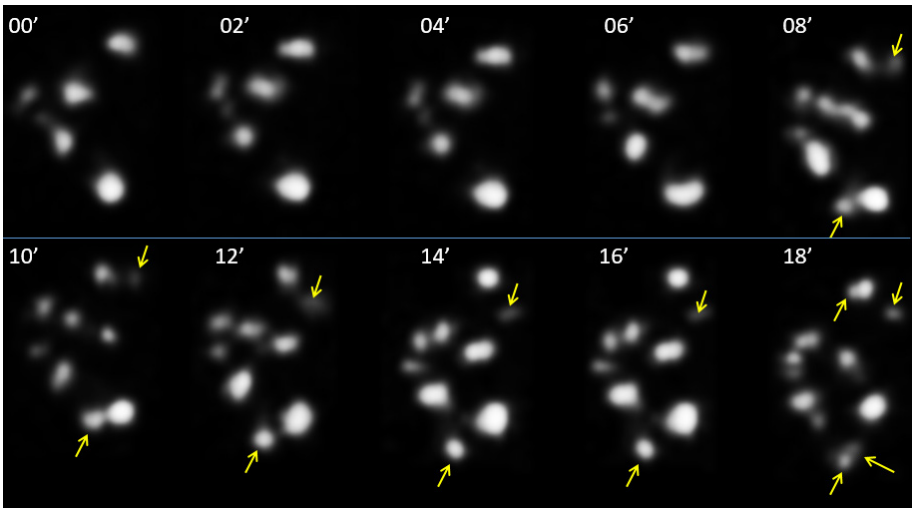

Figure 6.6. Emerging RPA43 positive units during early S phase - Short term observations. (Adapted from Smirnov et al., 2016b).

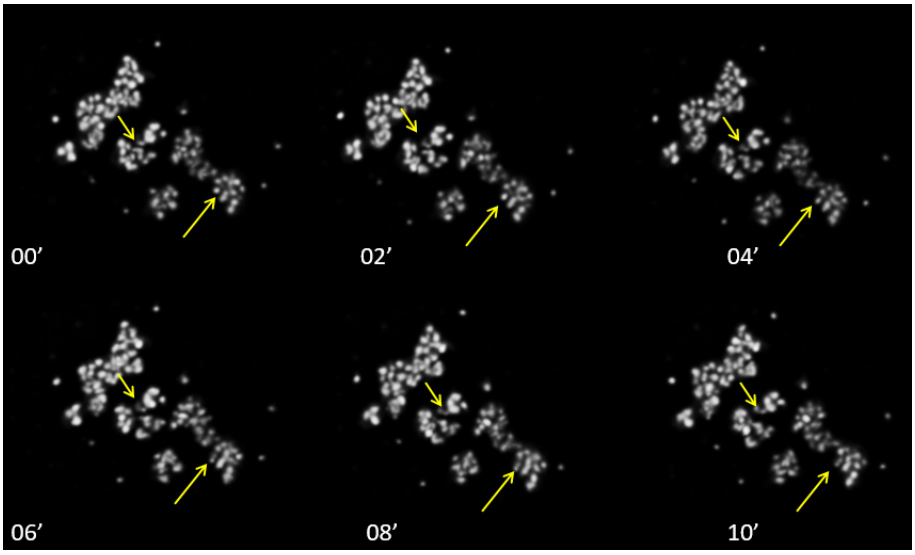

Figure 6.7. Emerging fibrillarin positive units during early S phase - The units retain fibrillarin. (Adapted from Smirnov et al., 2016b).

Based on previous research of our scientific group, we believe that transcription is suppressed in FC/DFC units involved in rDNA replication, which provide efficient separation of replication and transcription mechanisms in nucleoli. The results of our experiments confirm this hypothesis. Interesting finding is the fact that fibrillarin, which is associated with processed rRNA but not with rDNA (Dragon et al., 2002), remains in FC/DFC units throughout the S phase.

The absence of pol I in FC/DFC units is temporary and lasts relatively short. During this absence, the total number of FC/DFC units with a signal for pol I and fibrillarin is increased. This increase can be observed directly (figures 6.6 and 6.7) or by counting FC/DFC units before and after the start of the S phase (figures 6.8 and 6.9).

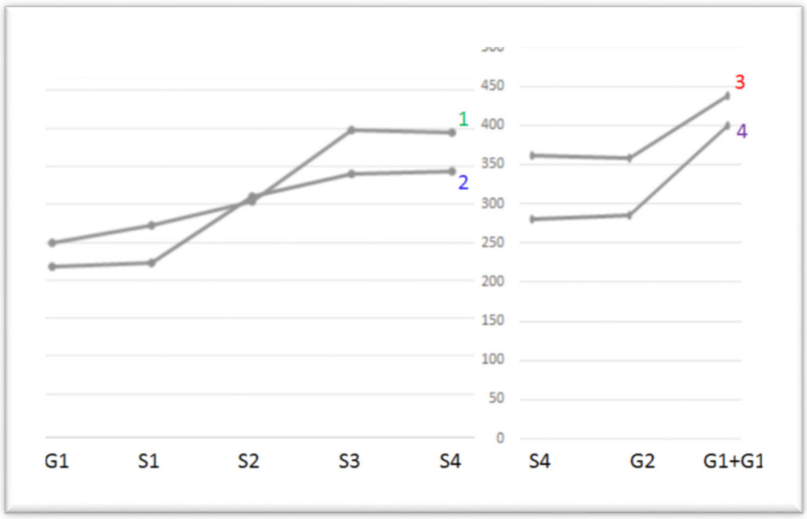

Figure 6.8. Counts of the pol I positive FC/DFC units in individual cells. The total number of pol I positive FC/DFC units increased sharply by about 70% during early to mid-S phase then do not changed from mid-S to the end of the G2 phase, and rose sharply again in the postmitotic cells during next G1 phase (Adapted from Smirnov et al., 2016b).

6.1.3. Regular changes, duplication and mobility of FC/DFC units

In this work, we assume that all DNA, including ribosomal genes, is duplicated between G1 and G2 phases. However, *in vivo* results of cell cycle observation showed that during S phase the number of FC/DFC units increases by about 70%, not by assumed 100% (figures 6.8 and 6.9). Duplication of units was completed only

after mitosis, after another sensible grow in the number of units at the beginning of the next G1 phase. Finally, each daughter cell in the mid-G1 phase gained as many units as the parent cell had at the same phase. The results obtained from multiple cell lines, such as HeLa and LEP, are congruent. Currently, it is assumed that FC/DFC units are individual transcriptionally active replications of rDNA (Haaf and Schmid, 1991; Haaf and Ward, 1996; Denissov et al., 2011).

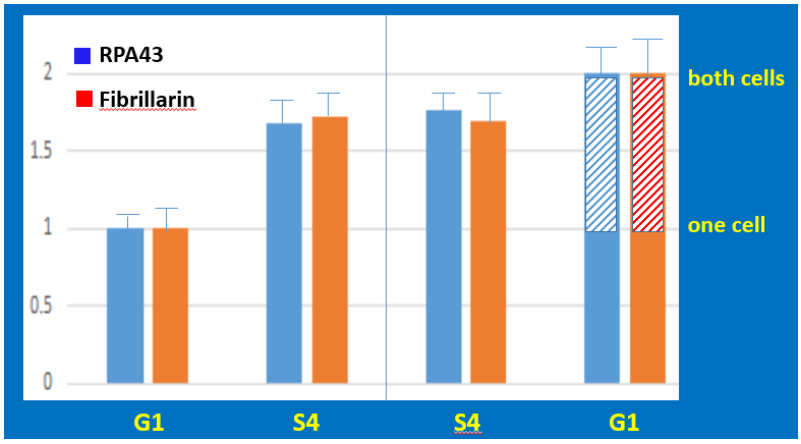

Figure 6.9. Dynamics of the number of FC/DFC units. The total number of FC/DFC units increased noticeably by approximately 70% during early-to-mid-S phase (”under-duplication”), and after remained the same during the end of S phase, rose again during next G1 phase in postmitotic cells by approximately 15% per cell (Adapted from Smirnov et al., 2016b).

The results of our experiments show that approximately 30% of the ribosomal genes remain active from the mid-S phase to the end of G2 phase and re-activated only in the cells of next generation at the start of the G1 phase (figure 6.10).

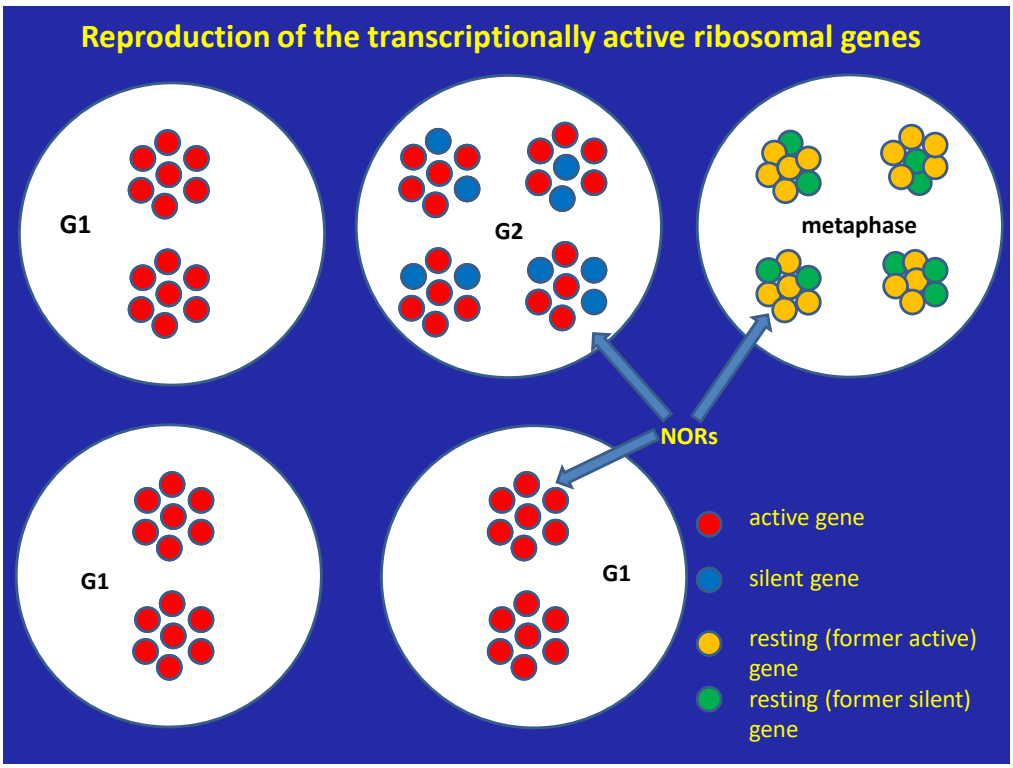

Figure 6.10. Hypothesis of reproduction of transcriptionally active ribosomal genes in cell cycle. Between the G1 and G2 phases, genes are duplicated, some of the genes remain transcriptionally active, some are silent. (the middle circle of the top line) During mitosis, transcription is temporarily stopped and former active as well as former silent genes are resting. In daughter postmitotic cells (down line), during the early G1 phase, genes are re-activated. Thus the stable state of activity is maintained through the consecutive cell cycles. The figure does not show the permanently inactive genes and NORs which are not associated with pol I (Adapted from Smirnov et al., 2016b).

The results obtained indicate that this mechanism is responsible for regulating the rDNA transcription intensity and, ultimately, ribosome production. The experiments results show that neither the signal strength of pol I in FC/DFC units nor their average size, measured from electron microscopic images, changed after replication of active rDNA.

Table 6.1. Relative increase in the number of FC/DFC units in presence of TSA and AzdC during S phase. Increase ranges between 60 and 80%, just as without the treatment, which indicates that silencing is resistant to the changes in the chromatin structure, sensitive to TSA and AzdC (Adapted from Smirnov et al., 2016b).

G1	S4	
	TSA	AzdC
1	1.68	1.74
1	1.59	1.70
1	1.84	1.64
1	1.65	1.64

Table 6.2. The number of FC/DFC units during S3 phase in presence of Roscovitine. The number of units does not change in presence of Roscovitine, which indicates that the silencing is not determined by phosphorylation of SL1. Roscovitine is an inhibitor of Cdc2/cyclin B kinase, which catalyses dephosphorylation of the SL1 transcription factor (Adapted from Smirnov et al., 2016b).

The number of units during S3 phase in presence of Roscovitine		
Control (0 min)	90 min	180 min
390	384	390
354	355	352
316	314	318
459	451	451

The transcriptional activity of FC/DFC unit remains at a stable level over most interphase (figure 6.11). Silencing of ribosomal genes after replication in mid-to-late S phase is very effective, because there is not increase in the number of pol I positive units and cannot be prevented by 5-aza-2'-desoxicytidine (AzdC), Trichostatin A (TSA) or Roscovitine ((R)-Roscovitine), (table 6.1 and table 6.2). The

permanently inactive ribosomal genes normally found in mammalian cells responded similarly (Santoro, 2005; Santoro, 2014; Conconi et al., 1989).

However, the distribution of transcriptional signal intensity showed no tendency to asymmetry either before or after early S phase. This indicates that the original number of active genes per FC/DFC unit (presumably only one gene) will resume shortly after replication. Alternatively, duplication of active genes may be complete but not always followed by duplication of FC/DFC units. In this case, some units (approximately 30% of the entire set in the studied cells) would contain more than one active gene by the end of interphase.

The results of our study also indicate that the restoration of rDNA activity after replication is not always symmetric, which means that one of daughter helixes may be silent. This hypothesis complements older results from our workplace of asymmetric NORs that regularly appear on r-chromosomes and cause mitotic asymmetry (Kalmarova et al., 2008).

Reproduction of FC/DFC units in nucleoli follows a special pattern that includes incomplete duplication during early S phase. Our data suggest that a large subset of the ribosomal genes remains transcriptionally silent from late S phase to mitosis but is re-activated in postmitotic daughter cells.

6.2. Fluctuations of pol I and fibrillarin contents of the nucleoli

6.2.1. Quick fluctuations of polymerase I and fibrillarin signal intensities in the FC/DFC units

Based on our previous experimental results, we decided to continue our research by studying the kinetics of the pol I and fibrillarin proteins during the cell cycle. Our findings are described in detail in the attached paper titled "Fluctuations of pol I and fibrillarin contents of the nucleoli". In the light of the latest research in the field of cell biology it starts to believe that many (perhaps even all) genes in the cell are not transcribed continuously, but in pulses of the transcriptional activity separated by periods of inactivity. We assume that pulse-like expression can also be found in ribosomal genes. However, given that the cell transcribes multiple copies of ribosomal genes at the same time, it is difficult to study their individual expression using standard methods, and by using these methods to determine whether it is a continuous or discontinuous process.

In our experiments, we worked with HT1080, HEP2 and HeLa human cell lines that had been transfected with plasmids containing the genes for GFP-RPA43 and RFP-fibrillarin fusion proteins. We also worked with another two cell lines derived from HT1080 cells. The first stably expressed GFP-RPA43 and RFP-PCNA fusion proteins and the second cell line stably expressed GFP-fibrillarin and RFP-PCNA fusion proteins. Both pol I and fibrillarin signals (which look like dots or beads at microscopic observations) (figure 6.12) as shown in our previous work, correspond to FC/DFC units. Thus, it allowed us to indirectly examine the state of the individual ribosomal genes.

Our first observations have shown that some FC/DFC units, probably units currently involved in replication, temporarily lose signal for pol I but not for fibrillarin. However, this is a rare phenomenon that probably reflects transcription pause in units that are involved in replication.

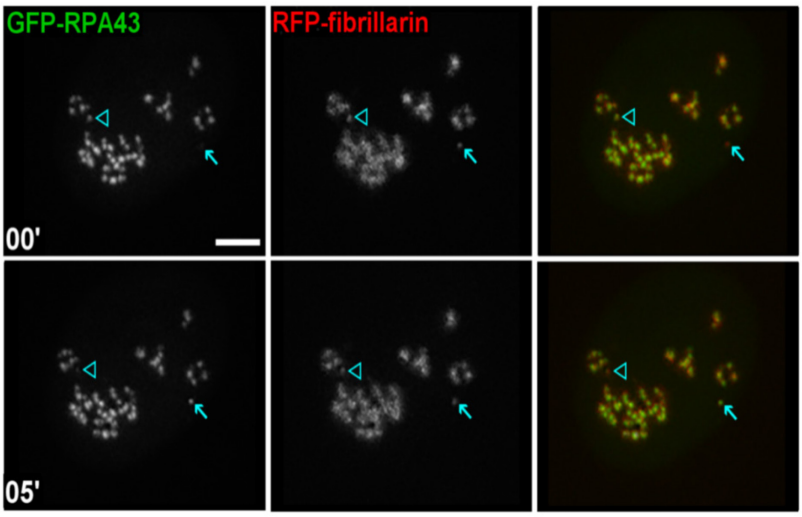

Figure 6.11. Kinetics of pol I and fibrillarin in FC/DFC units in HEP2 cells transfected with GFP-RPA43 and RFP-fibrillarin. Some FC/DFC units (apparently involved in replication) transiently lose pol I but not fibrillarin (Hornacek et al., 2017).

In following experiments, we measured the intensity of pol I and fibrillarin signals in individual FC/DFC units in one and two minute intervals. We have found that their intensities fluctuate in an antiphase manner. Pearson correlation coefficient (R_{Δ}) for the intensity of individual signals was variable but always negative, sometimes up to 98% (figure 6.13). The pol I signal intensity in neighboring FC/DFC units belonging to the same NOR does not correlate (figure 6.13), indicating that these fluctuations can not be attributed to measurement artefact or technical errors.

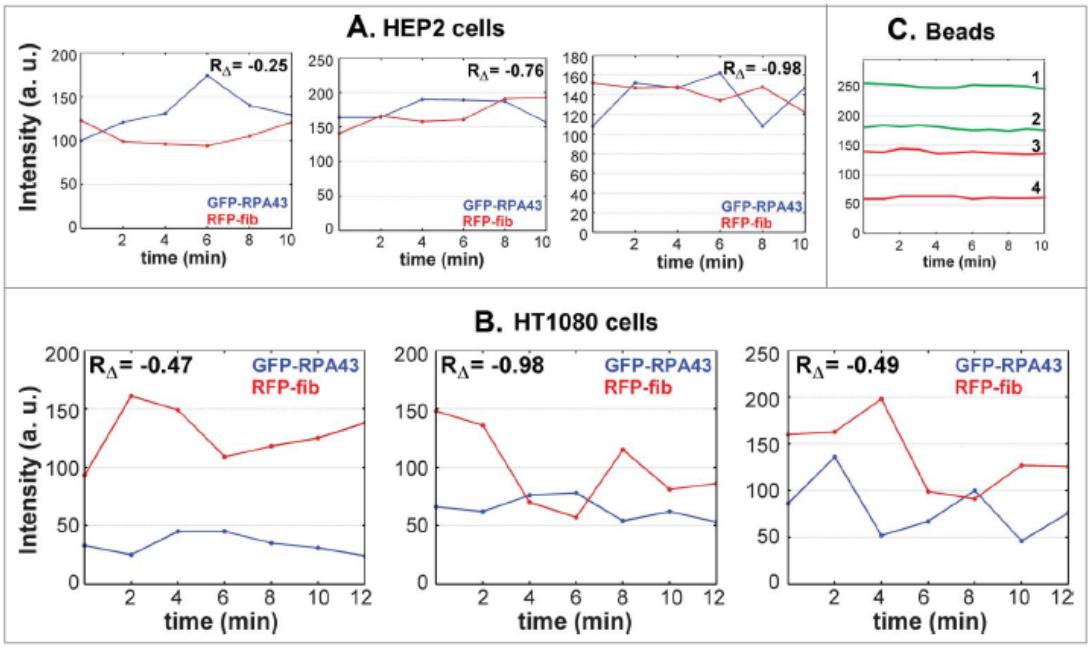

Figure 6.12. Fluctuations of pol I and fibrillarin signals. Intensities of pol I and fibrillarin signals in individual units (measured at 2-minute intervals) fluctuate in anti-phase manner; A - HEP cells, B - HT1080 cells, C - Beads, Control (Hornacek et al., 2017).

The mismatch of peaks of polymerase I signals intensity in several neighboring FC/DFC units that belonging to same NOR indicates that fluctuations are not due to local changes in pol I concentration.

The fact that neighboring FC/DFC units (belonging to the same nucleus and probably to the same chromosomes) are not synchronized is interesting. This means that they are not the result of changes in the surrounding environment.

When analyzing signal intensity variations in FC/DFC units, we did not detect any interaction between neighboring units (figure 6.14).

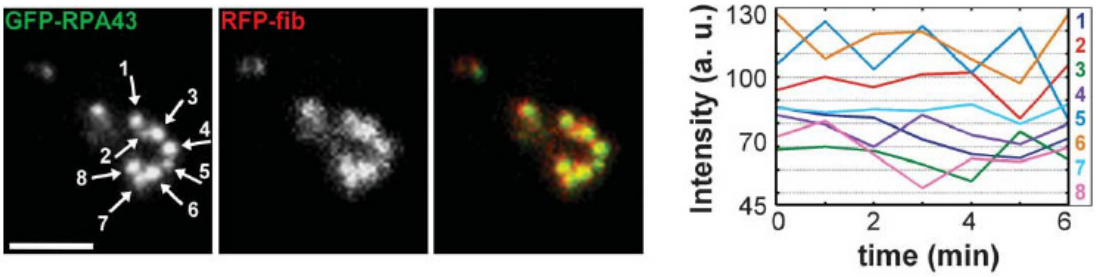

Figure 6.13. Intensity of pol I signals (GFP-RPA43) in 8 neighbouring FC/DFC units (a chain of units probably belonging to one NOR). Intensity of pol I signals does not corelate (Hornacek et al., 2017).

Previously, we found that the units are in constant motion, so the distance between neighbouring units is changing considerably. The graphs below show clearly that the signal in one unit is not affected by changing the distance to the nearest neighbouring units (left graph) or by changing the signal strength of these neighbouring units (right graph).

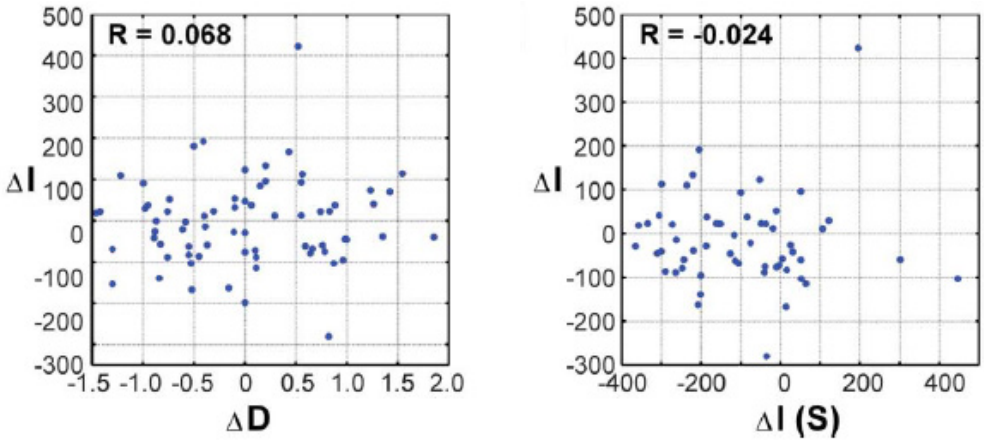

Figure 6.14. Variations of the signal intensity in 10 FC/DFC units. ΔD: variation of the average distance to the two nearest neighbours; ΔI: variation of the intensity of GFP-RPA43 signal in one unit; $\Delta I(S) = \Delta I_p + \Delta I_f$, i.e., sum of variations of signal intensity in the preceding (p) and following (f) unit (Hornacek et al., 2017).

By using spectral analysis of time series, we identified two main peaks that correspond to second and third minute of the periodogram. However, entire

ribosomal gene is not transcribed during this time, and therefore the periods are not directly correlated with transcription.

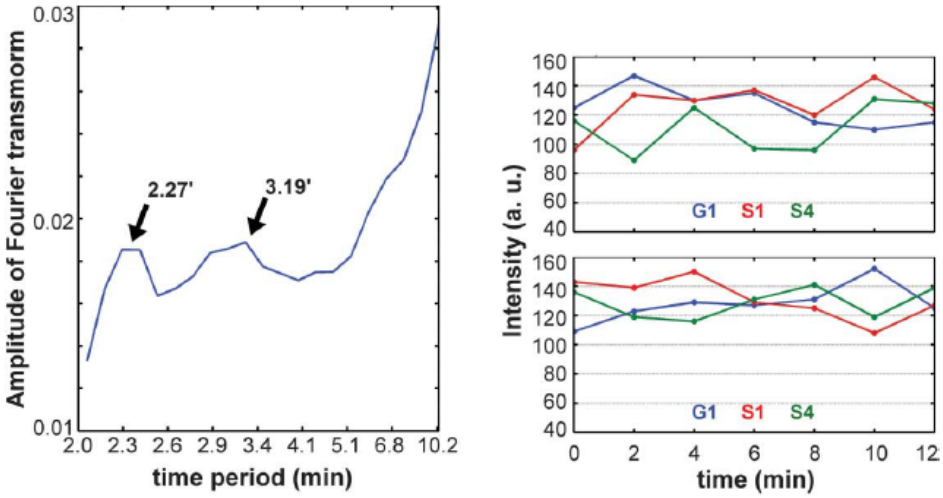

Figure 6.15. Characterization of rapid fluctuations. Periodogram of Fourier analysis of the time series (left) and examples of individual units showing that the fluctuations appear in G1, early S (S1) and late S (S4) phases of cell cycle (right), (Hornacek et al., 2017).

6.2.2. Slower fluctuations of polymerase I and fibrillarin signal intensities in the nucleoli

By measuring the intensity of signals every 5 minutes, we found that signal fluctuations for pol I and fibrillarin in the same unit do not correlate (figure 6.17B). Conversely, the pol I signals in different FC/DFC units, even in units belonging to different nucleoli, are synchronized (figure 6.17A and figure 6.17C). The same situation after data normalization can be seen in figure 6.16C – right graph. On the other hand, in longer periods there is no full synchronization between the signals for pol I and fibrillarin in the same unit. The periodogram has in this case three phases corresponding to fluctuations with periods of 10, 20 and 60 minutes with only slight variations (figure 6.16A). Similarly, in this case, the main source of fluctuation could not be a technical error because the intensity of the fibrillarin signal in different FC/DFC units was not synchronized. It seems that our work is related to the recent studies of discontinuous, bursting or pulsating expression of various genes in nucleoplasm (Golding et al., 2005; Chubb et al., 2006; Raj et al., 2006; Suter et al., 2011b).

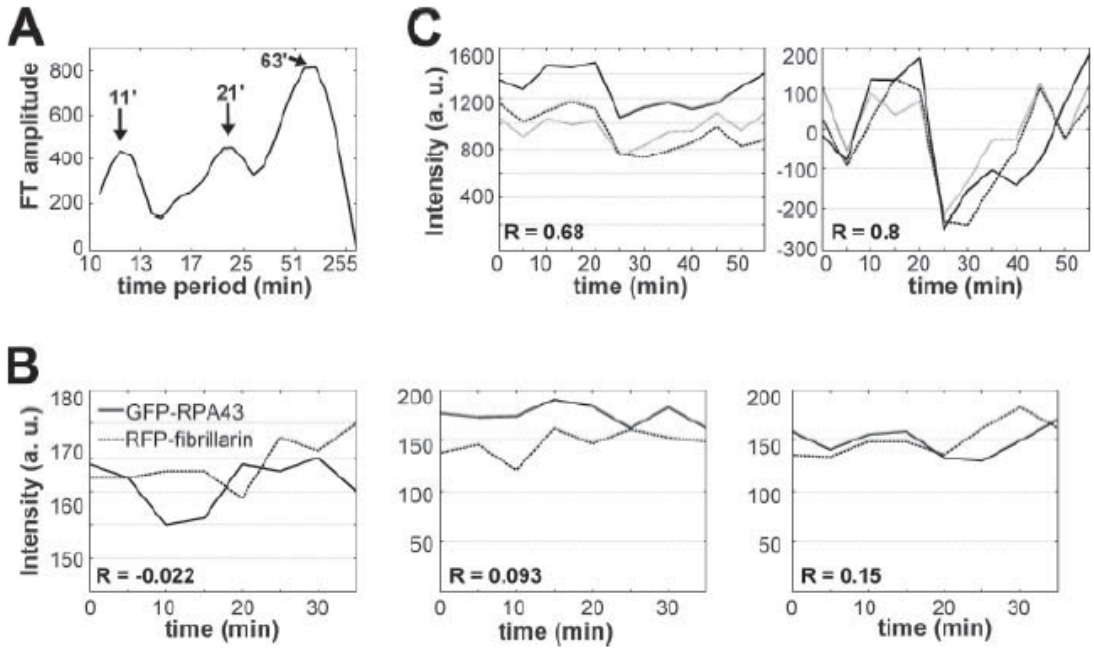

Figure 6.16. Slower fluctuations of pol I and fibrillarin signal intensities. (Hornacek et al., 2017).

6.2.3. Exchange rates of polymerase I and fibrillarin are related to their fluctuations in FC/DFC units

It is generally known that the exchange of nuclear proteins is very intense in the cell nucleus. We were interested in the recovery time of pol I and fibrillarin signals after photobleaching one of FC/DFC unit. We used a 2-channel FRAP method with 405 nm and 560 nm wavelengths. From the FRAP results for both proteins, we can conclude that the half-lives ($T_{1/2}$) for polymerase I and fibrillarin are about 20 seconds long and therefore are much shorter than one fluctuation period and also show a tendency to negative correlation.

In another experiment we used the FRAP method with prolonged pre-bleach period. The experiment was set up so that at the time of bleaching, the intensity of the pol I signal was increasing and intensity of fibrillarine signal was decreasing, or vice versa. After restoring the signal intensity, in the first case, the signal recovery for pol I was faster than for fibrillarin and in the second case it was inside out. Recovery times are thus modulated by fluctuation of polymerase I and fibrillarin contents in FC/DFC units. The observed changes are implicated in the regular exchange of both proteins in units.

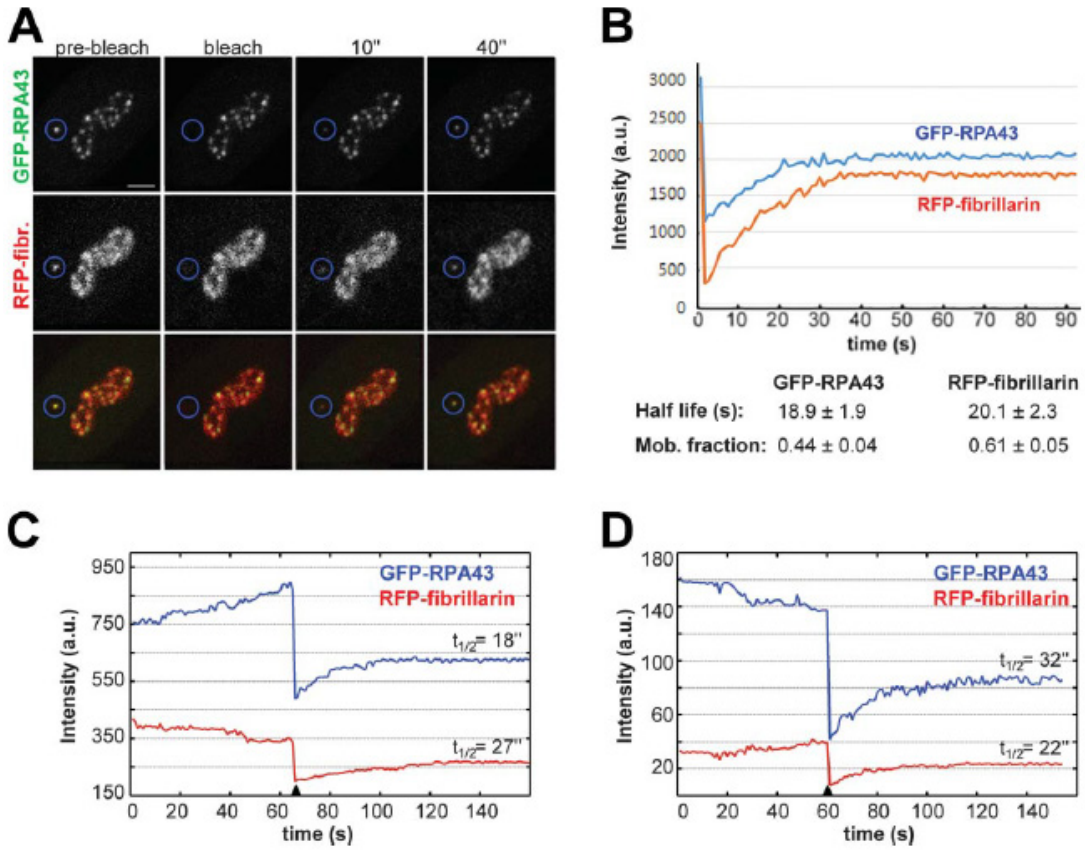

Figure 6.17. FRAP experiments. 2-channel FRAP (405nm and 560nm) on FC/FDC units in the transfected cells (A and B). FRAP with an extended pre-bleach period (C and D). (Hornacek et al., 2017).

6.2.4. Correlation of polymerase I and fibrillarin signals with transcription signal in the nucleoli

All previous experiments were performed on proteins. In order to link them directly to transcription, we studied co-localization of the incorporated FU (fluorouridine) with polymerase I or fibrillarin signal. The correlation was positive throughout the nucleolus which is not surprising at all, since nuclear transcription takes place in units where both proteins are accumulated. However, if the observation is limited to FC/DFC units alone, the correlation values for fibrillarin are rather low.

Co-localization of polymerase I and fibrillarin with incorporated fluorouridine showed that fluctuations in the levels of polymerase I correspond to the fluctuation of transcription intensity.

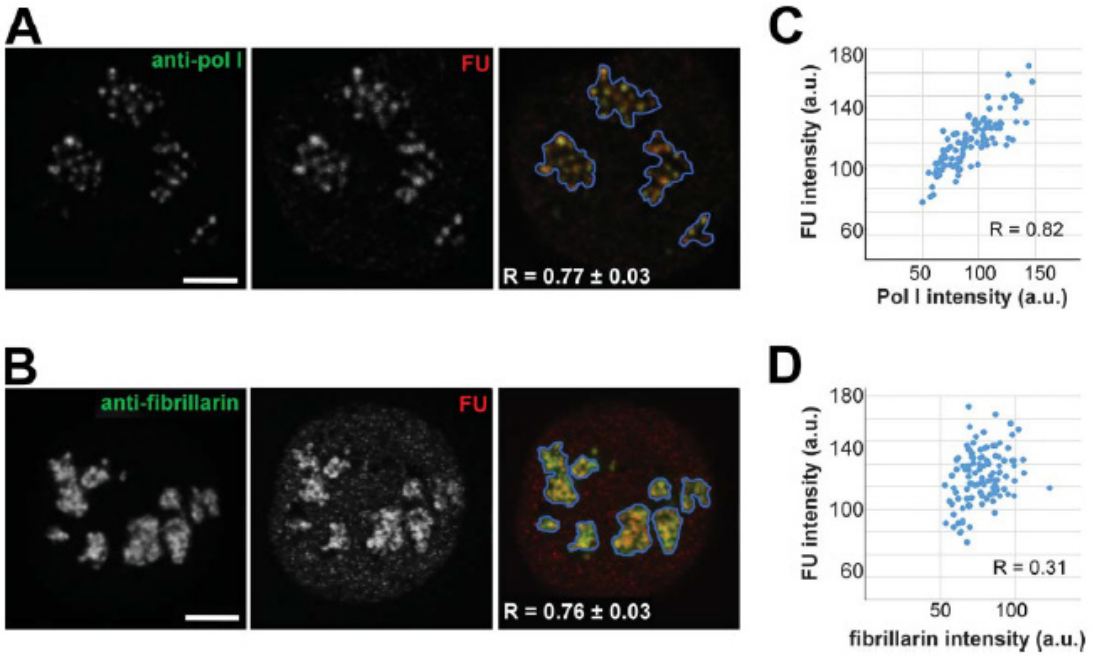

Figure 6.18. Colocalization of pol I and fibrillarin with incorporated FU. (Hornacek et al., 2017).

Based on the findings above, we were interested in what happens if we stop transcription with Actinomycin D (ActD). During the first hour of incubation with ActD, signal intensity for pol I continued to fluctuate and even show synchronization between the FC/DFC units (1 and 2) However, signal fluctuations for fibrillarin (3) were suppressed. We conclude that the fluctuation of polymerase I does not depend on elongation, whereas the fluctuation of fibrillarin does and therefore is probably associated with the processing.

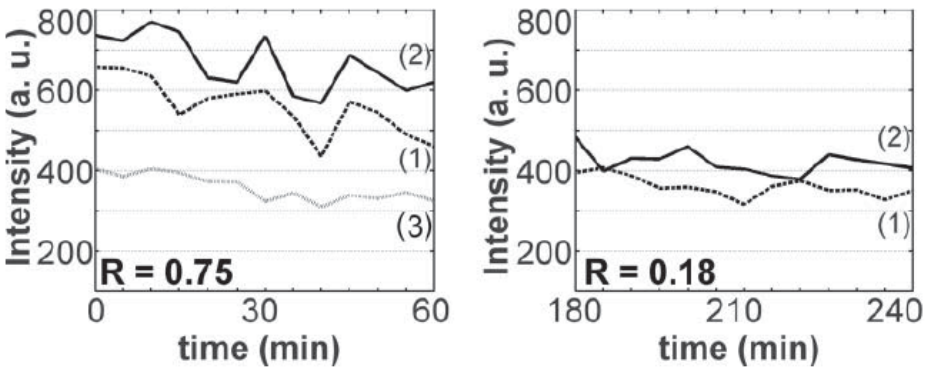

Figure 6.19. Fluctuations of pol I and fibrillarin signal intensity in FC/DFC units in HT1080 cells after treatment with 0.05 µg/ml Actinomycine D (ActD) for 10 minutes. (Hornacek et al., 2017).

7. DISCUSSION

In the introductory part we have shown that FC/DFC units of nucleoli play key role in cell metabolism being centers of ribosomal transcription and early rRNA processing. In the first experimental work included in the present thesis (entitled „Reproduction of the FC/DFC units in nucleoli“), we focused on the dynamics of these units in the course of the cell cycle. Using Correlative light and electron microscopy (CLEM) and specially produced cell lines, we showed that population of the units expressing both pol I and fibrillarin undergoes specific changes during 2 periods of the cell cycle interphase: early G1 and early S.

At early G1 phase, when nucleoli are re-assembled after mitosis, Nucleolar organizer regions (NORs) unfold into nucleolar necklaces with gradually multiplying beads so that each NOR produces one necklace (Figure 6.5). This process is concluded by mid G1. Then, the number of the beads, or FC/DFC units, does not change until the end of G1. Further significant changes take place during early S phase, when transcriptionally active ribosomal genes are replicated (Berger et al., 1997; Li et al., 2004). FC/DFC units occasionally lost their pol I signal, but not their fibrillarin signal (Figure 6.6; Figure 6.7). This lead us to think that such disappearance of pol I, not observed at other stages, is directly connected to replication of the ribosomal genes. According to our earlier hypothesis (Smirnov et al., 2014), transcription is suspended in the FC/DFC units involved in rDNA replicating, which provides a basis for efficient separation of replication and transcription machineries in nucleoli. Our present data agree with this hypothesis. Remarkably, fibrillarin, which is associated with processed rRNA, but not with rDNA (Dragon et al., 2002), is retained by the units throughout the S phase. The loss of pol I must be of a short duration, for at the same period we observe an increase in the total number of pol I and fibrillarin positive FC/DFC units (Figure 6.8; Figure 6.9). This increase may be detected directly (Figure 6.6; Figure 6.7) or by counting the units before and after early S phase (Figure 6.8; Figure 6.9). During the rest of S, as well as G2 and most of G1 phase, the number of FC/DFC units do not change.

In this work we assume that all DNA, including the ribosomal genes, is duplicated between G1 and G2 phases. But when we followed progress of individual

cells through the cell cycle, we found that in the course of S phase the number of FC/DFC units increased only by about 70% (Figure 6.8; Figure 6.9); the duplication was never completed until the end of interphase. But after mitosis, an additional multiplication of the units took place, so that each daughter cell by mid G1 acquired as many units as the maternal cell had at the same stage. Data obtained on HeLa and diploid LEP cells after synchronization agree with these findings (see Figure 6C, Figure 6D in publication „Reproduction of the FC/DFC units in nucleoli“ in supplementary).

Since there are good reasons to believe that FC/DFC units correspond, more or less precisely, to individual transcriptionally active repeats of rDNA (Haaf et al., 1991; Haaf and Ward, 1996; Denissov et al., 2011), our data suggest that about 30% of active ribosomal genes remain silent from the middle S till the end of G2 phase, and get re-activated in the cells of next generation, at early G1 phase (Figure 6.10). This may be important for maintaining optimal level of rDNA transcription. Production of ribosomes is regulated at different levels; it seems that one of the mechanisms prevents excessive production of the rDNA transcription centers after replication. Interestingly, neither intensity of pol I signal on the units, nor their average size measured on electron microscopic preparations, did change after replication of the active rDNA (Table 6.1, Table 6.2). This suggests that transcription activity per unit remains at a stable level for the most part of interphase.

The hypothetical silencing of some ribosomal genes after replication appears to be so efficient, it could not be prevented by 5-aza-2'-desoxicytidine (AzdC) and Trichostatin A (TSA), nor reversed by Roscovitine (Table 6.1, Table 6.2). Likewise, the permanently inactive ribosomal genes, which are usually present in mammalian cells (Santoro, 2005; Santoro, 2014; Conconi et al., 1989), did not respond to the treatment. Alternatively, duplication of the active genes may be complete, but not always followed by duplication of the FC/DFC units. In that case, some of the units (cca 30% of the entire pool in the studied cells), would accommodate more than one active gene until the end of interphase. But distribution of the signal intensity showed no tendency to asymmetry, neither before nor after early S phase. This suggests that an original number (probably just one) of active genes per unit is restored shortly after replication.

Our findings also indicate that restoration of rDNA activity after replication is not always symmetrical, i.e., one of the daughter helices may become silent. This hypothesis complements our data on asymmetrical NORs which regularly appear on certain r-chromosomes and cause a mitotic asymmetry (Kalmarova et al., 2008). Thus reproduction of FC/DFC units in nucleoli follows a peculiar pattern, which includes an incomplete duplication during early S phase. Our data suggest that a considerable subset of ribosomal genes remain transcriptionally silent from late S phase to mitosis, but become again active in the postmitotic daughter cells.

Our second study (entitled „Fluctuations of pol I and fibrillarin contents of the nucleoli“) was focused on the short-term dynamics of the FC/DFC units in the nucleoli of human derived cells. We followed in real time the fluorescent signals of RPA43 subunit of pol I and fibrillarin with intervals from 1 min to 1h. Since intensity of such signals positively correlates with the actual number of the fusion protein molecules within the region of interest (Golding et al., 2005), our data refer to the changing contents of nucleoli. Measuring the signal intensities of GFP-RPA43 and RFP-fibrillarin in the FC/DFC units, we observed specific fluctuations of two kinds. Firstly, in the short term experiments, when images were taken every 1–2 min, the pol I and fibrillarin signals fluctuated in anti-phase manner (Figure 6.12), and the intensities of pol I in the neighboring FC/DFC units belonging to the same NOR did not correlate (Figure 6.13), which shows that these rapid fluctuations cannot be attributed to artifacts or technical errors. Secondly, in the longer observations with intervals of 5 min, correlation of pol I and fibrillarin signals measured in the same unit became uncertain; but we found well defined synchrony of pol I signals in the units belonging to different nucleoli (Figure 6.16A, Figure 6.16C). Repeatability of fluctuations was monitored on a large number of measurements. Here again technical error could not be the chief source of the fluctuation, since the intensities of fibrillarin signal in different FC/DFC units were not synchronized. Results of this work seems to be related to the recent studies of discontinuous, bursting, or pulsing expression of various genes in the nucleoplasm (Golding et al., 2005; Chubb et al., 2006; Raj et al., 2006; Suter et al., 2011a). Various aspects of this phenomenon are discussed in our review (Smirnov et al., 2018). In the above mentioned studies, irregular pulses of activity, alternated with pulses of silence, were visualized in living cells by inserting into genome, downstream of the promoter, sequences encoding

stem loops of RNA, which could be detected with fluorescent bacteriophage coat proteins, such as MS2 or PP7 (Chubb et al., 2006; Bertrand et al., 1998). Alternatively, transcription output has been monitored by single molecule RNA fluorescence *in situ* hybridization (smFISH) (Femino et al., 1998; Mueller et al., 2013). Our work provides an approach to the study of irregular activity of ribosomal genes. Indeed, since we found high correlation of pol I and incorporated FU signals within FC/DFC units (Figure 6.18), which probably correspond to individual transcriptionally active units of rDNA (Haaf et al., 1991; Haaf and Ward, 1996; Denissov et al., 2011), the observed fluctuations of RPA43 signal follow pulsing activity of ribosomal genes. Remarkably, distribution of pol I and FU signal intensities measured in FC/DFC units showed considerable positive skewness (see Results, section 3 in publication „Fluctuations of pol I and fibrillarin contents of the nucleoli“ in supplementary) and this agrees with the models of pulse-like, rather than continuous, activity of the promoters (Bahar Halpern et al., 2015).

Different kinetics of the transcription output is peculiar to a specific sort of gene or current status of the cell. Our study indicates that each ribosomal gene may be engaged simultaneously in at least two kinds of fluctuations. In the first kind, which is characterized by periods of about 2–3 min (Figure 6.12, Figure 6.13, Figure 6.14, Figure 6.15), the pol I signals, as well as the corresponding transcription rates, in the neighboring FC/DFC units fluctuated independently. Such behavior, supposedly caused by inherent stochasticity of the transcription, was termed “intrinsic” (Elowitz et al., 2002; Elgart et al., 2011; Gillespie, 1976; Paulsson, 2005; Shahrezaei, 2008; Sherman and Cohen, 2014; Swain, 2002). But the second kind of fluctuations, in which the units belonging to different nucleoli showed a pronounced synchrony (Figure 6.16C), apparently depends on variations in the state of cell nucleus, especially in the levels of pol I, its co-enzymes, and RNA nucleotides. Such kind of pulsation may be related to the “extrinsic” (Elowitz et al., 2002; Elgart et al., 2011; Swain et al., 2002). Our data about the synchronous fluctuations of pol I signal seem to agree with the results of direct measurements of rDNA transcription in the entire nucleoli by label-free confocal Raman microspectrometry (Pliss et al., 2015). Notably, in this work, as well as in ours, the extended periods of silence known from the studies of other genes were not detected (Chubb et al., 2006). Perhaps such periods just cannot be discovered without monitoring transcription of individual

rDNA units. But it is also likely that production of rRNA molecules pauses for no longer than 1–2 min.

Several models were devised in effort to understand the nature of transcription bursting discovered in cell populations. Some authors analyzing the results of smFISH (Femino et al., 1998; Mueller et al., 2013; Zenklusen et al., 2008), postulate one state of promoter, whose activity is well characterized by a Poisson distribution. In other models (Raj and van Oudenaarden, 2008; Singer et al., 2014; Sherman et al., 2015), the promoter switches stochastically between an active state, in which mRNA is produced with constant probability, and an inactive state, in which transcription does not occur. The extra state increases the potential variability of output from different cells in population or tissue. A still more complicated model of the bursting gene expression is suggested in recent study of actin gene in myxamoeba *Dictyostelium* (Corrigan et al., 2016). The authors interpreted their data as produced by a wide „spectrum" of activity states with variable rates of initiation. Such kind of model may agree with the complex kinetics of pol I signal intensity in FC/DFC units observed in our work (Figure 6.13), although in the case of rDNA the multiple genes of similar structure are situated in the same cell nucleus, where some aspects of their activity may be synchronized.

Since the observed fluctuations did not depend on rRNA elongation (Figure 6.19A), and their amplitudes were relatively low, we suppose that the fluctuating components of transcription machinery are accumulated around the promoters, and the observed variations of the gene output depend on a complex periodicity in the assembly of pre-initiation complex. Moreover, the observed correlation between the exchange rate of pol I and the phase of fluctuation in individual FC/DFC units (Figure 6.17C, Figure 6.17D) indicates that the rDNA promoter may exist in multiple states with different probabilities of initiation. Our *in vivo* measurements of pol I and fibrillarin signals in FC/DFC units of mammalian cells revealed two kinds of fluctuations, short-term (with periods of about 2-3 min) and long-term (with period up to 60 min); the second kind apparently corresponds to the fluctuations of rDNA transcription and early rRNA processing. Thus our results indicate that the synthetic activity of pol I is discontinuous, and that it follows a complex kinetic pattern.

8. CONCLUSIONS

The studies presented in our published works were designed to contribute to the understanding of the complex nucleolar dynamics. Firstly, we examined the changes of the FC/DFC units in the course of the cell cycle, and we suggested a scheme of their reproduction. Secondly, we found fluctuations of the key enzymes involved in the rDNA expression within the FC/DFC units. Some of these fluctuations, with periods ranging from 10 min to 1 h, probably reflect discontinuity of rDNA transcription and early steps of rRNA processing.

I.) Our study of reproduction of the FC/DFC units showed the following findings:

- FC/DFC units of mammalian nucleoli correspond to the pol I positive foci observed in confocal images of nucleoli.
- Total number of the units in the cell (both pol I and fibrillarin positive) increases by 60-80% in the course of early S phase but does not change from mid S to the end of interphase.
- Duplication of the units is not completed until the end of the interphase. The daughter postmitotic cells replenish the deficit of the units during early G1 phase.
- During early S phase, some of the FC/DFC units lose pol I, but fibrillarin is retained.
- TSA, AzdC, and Roscovitine treatment during the middle to late S phase did not increase the number of pol I positive units

Our data suggest that a subset of ribosomal genes remain transcriptionally silent from late S phase to mitosis.

II.) The results of our study of polymerase I kinetics and fibrillarin in FC/DFC nucleolar units showed two types of signal intensity fluctuations: rapid and slow

1.) <u>Rapid fluctuations</u>

- The periods are 2 to 3 minutes long.
- Signals of polymerase I and fibrillarin fluctuate there in an antiphase manner.
- Signals of polymerase I in the neighbouring FC/DFC units are not synchronized.
- Rapid fluctuations correlate with the exchange times of both proteins in FC/DFC units.

2.) <u>Slow fluctuations</u>

- The periods are cca 10, 20 and 60 minutes.
- Polymerase I signal in units belonging to different nucleoli are synchronized.
- The intensity of the polymerase I and fibrillarin signals measured in the same unit does not correlate.
- The intensity of the polymerase I signals strongly correlates with the transcription intensity within the FC/DFC units.

<u>Our data suggest that transcription of ribosomal genes is discontinuous.</u>

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https://github.com/vmodrosedem/segmentation-correlation

10. SUPPLEMENTARY

PAPER I

„Nucleolar DNA: the host and the guests“

2016

PAPER II

„Reproduction of the FC/DFC units in nucleoli“

2016

PAPER III

„Fluctuations of pol I and fibrillarin contents of the nucleoli“

2017

PAPER IV

„Discontinuous transcription”

2018