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CDK9 LINKS RNA POLYMERASE II TRANSCRIPTION TO PROCESSING OF RIBOSOMAL RNA

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Dedicated to my Grandmother (†)

AFFIDAVIT (EIDESSTATTLICHE VERSICHERUNG)

Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit mit dem Titel

„CDK9 LINKS RNA POLYMERASE II TRANSCRIPTION TO PROCESSING OF RIBOSOMAL RNA“

von mir selbstständig und ohne unerlaubte Hilfsmittel angefertigt worden ist, und ich mich dabei nur der ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe.

Die Dissertation ist weder in der jetzigen noch in einer abgewandelten Form einer anderen Prüfungskommission vorgelegt worden.

Ich erkläre weiter, dass ich mich anderweitig einer anderen Doktorprüfung ohne Erfolg nicht unterzogen habe.

München, 27. September 2012

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Kaspar Burger

SYNOPSIS

The nucleolar process of ribosome biogenesis is deregulated in many tumors, but has been disregarded in cancer research until recently. Mutations of ribosomal proteins have been shown to be causal for proliferative diseases and cancer. Key features of ribosome biogenesis, such as transcription and processing of ribosomal RNA (rRNA) are adaptable to the cell's proliferation state. In fact, the structural and functional integrity of the human nucleolus is tightly connected to cell cycle regulation, cell growth, and proliferation. Ongoing ribosome biogenesis is a prerequisite for destabilization of the tumor suppressor p53, a key cell cycle regulator. Various kinds of stress, including chemotherapeutic drugs, inhibit ribosome biogenesis and stabilize p53. Thus, the nucleolus is a highly sensitive sensor of cellular stress.

Previous work showed that the serine/threonine kinase-inhibitor Flavopiridol (FL) causes an efficient and specific block of ribosome biogenesis at the level of 47S rRNA processing and induces nucleolar stress. In the work described here, the molecular mechanism of FL-mediated 47S rRNA processing inhibition was analyzed. An RNAi screen identified the cyclin-dependent kinase 9 (Cdk9) as critical regulator of 47S rRNA processing. Cdk9 facilitates 47S rRNA processing by its function in RNA polymerase II (RNAP II) elongation. Inhibition of Cdk9-mediated RNAP II transcription depletes a set of small nucleolar RNAs (snoRNAs) critical for 47S rRNA processing. Precisely, Cdk9 activity is required for expression of rRNA processing-regulating snoRNAs like SNORD3 (U3), SNORD14A (U14A), SNORA73A (U17a), SNORD22 (U22), and SNORD118 (U8). In particular, depletion of U8 snoRNA causes a specific processing defect of the 47S rRNA primary transcript in the 3' external transcribed spacer (3'ETS) region. Defective 3'ETS processing, in turn, negatively feeds back on RNA polymerase I (RNAP I) transcription. The requirement of Cdk9 for rRNA processing is conserved in yeast. Thus, Cdk9 links RNAP II transcription to rRNA processing by facilitating the biogenesis of critical snoRNAs.

In a second project, I assessed the impact of 4-thiouridine (4sU) on rRNA synthesis. The uridine analog is frequently used in nascent RNA labelling experiments, but its biological activity has been poorly characterized, so far. My results show that 4sU treatment is sensed by the nucleolus. 4sU globally inhibits rRNA synthesis and triggers a nucleolar stress response.

Taken together, the data presented in this thesis shed light on regulation of mammalian ribosome biogenesis. My work suggests that inhibition of Cdk9-mediated snoRNA biogenesis is a promising approach to establish ribosome biogenesis as process of therapeutic relevance. The results may stimulate the development of small molecule inhibitors for non-genotoxic inhibition of ribosome biogenesis.

CONTENT

1. INTRODUCTION

1.0 Opening remark

Biology is the leading science of the 21st century. A large amount of exponentially growing knowledge has been accumulating within the last decades. Life sciences explore complex phenomena of life, covering all facets of biology from macroscopic analysis of populations and organisms to basic mechanisms and molecules at cellular and molecular level. Biomedical research not only contributes to the improved understanding of life, it also aims to transfer knowledge into clinical applications. There is a continuous medical need for the development of new therapeutic strategies. For example, > 10 % of deaths in high-income countries can be allocated to cancer. Increasing incidence rates underscore the importance to discover novel approaches to treat cancer (WHO, Fact sheet No. 310, 2011; RKI, Krebs in Deutschland, 2010).

Ribosome biogenesis is a highly conserved process in the nucleolus of cells, which is tightly connected to cell growth and proliferation. Recent findings suggest that changes in ribosome biogenesis rate interfere with cell cycle regulation and have implications for tumor formation (Ruggero and Pandolfi, 2003). My thesis explores molecular principles of ribosome biogenesis regulation. Before introducing the theme in detail, key principles of the cell cycle for regulation of cell growth and proliferation will be presented.

1.1 The cell cycle - basic principles and regulation

A normal cell proceeds through repetitive, but limited phases of growth and division (Fig. 1A). The cell cycle can be divided into two distinct stages: interphase and M-phase. Quiescent cells that have stopped dividing can also rest in G_0-phase. Interphase is further divided into G_1-phase, S-phase, and G_2-phase. In G_1-phase, the cell's metabolic activity is high, producing ribosomes and enzymes for DNA replication, the major event in S-phase. Another biosynthetic period in G_2-phase is

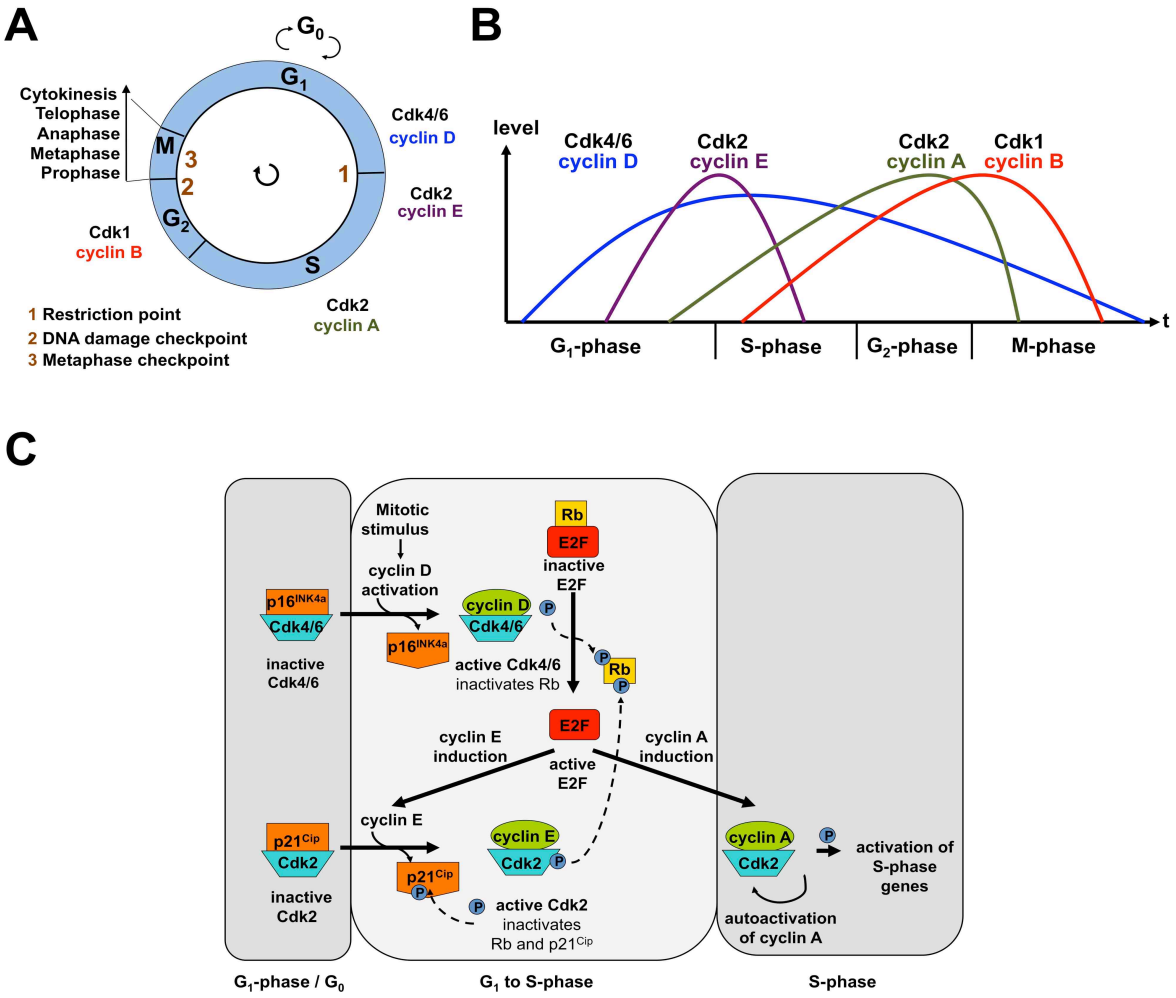

Figure 1. **The cell cycle: major checkpoints and cyclin expression. (A)** The cell cycle comprises three major checkpoints: (1) the restriction point, which monitors G_1- to S-phase transition, (2) the DNA damage checkpoint, which is required for G_2- to M-phase transition, and (3) the metaphase/spindle checkpoint, which controls chromosomal separation. Relevant Cdk/cyclin complexes are indicated. **(B)** Cyclin expression during the cell cycle. Cyclin D, cyclin E, cyclin A, and cyclin B levels are plotted as a function of time (adapted from Bardin and Amon, 2001). **(C)** The restriction point. Transition from G_1- to S-phase is under control of Cdk inhibitory proteins from the Cip/Kip (e.g. $p21^{Cip}$) and INK4a (e.g. $p16^{INK4a}$) families, which bind G_1-phase Cdk4/6 and Cdk2. Upon growth stimulation, cyclin D is induced and activates Cdk4/6, which then activates E2F, cyclin E, and cyclin A. Active Cdk2 ensures progression to S-phase. Cdk2 inhibits $p21^{Cip}$ and Rb and induces S-phase genes.

required to prepare for the actual cell division in M-phase. M-phase is discriminated into mitosis (chromosomal segregation and nuclear division) and cytokinesis (division of the cytoplasm). Progression through the cell cycle is strictly driven by interplay of two classes of proteins: cyclins and cyclin-dependent kinases (Cdks), which form heterodimers in different combinations, with cyclins being the regulatory component and Cdks being the catalytical active component. In mammals, more than ten different Cdks and various cyclins have been discribed. While Cdks are constitutively expressed, expression of cyclins is phase-specific and, in some cases,

mutually exclusive (Fig. 1B) (Grana et al., 1994; MacLachlan et al., 1995; Peng et al., 1998; Rickert et al., 1996). Thus, distinct Cdk/cyclin complexes exist at distinct cell cycle phases, phosphorylating distinct downstream effectors, which regulate formation and activity of subsequent Cdk/cyclin complexes. The Cip/Kip and INK4a inhibitory proteins negatively regulate cell cycle progression by binding Cdk/cyclin complexes (Sherr and Roberts, 1999). These regulatory principles apply at three critical stages within the cell cycle, the checkpoints.

The major checkpoint is called 'restriction point' and regulates transition from G_1- to S-phase (Fig. 1C). This checkpoint is of particular importance, since transition into S-phase is irreversible. The cell cycle has to be completed after crossing the restriction point. INK4a proteins such as $p16^{INK4a}$ block the cell cycle by binding Cdk4/cyclin D complexes (Sherr, 1996). Once the cell receives a mitotic stimulus, cyclin D expression is induced. Cdk4 now preferentially binds cyclin D, forming an active complex. Cdk4/cyclin D phosphorylates and inactivates the Retinoblastoma protein (Rb), an inhibitor of the E2F transcription factor family (Morgan, 1997; Wang et al., 2001). Active E2F is released from phosphorylated Rb and induces expression of cyclin E and cyclin A (Hatakeyama and Weinberg, 1995; Weinberg, 1995; Dyson, 1998). Cyclin E liberates Cdk2 from Cip/Kip family Cdk-inhibitors such as $p21^{Cip}$. Active Cdk2/cyclin E further promotes transition from G_1- to S-phase, since inhibition of $p21^{Cip}$ and Rb is manifested by Cdk2/cyclin E phosphorylation. Induction of cyclin A by E2F activates S-phase specific genes (Lees et al., 1993; DeGregori et al., 1995). Cyclin A induction is also self-stimulatory for formation of active Cdk2/cyclin A (Grana and Reddy, 1995; Pines, 1995; Sherr, 2000).

Two further checkpoints need to be passed to complete the cell cycle. Entry to mitosis is regulated at the end of G_2-phase, when replication is completed. This checkpoint is called DNA damage checkpoint. It ensures that no DNA damage, which frequently occurs in replication, is present before cell division. G_2- to M-phase transition is regulated by the DNA damage sensitive CHK1 kinase, which both activates the Wee1 kinase and inactivates the Cdc25 phosphatase. Active Wee1 phosphorylates the Cdk2/cyclin B complex and thereby restricts G_2- to M-phase progression. After DNA repair has been completed, CHK1 kinase is inactive and can no longer activate Wee1 and inhibit Cdc25, respectively. Dephosphorylation of the Cdk2/cyclin A complex by Cdc25 enables cell cycle progression (Gould and Nurse, 1989; Lundgren et al., 1991; Walworth et al., 1993; Cuddihy and O'Connell, 2003). The third major checkpoint is located in metaphase of mitosis. It ensures that all chromosomes are fully condensed before segregation in anaphase. Once

chromosomes are arranged properly at the mitotic plate, the anaphase-promoting complex (APC/C) is active. APC/C degrades securin, an inhibitor of separase, which subsequently cleaves cohesin, thereby releasing the chromosomes for segregation (Zachariae and Nasmyth, 1999).

1.2 The nucleolus is embedded in the cell cycle

Cell growth and proliferation is an enormous logistic task. The whole genome has to be copied and a huge amount of biosynthesis has to be performed to produce two daughter cells, which equal the mother cell. In fact, a proliferating cell consumes up to 80 % of its energy for the production of ribosomes (Thomas, 2000). Given that ribosome biogenesis is a prerequisite for cell growth and proliferation, it is evident that ribosome biogenesis and the cell cycle are co-regulated and influence each other. Before highlighting various connections between the nucleolus and cell cycle control, the structural und functional principles of ribosome biogenesis will be introduced.

1.2.1 The nucleolus provides a structural frame for ribosome biogenesis

Ribosome biogenesis takes place in the nucleolus (Brown and Gurdon, 1964; Miller and Beatty, 1969). The interphase nucleolus is a membrane-free sub-cellular component, which forms around nucleolar organizer regions (NORs) within the nucleus. NORs contain ribosomal DNA (rDNA) genes coding for ribosomal RNA (rRNA) (Perry, 1962; Ritossa and Spiegelmann, 1965). NORs are located on five acrocentric chromosomes 13, 14, 15, 21, and 22. Mammalian nucleoli can differ significantly in number and size; varying from 0.5 to 9 μm. Nucleoli can also fuse. Every mammalian nucleolus comprises a regular organization, the tripartite structure (Fig. 2). This organization consists of the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The nucleolar structure is largely defined by its composition and contrast in electron micoscropy (EM). The FC in the heart of the nucleolus is a fibrillar region with low contrast in EM. It is partially surrounded by the DFC, a region of compact texture and high contrast. FC and DFC are embedded in the GC, a granular structure establishing the bulk of a nucleolus. The mammalian nucleolus is surrounded with a ring of condensed heterochromatin, the perinucleolar chromatin. Interestingly, a nucleolar tripartite structure is not conserved throughout eukaryotes. In *S. cerevisiae*, for example, a bipartite structure consisting of fibrillar strands and granules has evolved. Yeast nucleoli lack perinucleolar chromatin (Hernandez-Verdun et al., 2010).

Evolution of the tripartite structure is provoked by the increasing size of intergenic

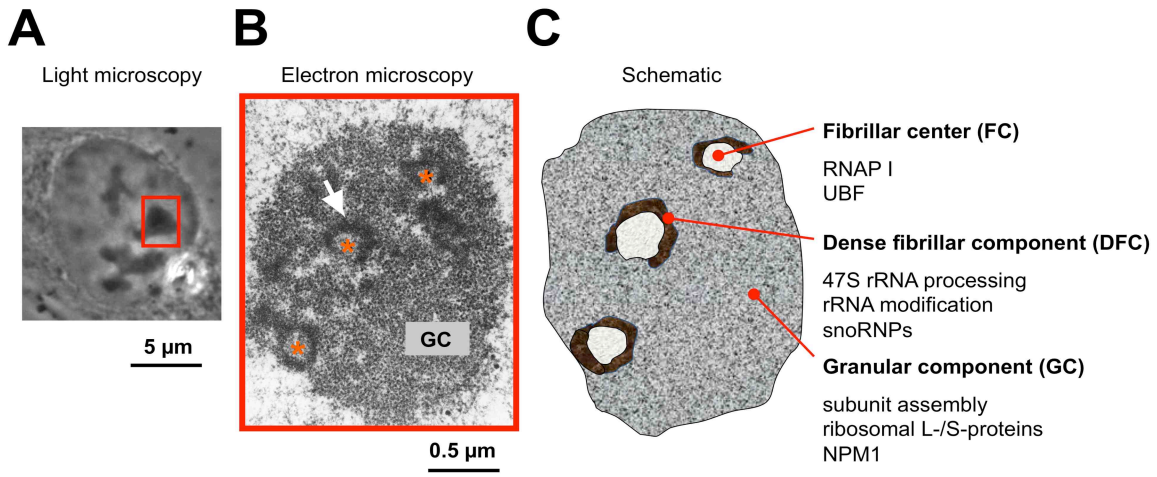

Figure 2. **The nucleolar tripartite structure. (A)** Light microscopy image of a human nucleus (U2OS cell, para-formaldehyde fixed). The nucleolus is visible as a dark-staining amorphic structure (red box). **(B)** Electron microscopy image of the human nucleolus (HeLa cell, glutaraldehyde and osmic acid fixed, contrasted with uranyl acetate). The nucleolar tripartite structure is visible. Star: fibrillar center (FC), Arrow: dense fibrillar component (DFC), GC: granular component. Scale bar: 0.5 µm (figure taken from Hernandez-Verdun et al., 2010). **(C)** Scheme of the human nucleolus. The tripartite structure and its major nucleolar functions are illustrated. RNAP I: RNA polymerase I, UBF: upstream binding factor, snoRNP: small nucleolar ribonucleoprotein, NPM1: Nucleophosmin.

non-transcribed rDNA spacers, necessitating a third nucleolar component to maintain a correct spatio-temporal regulation of ribosome biogenesis (Thiry and Lafontaine, 2005). Indeed, analysis of protein and RNA compositions show that distinct steps of ribosome biogenesis can be allocated to distinct nucleolar components. The FC contains non-transcribed rDNA, inactive RNAP I, and parts of the transcription machinery, such as upstream binding factor (UBF) or Topoisomerase II. Active rDNA transcription takes place at the interface between FC and DFC and produces large amounts of nascent, unprocessed rRNA precursors (pre-rRNAs). Processing and modification of pre-rRNAs mainly takes place in DFC, where small nucleolar ribonucleoprotein complexes (snoRNPs) concentrate. Most ribosomal proteins are present in GC, the major site of ribosome subunit assembly (Boisvert et al., 2007).

1.2.2 The nucleolar structure is modulated by the cell cycle

Nucleoli are dynamic structures and subject to strong morphological changes during the cell cycle. Assembly, maintenance and disassembly are closely linked to the transcriptional activity of RNAP I. Continuous synthesis of nascent pre-rRNA is the basis for the nucleolar structure and ribosome biogenesis during interphase (Dimario, 2004). In mitosis, however, nucleoli are disassembled. The Cdk1/cyclin B complex inactivates RNAP I transcription in mitosis (Heix et al., 1998). Most

components of the RNAP I transcription machinery remain associated with rDNA during mitosis, whereas nucleolar processing components and partially processed pre-rRNAs translocate from the NOR to the cytoplasm or localize in the perichromosomal region (PR) (Roussel et al., 1996; Gautier, 1994; Leung et al., 2004). Cdk1/cyclin B levels decrease during telophase and early G_1-phase, which reactivates RNAP I transcription and triggers nucleolar reassembly at the end of mitosis (Roussel et al., 1996) (Fig. 3). Nucleolar processing components and partially processed pre-rRNAs reassemble themselves in prenucleolar bodies (PNBs), which concentrate and release nucleolar components to nascent nucleoli in a defined order (Savino et al., 2001). Thus, the nucleolus is a cell cycle-dependent structure and 'formed by the act of building a ribosome' (Mélèse and Xue, 1995).

1.2.3 The nucleolus influences cell cycle progression

The nucleolus sequesters a number of cell cycle regulating factors during interphase. Nucleolar sequestration prevents them from being active throughout the entire cell cycle. Human telomerase reverse transcriptase (hTERT) is sequestered in the nucleous by nucleolin. hTERT is released from nucleolin at late stages of S-phase to catalyze telomere replication (Wong et al., 2002; Khurts et al., 2004). The protein phosphatase 1 gamma isoform (PP1γ) is also concentrated in nucleoli during interphase. PP1γ is released from the disassembling nucleolus to the cytoplasm at the beginning of mitosis. PP1γ binds mitotic chromosomes in anaphase to control chromosome condensation (Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2006; Trinkle-Mulcahy and Lamond, 2006). Similarly, Cdc14, a protein phosphatase crucial for promoting mitotic exit, is anchored in the nucleolus. Cdc14 binds the nucleolar

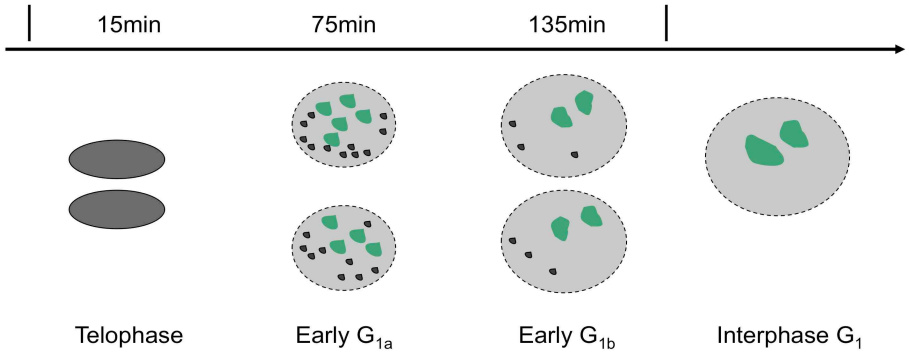

Figure 3. **Scheme of nucleolar reassembly in early G_1-phase.** The nucleolus remains disassembled until the end of telophase, when RNAP I transcription is reinitiated in nucleolar organizing regions (NORs) and the chromatin is still condensed (dark grey area). Mitotic chromatin decondenses in early G_{1a}-phase (light grey area), the nucleus is assembled (broken line), numerous perinucleolar bodies (PNBs) form (dark foci) and the transcriptional active NORs recruit processing factors to the dense fibrillar component (DFC) (green). Transfer of processing proteins from PNBs to the DFC is almost completed in early G_{1b}-phase and NORs regroup into nucleoli. Human nucleolar reassembly takes about two hours (adapted from Hernandez-Verdun et al., 2010).

factor Net1 until onset of anaphase. Binding to Net1 prevents an uncoordinated mitotic exit (Visintin et al., 1998; Shou et al., 1999). In anaphase, Net1 is phosphorylated by the Cdk1/cyclin B complex and looses its affinity to Cdc14, which is now able to dissolve chromosomal linkages by promoting cohesin cleavage (Azzam et al., 2004; D'Amours et al., 2004). Depletion of ribosomal processing factors Nop15 or Rrp14 causes a cytokinesis defect and a mislocalization of the mitotic spindle followed by a mitotic arrest (Oeffinger and Tollervey, 2003; Oeffinger et al., 2007). The examples characterize the interphase nucleolus as a storage domain, which is dissolved in mitosis to allow the coordinated release of critical factors that regulate cell cycle progression.

1.3 Ribosome biogenesis at the molecular level

Synthesis of ribosomes is the major function of the nucleolus. Nobel prize laureate Dr. Barbara McClintock connected the nucleolus with gene activity already in 1934. It took many decades until the understanding of ribosome biogenesis has evolved into one of the most complex processes of life. Ribosome biogenesis is highly coordinated and involves all three RNA polymerases. rRNA is synthesized, processed, modified, and assembled into ribosomal subunits. Mammalian 80S ribosomes consist of four rRNAs (28S, 18S, 5.8S rRNA transcribed by RNAP I, and 5S rRNA transcribed by RNAP III) and 79 ribosomal proteins (RNAP II transcripts) (Warner, 1999). The multiple steps of ribosome biogenesis are discussed next.

1.3.1 RNAP I transcription

rRNA transcription initiates ribosome biogenesis. Although rDNA comprises only 0.4 % of the human genome, the fraction of rRNA within the transcriptome can be up to 90 %, implicating that rDNA genes are heavily transcribed (Rabani et al., 2011). In total, about 400 rDNA tandem repeats ensure continuous biogenesis of one to two million ribosomes per cell (Prieto and McStay, 2008; Drygin et al., 2010). Many rDNA repeats are transcriptional inactive, which allows adaption to changing growth conditions and genome maintenance (Birch and Zomerdijk, 2008; Stefanovsky and Moss, 2006; Ide et al., 2010). Each rDNA repeat has a size of approximately 44 kilobases (kb), composed of a 14 kb coding region and a 30 kb long intergenic spacer sequence. Transcription of rDNA repeats produces a polycistronic 47S rRNA primary transcript, which contains 18S, 5.8S, and 28S rRNA and is performed by RNAP I, a 14-subunit enzyme with high structural similarity to RNAP II and III (Fatica and Tollervey, 2002; Kuhn et al., 2007; Werner et al., 2009).

Initiation is the critical and most regulated step in RNAP I transcription (Fig. 4, upper panel). Initiation of mammalian RNAP I transcription requires four essential transcription factors: transcription initiation factor 1A (TIF-1A), the selectivity factor 1 (SL1) complex, upstream binding factor (UBF), and transcription termination factor 1 (TTF-1). The latter also terminates RNAP I transcription. TIF-1A is associated with RNAP I and is required to render RNAP I competent for initiation (Peyroche et al., 2000). The SL1 complex consists of multiple subunits, including TATA-binding protein (TBP), and binds the core promoter. The SL1 complex is crucial for start site selection (Russel and Zomerdijk, 2006). UBF binds the upstream control element (UCE) as homodimer and facilitates RNAP I recruitment and promoter escape. UBF also stabilizes SL1 binding (Jantzen et al., 1990; Panov et al., 2006; Stefanovsky et al., 2006; Kuhn and Grummt, 1992). RNAP I is recruited to the promoter by synergistic binding of the SL1 complex and UBF. Recently, it was shown that actively transcribed rDNA repeats contain nucleosomes and remodelling factors, which regulate chromatin accessibility (Jones et al., 2007). Antisense transcription of rDNA produces a short non-coding RNA, which binds the promoter to recruit enzymes for epigenetic modification and thereby regulate initiation (Schmitz et al., 2010).

1.3.2 rRNA processing and maturation

RNAP I synthesizes a nascent 47S rRNA primary transcript, which is bound co-transcriptional by large and small ribosomal L- and S-proteins and processing factors to form a large and highly dynamic ribonucleoprotein complex, the 90S pre-

ribosome. Several rRNA synthesis steps happen simultaneously within the 90S pre-ribosome. More than 200 factors such as small nucleolar RNAs (snoRNAs), RNA helicases, nucleases, GTPases, and export factors are required to assemble, process, and mature ribosomes by continuous association and dissociation from immature ribosomes (Staley and Woolford, 2009). The 90S pre-ribosome is the stage of 47S rRNA modification and processing. Distinct sites of the nascent pre-rRNA precursor get methylated at the 2' oxygen of the ribose (2'-O-Met) and a subset of uridines is converted to pseudouridines (Ψ) (Cavaillé et al., 1996; Ganot et al., 1997; Weinstein and Steitz, 1999; Kiss, 2002). Both modifications are introduced by roughly 70 snoRNPs (Balakin et al., 1996; Tran et al., 2003). Each snoRNP contains one modifying enzyme (Fibrillarin for 2'-O-Met, Dyskerin for Ψ), one snoRNA, and additional stabilizing proteins. SnoRNAs can be divided into two families according to structural similarities, conserved sequence elements, and associated proteins. Box C/D snoRNAs guide rRNA methylation and form a box C/D snoRNP. Box H/ACA snoRNAs guide rRNA pseudouridylation and form a box H/ACA snoRNP (Kiss, 2002; Decatur and Fournier, 2003). Extensive rRNA modification occurs at catalytical active rRNA regions within the decoding and peptidyl transferase centers of the ribosome (Bakin et al., 1994). Modifications are guided by complementary base pairing of snoRNAs and facilitate rRNA folding to stabilize nascent pre-rRNA.

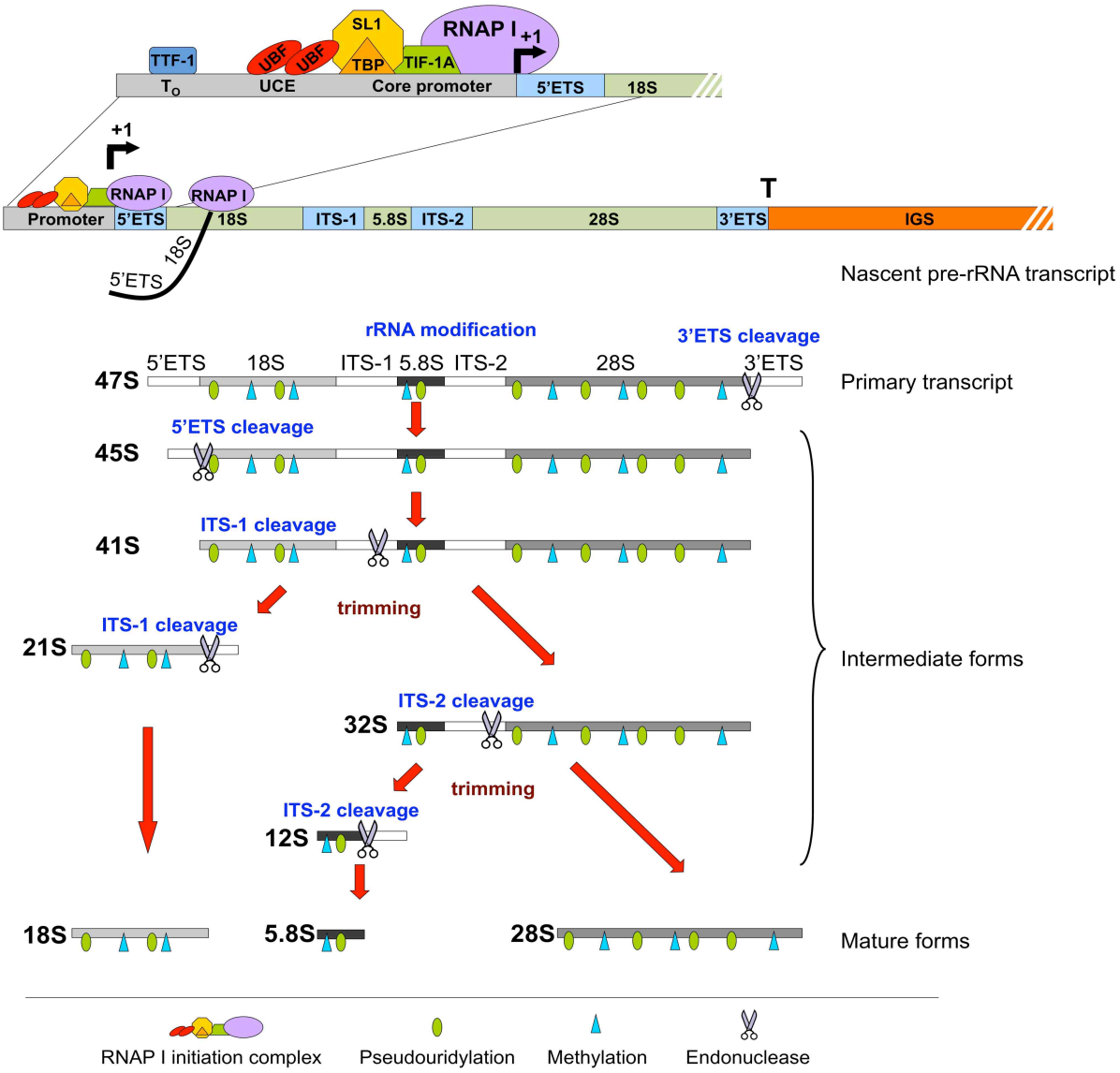

Figure 4. **Transcription and processing of mammalian ribosomal RNA.** RNAP I transcription occurs on tandem rDNA repeats. Each rDNA repeat contains a promoter region, a transcription start site (+1), a polycistronic transcription unit, and a termination site (T). RNAP I initiation is facilitated by four general transcription factors: TTF-1, UBF, the SL1 complex, and TIF-1A. The polycistronic transcription unit encodes a 47S precursor rRNA, which contains a 5' external transcribed spacer (5'ETS), 18S rRNA, an internal transcribed spacer 1 (ITS-1), 5.8S rRNA, an internal transcribed spacer 2 (ITS-2), 28S rRNA, and a 3' external transcribed spacer (3'ETS). The 47S rRNA primary transcript undergoes co-transcriptional rRNA modifications (methylation, pseudouridylation) and a cascade of endonucleolytic cleavages (scissors) and exonucleolytic degradation steps (trimming). UCE: upstream control element, IGS: intergenic spacer (adapted from Mullineux and Lafontaine, 2012).

SnoRNPs bind the pre-rRNA modification region surrounding the nucleotide to be modified (Kiss-László et al., 1996; Bachellerie et al., 2002).

Little is known about enzymes that catalyze mammalian rRNA processing. Endo- and exonucleases cleave and trim external and internal transcribed spacers within polycistronic 47S rRNA (Kos and Tollervey, 2005; Venema et al., 1997). 47S rRNA processing is characterized by a cleavage cascade and stepwise production of intermediate rRNA forms, which finally mature to 18S, 5.8S, and 28S rRNA

(Hadjiolova et al., 1993) (Fig. 4, lower panel). In yeast, there are two independent and spatially separated processing machineries. While 40S maturation is facilitated by the small subunit processome (SSU), 60S maturation requires different processing and assembly factors (Dragon et al., 2002; Grandi et al., 2002). The yeast 35S rRNA primary transcript is initially cut by the t-UTP-complex. The t-UTP-complex contains SNORD3 (U3) snoRNA, which catalyzes 5'ETS processing of the primary transcript and directs correct folding of cleaved rRNA (Gallagher et al., 2004; Gerbi et al., 2001). 3'ETS processing of 35S rRNA is performed by the Rnt1 endonuclease. Rnt1 cleavage produces a 27S rRNA intermediate, which is then cleaved by RNase MRP in ITS-1 and trimmed by 3'-5' exonuclease Rex1. Additional trimming of premature 25S rRNA by 5'-3' exonucleases Xrn1/Rat1 produces mature 25S rRNA. Rex1 is also involved in trimming of premature 5.8S rRNA (Fatica and Tollervey, 2002; Peculis and Steitz, 1993). Some processing factors in yeast have homologs in higher eukaryotes. For example, human ribosomal processing factors Pes1, Bop1, and WDR12 form a trimeric complex, PeBoW, which is essential for ITS-2 processing of intermediate 32S rRNA into mature 28S rRNA (Lapik et al., 2004; Hölzel et al., 2005; Grimm et al., 2006; Hölzel et al., 2007; Rohrmoser et al., 2007). Nucleophosmin (NPM1) and Nucleostemin (NST) also modulate ITS-2 processing (Haindl et al., 2008; Itahana et al., 2003; Romanova et al., 2009).

On its transport to the nucleoplasm, the 90S pre-ribosome splits into pre-60S and pre-40S particles, which further mature in the nucleoplasm. 60S and 40S ribosomal subunits are exported to the cytoplasm and form mature 80S ribosomes for translation of messenger RNAs (mRNAs) (Gadal et al., 2001; Thomas and Kutay, 2003; Tschocher and Hurt, 2003). Importantly, recent findings suggest that ribosomes are more than just machines that translate mRNA. Ribosomal protein composition affects gene regulation and tissue development. For example, a mutation in ribosomal protein L38 reduces the translational efficacy for Hox factors in a tissue specific manner (Kondrashov et al., 2011). Great heterogeneity in ribosomal protein expression suggests the existence of ribosomes, which are specialized for translation of mRNAs in a tissue specific manner.

1.3.3 The multifunctional nucleolus

The nucleolus is involved in processing and maturation of various RNAs and RNPs. First, 5S rRNA, a product of RNAP III transcription, enters the nucleolus in complex with ribosomal protein L5 for maturation and incorporation into the ribosome (Steitz et al., 1988). The transfer RNA (tRNA) processing component RNase

P localizes in the nucleolus, suggesting a role for tRNA splicing (Bertrand et al., 1998). Adenosine deaminase ADRA1 is active in the nucleolus, which assigns RNA editing as a nucleolar function (Vitali et al., 2005). The nucleolus seems also to be involved in RNA interference (RNAi). Endogenous short interfering RNAs (siRNAs) co-localize in the nucleolus together with components of the RNAi processing machinery (Pontes et al., 2006). The nucleolus is also required for assembly and maturation of various RNPs, such as the U6 sliceosomal small nuclear RNP, telomerase, or the signal recognition particle (SRP), thereby contributing to splicing, telomere replication, and peptide sorting (Ganot et al., 1999; Mitchell et al., 1999; Jacobson and Pederson, 1998; Politz et al., 2000). In summary, the nucleolus is a multifunctional compartment.

1.3.4 Surveillance of ribosome biogenesis

Nucleolar ribosome biogenesis is a complex, non-linear, and error-prone process. Up to 80 % of pre-rRNAs in the cell are produced incorrectly. Defective pre-rRNAs need to be eliminated to ensure biogenesis of high quality ribosomes. A robust surveillance mechanism is required to prevent production of misassembled ribosomes with poor translational capacity. Defective pre-rRNAs are 3′-polyadenylated by interaction with accessory factors like the TRAMP complex, the Nrd1 complex, or the Ski7 complex (LaCava et al., 2005; Orban and Izaurralde, 2005). Polyadenylation recruits the exosome and triggers degradation of defective pre-rRNA. The exosome is a 400 kDa multi-enzyme complex with 3′-5′ exonuclease activity. The exosome consists of nine core subunits and one catalytical active subunit (Kuai et al. 2004; Allmang et al. 1999). Recently, it was shown that three distinct exosome isoforms, differing in their cellular localization and 3′-5′ exonuclease subunit, exist in mammalian cells. While hRrp6 is the catalytic subunit of nucleolar exosomes, hDIS3 and hDIS3L are present in nucleoplasmatic and cytoplasmic exosomes, respectively. The putative RNA helicase hMtr4 is also required for nuclear exosome activity; it unwinds defective pre-rRNAs (Tomecki et al., 2010; Staals et al., 2010; Lubas et al., 2011). The exosome is part of the major quality control pathway for rRNAs, but it also facilitates degradation of defective or dispensable mRNAs and maturation of other non-coding RNAs such as snoRNAs, snRNAs, and tRNAs (Anderson and Parker, 1998; Mukherjee et al., 2002; Wyers et al., 2005).

1.4 RNAP II transcription

A basic overview of RNAP II transcription and its regulation is described next, as the transcriptional activity of RNAP II will be a key subject of this thesis. RNAP II transcribes mRNAs for 79 ribosomal proteins and > 200 processing and synthesis factors for ribosomes including snoRNAs. Eukaryotic RNAP II consists of 12 subunits (Rpb1-12) (Cramer, 2004). The 10-subunit core complex shows high conservation with RNAP I and III. RNAP II has evolved two additional subunits, Rpb4 and Rpb7, which are potential paralogs to RNAP I subunits (Edwards et al., 1991; Cramer, 2002). The largest subunit of RNAP II (Rpb1) harbors a repetitive carboxy-terminal domain (CTD). The RNAP II CTD is flexible and locates in close proximity to the RNA exit pore and nascent mRNA (Noble et al., 2005; Armache et al., 2005). The CTD is unique to RNAP II and comprises an evolutionary conserved, tandemly arranged repetitive consensus amino acid sequence, the $Y_1S_2P_3T_4S_5P_6S_7$ heptad. The mammalian CTD is composed of 52 heptad repeats (Chapman et al., 2008).

RNAP II transcription can be roughly divided into three distinct stages: initiation, elongation, and termination. These stages are repeated in a transcription cycle (Fig. 5). Interestingly, the phosphorylation patterns of the CTD vary upon progression through the transcription cycle. RNAP II initiation is orchestrated by the interplay of three distinct classes of regulatory factors. First, sequence-specific transactivators bind enhancer sequences. Second, transcriptional co-activators such as the Mediator complex or chromatin remodellers and histone modifying enzymes are recruited, making the promoter accessible for the third class of regulatory factors, the general transcription factors (GTFs) (Hahn, 2004). Six GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) sequentially bind the promoter and recruit RNAP II to form the pre-initiation complex (PIC) (Orphanides et al., 1996; Ptashne, 1986). Prior to recruitment, RNAP II CTD is phosphorylated at position serine-5 by the Cdk8/cyclin C complex, a cofactor of the Mediator. Cdk8 prevents premature PIC formation, which only forms after dephosphorylation of serine-5 by the Fcp1 phosphatase (Hengartner et al., 1998; Kamenski et al., 2004). Several critical events are required in order to release RNAP II from the promoter and the transit into elongation. In brief, XPB/D helicase activity in TFIIH melts 11-15 base pairs of DNA to make the template accessible for transcription. Cdk7 kinase activity in TFIIH re-phosphorylates the CTD at serine-5 (Hengartner et al., 1998). Serine-5 phosphorylation triggers a partial PIC disassembly and allows RNAP II to escape from the promoter, entering a short critical phase of instable abortive initiation,

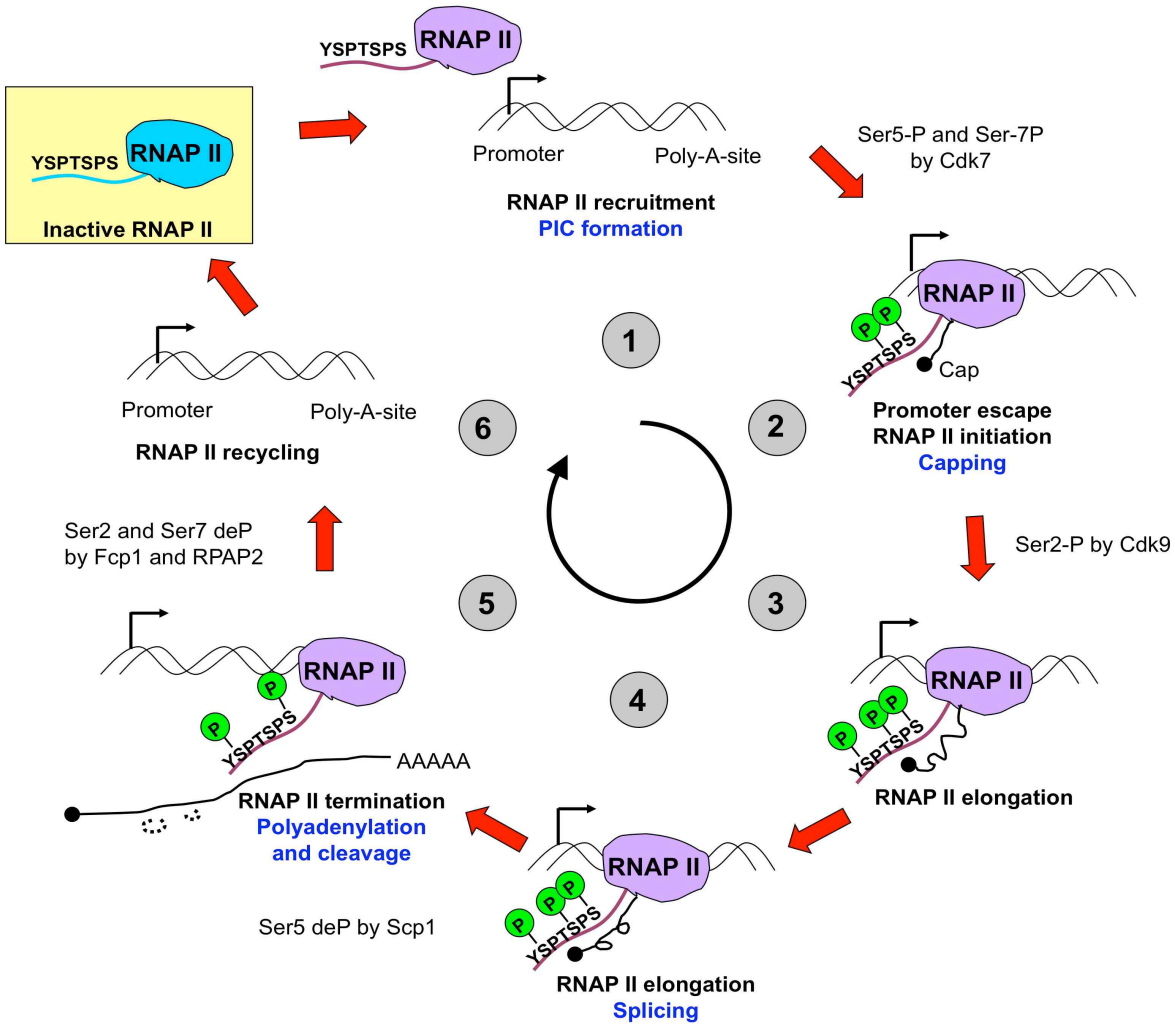

Figure 5. **The RNAP II transcription cycle. (1)** RNAP II recruitment and PIC formation. RNAP II forms a pre-initiation complex (PIC) together with general transcription factors (GTFs) and the Mediator complex. **(2)** Promoter initiation and escape. Cdk7 phosphorylates the RNAP II carboxy-terminal domain (CTD) at serine-5 and -7, which destabilizes the PIC and initiates transcription. RNAP II initial transcription is abortive and pauses before elongation. The nascent pre-mRNA is capped. **(3)** RNAP II elongation. Cdk9 phosphorylates the RNAP II CTD at serine-2 and -5 and triggers transition from promoter proximal pausing to elongation. **(4)** Co-transcriptional pre-mRNA splicing occurs at hyperphosphorylated CTD, which recruits splicing factors and is crucial for small nuclear (sn)RNA transcription. **(5)** Termination of RNAP II transcription. RNAP II reaches a poly-A-site and RNAP II phosphatase Scp1 dephosphorylates the CTD at position serine-5. Pre-mRNA is cleaved and RNAP II dissociates from the template. **(6)** RNAP II recycling. CTD phosphatases Fcp1 and RPAP2 dephosphorylate the CTD at positions serine-2 and -7. Hypophosphorylated RNAP II dissociates from the DNA, forming an inactive pool.

which is immediately stabilized by the increasing length of the nascent transcript (Pal and Luse, 2003; Kireeva et al., 2000). Promoter proximal pausing of RNAP II enables capping and stabilizes nascent mRNA. RNAP II CTD phosphorylation at serine-5 was also shown to be required for recruitment of the capping enzyme (Yamaguchi et al., 1999; Wada et al., 1998; Pei and Shuman, 2002).

RNAP II is now competent for productive elongation, but is still retracted by negative transcription elongation factor (NELF) and the Spt4/5 heterodimer. Productive elongation is stimulated by the positive transcription elongation factor b

(pTEFb), which consists of Cdk9 and cyclin T1 (Marshall et al., 1996; Price, 2000). pTEFb phosphorylates both NELF, Spt4/5, and the RNAP II CTD at positions serine-2 and -5. NELF dissociates from RNAP II after pTEFb phosphorylation (Fujinaga et al., 2004; Yamada et al., 2006; Cho et al., 2001; Marshall et al. 1996; Andrulis et al., 2000; Czudnochowski et al., 2012). Thus, phosphorylation of the CTD at position serine-2 is a hallmark for elongating RNAP II. A number of additional factors, such as TFIIF or the Elongator complex promote RNAP II elongation by chromatin remodelling (Cheng and Price, 2007; Close et al., 2006). Splicing of precursor-mRNAs (pre-mRNAs) is a second important co-transcriptional event. It is not entirely clear, whether co-transcriptional splicing requires active RNAP II elongation or is linked to the CTD solely as a mechanical binding platform, independent of mRNA synthesis (Kornblihtt, 2004). The splicing machinery can bind nascent mRNA either directly or via the CTD (Moore et al., 2006; de la Mata and Kornblihtt, 2006). Occasionally during elongation, RNAP II backtracks in the gene. Backtracking involves TFIIS and may increase RNAP II transcriptional fidelity (Cheung and Cramer, 2011). RNAP II CTD is also phosphorylated at position serine-7. The CTD serine-7 phosphorylation is introduced by Cdk7 (Chapman et al., 2007; Akhtar et al., 2009). It has been shown that serine-7 phosphorylation recruits the Integrator complex and is specifically required for small nuclear RNA (snRNA) transcription (Baillat et al., 2005; Egloff et al., 2007).

RNAP II termination requires a poly-A-site, cleavage and processing factors like Pcf1, Sen1, Nab3, or Nrd1 (Zaret and Sherman, 1982; Birse et al., 1998; Steinmetz et al., 2001). CTD phosphorylations are sequentially removed by phosphatases like Scp1, Fcp1, and RPAP2 (Proudfoot, 1997; Vasiljeva et al., 2008; Yeo et al., 2005; Egloff et al., 2012). Removal of c-Abl-mediated CTD phosphorylation at position tyrosine-1 is required for the interaction of the RNAP II CTD with termination factors (Mayer et al., 2012). There are two alternative models for RNAP II termination: the allosteric model, in which RNAP II undergoes a conformational change at the poly-A-site, leading to loss of processivity and gradual termination (Greenblatt et al., 1993). In the torpedo model, RNAP II is targeted by the 5′-3′ exonuclease Rat1 (torpedo), which attacks the 5′ end of the nascent transcript after endonucleolytic cleavage (Conelly and Manley, 1988; Kim et al., 2004). Rat1 catches up to RNAP II and triggers its release from the gene. Interestingly, RNAP II termination is coupled to 3′ end processing by interdependent recruitment between Rat1 and 3′ end processing factors like Pcf1 (Luo et al., 2006). Once terminated, RNAP II is subsequently available for another round of transcription.

1.5 SnoRNA biogenesis and pre-mRNA splicing are coupled events

Rat1 not only is required for rRNA synthesis and RNAP II termination, it also contributes to snoRNA biogenesis (Petfalski et al., 1998). In higher eukaryotes, most snoRNAs are co-transcribed within pre-mRNA and released from excised and debranched introns by exonucleolytic trimming (Kiss and Filipowicz, 1995; Richard and Kiss, 2006) (Fig. 6). The mature mRNAs of these pre-mRNAs often encode for proteins involved in ribosome biogenesis (Kiss, 2002). For example, box C/D snoRNA U14A is encoded in the Hsc70 pre-mRNA (Liu and Maxwell, 1990; Watkins et al., 1996). Four snoRNA-stabilizing proteins associate with the intronic pre-snoRNA sequence during pre-mRNA splicing (15.5kD, Nop56, Nop58, and Fibrillarin with box C/D snoRNAs; Nop10, Nhp2, Dyskerin (DKC1), and Gar1 with box H/ACA snoRNAs) (Hirose et al., 2003; Richard and Kiss, 2006; Darzacq et al., 2006). Assembly of pre-snoRNP complexes protects the pre-snoRNA from exonucleolytic degradation. Box C/D snoRNPs require the Hsp90 chaperone and its co-chaperone, the R2TP (Rvb1, Rvb2, Tah1, and Pih1)/Prefoldin-like (hereinafter R2TP) complex for correct folding, assembly, and stabilization. Box H/ACA snoRNP assembly also requires the Naf1 assembly factor (Hoareau-Aveilla et al., 2006; Ballarino et al., 2005). Thus, RNAP II transcription and co-transcriptional splicing actively promote snoRNA biogenesis.

1.6 Ribosome biogenesis in context of cancer

Ribosome biogenesis is upregulated upon growth factor stimulation and downregulated upon nutrient starvation (Drygin et al., 2010). In cancer cells, ribosome synthesis is altered in conditions of derailed growth control. Nucleolar morphology has been used as a diagnostic marker for the proliferative state of cancer

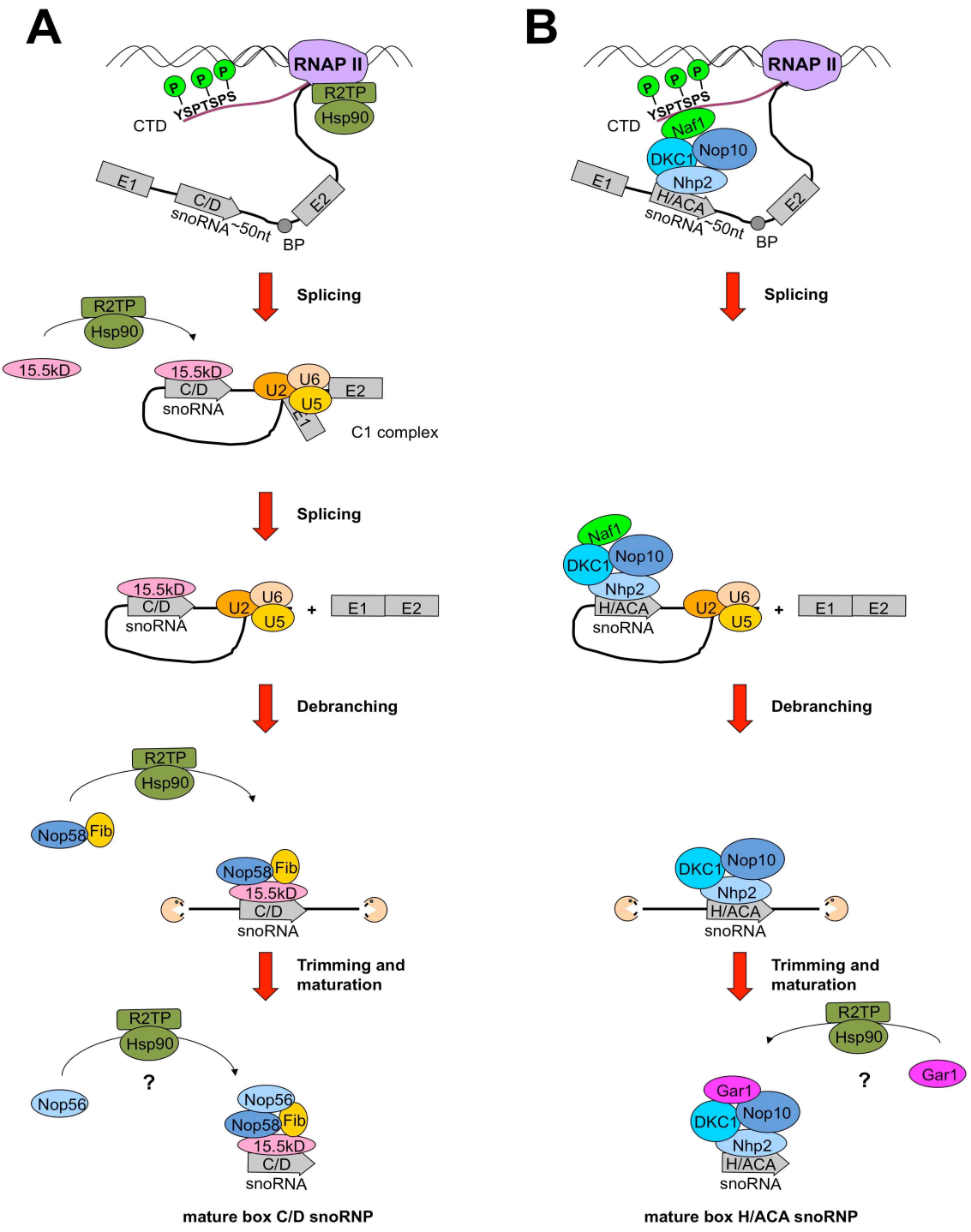

Figure 6. **SnoRNP assembly is coupled with pre-mRNA transcription and splicing. (A)** Assembly of box C/D small nucleolar ribonucleoproteins (snoRNPs) is dependent on the Hsp90/R2TP chaperone and active splicing. Hsp90/R2TP facilitates assembly of RNAP II and folding and assembly of box C/D core proteins 15.5kD, Fibrillarin (Fib), Nop58, and Nop56. Most box C/D snoRNAs are located about 50 nucleotides (nts) upstream of the branch point (BP) and are processed in a splicing-dependent manner. SnoRNPs assemble by successive binding of 15.5kD, Fib, Nop58, and Nop56. 15.5kD binds before splicing and debranching. Fib and Nop58 bind before processing. Binding of Nop56 completes snoRNP assembly. **(B)** Transcription-dependent assembly of box H/ACA snoRNPs. Assembly of pre-snoRNPs occurs co-transcriptionally. Nucleolar assembly factor 1 (Naf1) interacts with the RNAP II carboxy terminal domain (CTD) and facilitates recruitment of box H/ACA core proteins Dyskerin (DKC1), Nop10, and Nhp2 to nascent snoRNA. Gar1 core protein binds the pre-snoRNP probably after splicing and debranching. Note that a fraction of box C/D snoRNPs, which have suboptimal intronic positions and carry stem-loop structures, might follow a transcription-dependent pathway similar to box H/ACA snoRNP assembly (Hirose et al., 2003) (adapted from Richard and Kiss, 2006).

cells > 100 years ago (Pianese, 1896). In fact, an increase in nucleolar size correlates with increased ribosome biogenesis to meet high demands of protein synthesis for elevated tumor growth (Derenzini et al., 1998). Next, key principles of tumor formation are introduced before deregulation of ribosome biogenesis in cancer will be reviewed.

Cancer is a proliferative disease, defined as a neoplasm forming malignant tumors. Cancer cells have escaped the cellular mechanisms that restrict cell growth and proliferation. They are resistant to programmed cell death and have the ability to invade other tissues, form metastases, and spread around the body. Malignancy is achieved by cellular transformation, a multi-causal process of stepwise accumulation of somatic mutations. Which mutations drive transformation? Key regulators of the cell cycle and cell growth are frequently mutated in tumors (Croce, 2008). Two types of genes influence cell growth: proto-oncogenes and tumor suppressor genes. Proto-oncogenes can become hyperactive oncogenes by dominant gain of function mutations; tumor suppressor genes drive transformation by recessive loss of function mutations. Proto-oncogenes support growth by driving the cell cycle or stimulating signalling pathways. Typical examples of proto-oncogenes encode transcription factors such as c-Myc, kinases involved in signal transduction (e.g. Src, Raf, ERK or Abl), or cell cycle regulators like E2F. Tumor suppressors restrict growth. They decelerate cell cycle progression and are pro-apoptotic. One of the most important tumor suppressors is the p53 protein. p53 is regarded as the guardian of the genome, which senses DNA damage by induction of G_1-arrest or apoptosis (Carson and Lois, 1995; Levine 1997). The Rb protein is another major tumor suppressor that inhibits E2F at the restriction point (Sherr, 2000). Importantly, ribosome biogenesis is regulated by prominent proto-oncogenes and tumor suppressor genes, which are frequently mutated in cancer (Table 1).

Stimulatory Genes	Function in ribosome biogenesis	Inhibitory Genes	Function in ribosome biogenesis
CK II	Hyperphosphorylation of TIF-1A, UBF, SL1	*RB*	Inhibition of UBF
ERK	Hyperphosphorylation of TIF-1A, UBF	*TP53*	Inhibition of initiation complex formation
S6K	Hyperphosphorylation of TIF-1A	*PTEN*	Repression of PI3K signalling
	Induction of 5' TOP mRNA expression	*p14ARF*	Inhibition of rRNA processing
PI3K	Induction of 5' TOP mRNA expression		
mTOR	Induction of 5' TOP mRNA expression		
C-MYC	Induction of UBF		
	Enhancing of SL1 recruitment		
	Induction of processing factor expression		
NPM1	Induction of SL1 expression		

Table 1. **Cancer genes and ribosome biogenesis regulation.** Mutations in major proto-oncogenes and tumor suppressor genes deregulate RNAP I transcription, rRNA processing, and translation.

1.6.1 Deregulation of RNAP I transcription in cancer

RNAP I transcription initiation is strongly regulated by UBF and TIF-1A. High levels of hyperphosphorylated UBF and TIF-1A are present in growth favourable conditions, driving RNAP I transcription, ribosome biogenesis, and proliferation. Three serine/threonine kinases achieve hyperphosphorylation of UBF and TIF-1A: casein kinase II (CK II), S6 kinase (S6K) and extracellular signal-regulated kinase (ERK) (Voit et al., 1992; Stefanovsky et al., 2001; Zhao et al., 2003; Panova et al., 2006). Consequently, UBF is frequently upregulated in cancer, but downregulated and hypophosphorylated in quiescent cells (Drygin et al., 2010). CK II, ERK, and the various components of the receptor tyrosine kinase signalling pathway (e.g. Ras, Raf, or MEK) are also frequently mutated in human cancer cells, allowing self-stimulatory ribosome biogenesis induction independent from extracellular growth factors (Huang et al., 2002; Guerra and Issinger, 2008; McCubrey et al., 2007). c-Myc binds the RNAP I promoter to stimulate rRNA transcription (Arabi et al., 2005; Grandori et al., 2005). c-Myc also induces UBF and is upregulated in many tumors (Poortinga et al., 2004; Meyer and Penn, 2008). p53 represses rRNA transcription *in vitro* by inhibiting both the SL1 complex and UBF, which prevents PIC formation (Zhai and Comai, 2000). Alterations in the p53 status are found in > 50 % of all tumors (Sigal and Rotter, 2000; Toledo and Wahl, 2006). Rb binds UBF and thereby diminishes rRNA transcription (Cavanaugh et al., 1995; Voit et al., 1997). Consequently, loss of function mutations in Rb favour ribosome biogenesis. Taken together, RNAP I initiation is strongly influenced by major proto-oncogenes and tumor suppressor genes.

1.6.2 Deregulation of rRNA processing and protein synthesis in cancer

c-Myc also drives ribosome biogenesis by induction of various genes required for rRNA processing, ribosomal maturation, and translation (Boon et al., 2001; Schlosser et. al., 2003). The tumor suppressor PTEN negatively regulates ribosome biogenesis. PTEN phosphatase downregulates phosphatidylinositol 3-kinase (PI3K) signalling. PI3K activates the mammalian Target of Rapamycin (mTOR) kinase, which then activates S6-kinase (S6K) and drives translation initiation. Active S6K phosphorylates ribosomal protein S6, which enhances translation of 5'-terminal oligopyrimidine tract mRNAs (5'TOP mRNAs) coding for transcription factors, which, in turn, enhance expression of ribosomal processing factors in a self-stimularory manner (Thomas et al., 1979; Terada et al., 1994; Ruggero and Pandolfi, 2003). Mutations in PTEN and S6K can trigger constitutive PI3K signalling, which

stimulates ribosome biogenesis and tumorigenesis (Salmena et al., 2008; Yin and Shen, 2008). Overexpression of $p14^{ARF}$ protein was shown to inhibit rRNA processing (Sugimoto, et al., 2003). In line with that, rRNA processing is enhanced in $p19^{ARF}$-deficient mice (Apicelli et al., 2008). Depletion of NPM1 also inhibits rRNA processing (Itahana et al., 2003). Not surprisingly, PTEN, $p14^{ARF}$, and NPM1 are frequently mutated in tumors (Ruggero and Pandolfi, 2003). Taken together, numerous examples demonstrate various options of ribosome biogenesis regulation to be altered in cancer. With no doubt, deregulation of ribosome biogenesis occurs in tumor cells and is crucial to maintain increased proliferation rates (White, 2005). From a therapeutic point of view, it would be desirable to develop strategies to downregulate ribosome biogenesis and restrict tumor growth. How could ribosome biogenesis be linked with cancer therapy?

1.7 The nucleolus as stress sensor

There is increasing evidence that the nucleolus is a sensitive stress sensor. The nucleolar stress response is directly connected to p53 levels (Fig. 7). In unstressed conditions, the nucleolar structure remains intact, ribosome biogenesis is functional and p53 levels are maintained low by Hdm2, an E3-ubiquitin ligase that continuously binds p53, ubiquitinates it, and targets it for proteasomal degradation (Stommel et al., 1999; Ljungman, 2000; Xirodimas et al., 2001). In fact, the nucleolus directly regulates p53 levels, since it is the site of p53 ubiquitination. p53 requires a transit through a structural and functional intact nucleolus to be degraded (Boyd et al., 2011). In case of nucleolar stress, however, the nucleolus disintegrates, which is accompanied by a translocation of various nucleolar proteins to the nucleoplasm (Rubbi and Milner, 2003; Hernandez-Verdun et al., 2010). Subsequently, Hdm2 is bound and inactivated by translocated ribosomal proteins, most prominently by L5, L11, and L23 (Lohrum et al., 2003; Dai et al., 2004a; Bhat et al., 2004; Dai et al., 2004b). The ribosomal protein L11 was found to be crucial for p53 induction. Similarly, other nucleolar factors such as $p14^{ARF}$, NPM1, or NST are capable of inactivating HDM2 and thereby stabilizing p53 (Tao and Levine, 1999; Kurki et al., 2004; Tsai and McKay, 2005). Unincorporated L26 protein can promote p53 translation (Takagi et al., 2005; Chen and Kastan, 2010). Various forms of nucleolar stress, including heat, hypoxia, or UBF depletion cause nucleolar disruption and thereby stabilize p53 (Rubbi and Milner, 2003). Consequently, functional ribosome biogenesis is a prerequisite for low levels of p53. The interplay between p53 and the nucleolus also

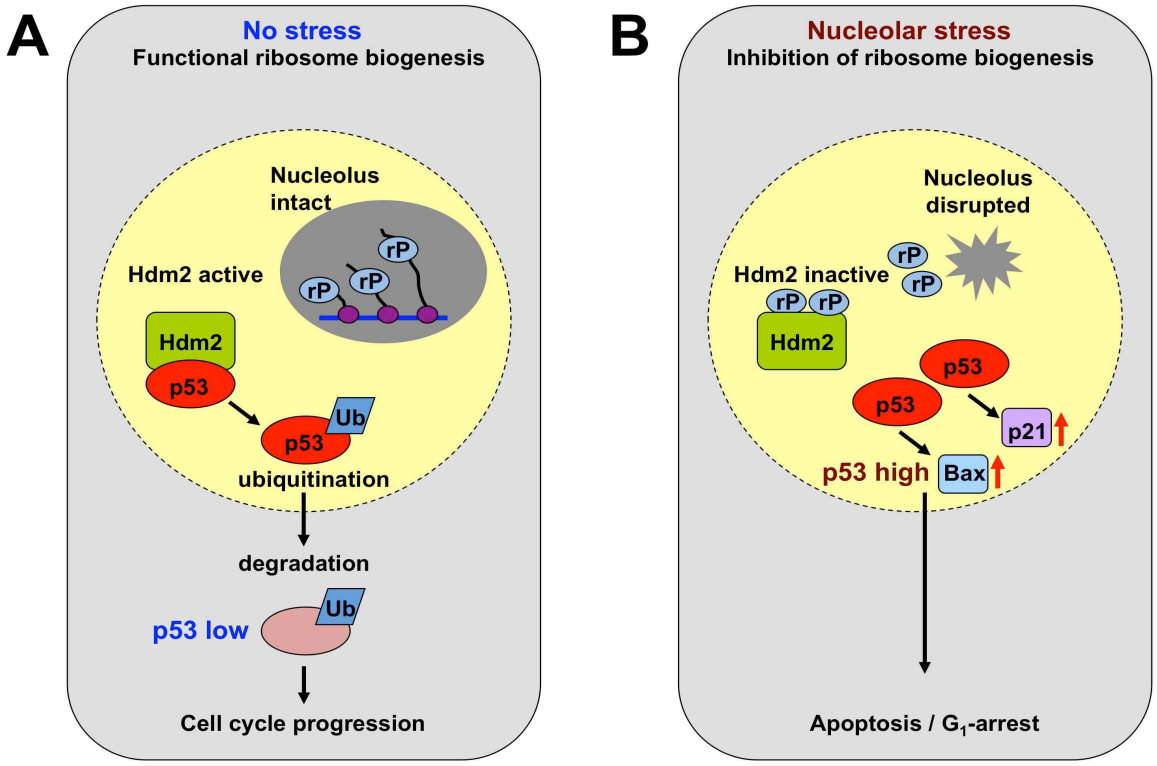

Figure 7. **The nucleolus as stress sensor. (A)** Nucleolar integrity is maintained under normal conditions. Ribosome biogenesis is functional and active Hdm2, which ubiquitinates p53 for proteasomal degradation, keeps p53 levels low. **(B)** The nucleolus disrupts under stress. Nucleolar disintegration induces nucleoplasmatic translocation of nucleolar ribosomal proteins (rP), which bind Hdm2 and inhibit its activity. p53 no longer can be actively degraded, accumulates in the nucleus, and triggers cell cycle arrest and apoptosis by inducing critical downstream targets (e.g. p21 or Bax) (adapted from Drygin et al., 2010).

explains cell cycle-dependent variations in p53 levels, which are relatively low throughout the cell cycle, but increased in early G_1-phase, when a functional nucleolar structure has not been reassembled, yet (Klein and Grummt, 1999; David-Pfeuty, 1999).

1.8 The Cdk-inhibitor Flavopiridol impairs ribosome biogenesis

In 2005, the nucleolar proteome became available. 692 proteins were found to reproducibly localize in the nucleolus, confirming earlier studies in yeast (Andersen et al., 2005; Huh et al., 2003). Interestingly, 7 % of the nucleolar proteome are associated with cell cycle regulating proteins, including 15 kinases and phosphatases with potential regulatory function in ribosome biogenesis. Inhibition of kinase activity is a promising and successfully proven approach for the development of novel small molecule inhibitors. Non-genotoxic small molecule inhibitors like Flavopiridol (FL) are superior to a wide range of commonly used drugs, which often intercalate or alkylate DNA and cause frameshift mutations, lesions, or cross-links.

FL is a semi-synthetic flavonoid, derived from *Amoora rohituka* and *Dysoxylum binectariferum*. In 1992, Kaur and colleagues found that FL inhibits cell cycle progression. Cell cycle inhibition was initially explained by inactivation of Cdk1 and Cdk2 (Carlson et al., 1996). *In vitro* studies with recombinant Cdks revealed that FL is a pan-specific Cdk-inhibitor, which inhibits different Cdks at different concentrations (Newcomb, 2004). FL inactivates Cdk1, Cdk2, and Cdk4 with a dissociation constant (K_i) of around 50 nM (Carlson et al., 1996). Cdk7 and Cdk8 are less potently inhibited (Losiewicz et al., 1994). Later on, it became evident that Cdk9 is the primary target of FL. Cdk9 is inactivated by FL *in vitro* with an K_i of 3 nM, a five to ten fold lower concentration than required for an effect on any other Cdk (Chao et al., 2000; Chao and Price, 2001; Schmerwitz et al., 2011).

Many small molecules induce cell cycle arrest or apoptosis by p53 induction. Do they inhibit ribosome biogenesis as well? Indeed, 47S rRNA processing is highly sensitive to the Cdk-inhibitors. It has been shown that Cdk-inhibitors like 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) or Roscovitine strongly inhibit processing of the 47S rRNA primary transcript, whereas transcription rates apparently remain unaffected (David-Pfeuty et al., 2001; Sirri et al., 2002; Schlosser et al., 2003; Louvet et al., 2005). In my previous work, I have extended these findings by testing a comprehensive panel of 36 drugs on their inhibitory potential on ribosome biogenesis. I found that > 50 % of drugs interefere with rRNA synthesis. Interestingly, low nanomolar concentrations of FL severely block 47S rRNA processing, cause nucleolar disruption, and induce p53 (Burger et al., 2010). FL is currently tested in clinical trials for therapy of relapsed chronic lymphocytic leukaemia (CLL). Patients with a genetically high-risk CLL are highly responsive to FL (Byrd et al., 2007; Phelps et al., 2009; Lin et al., 2009). In addition, FL causes haematological improvement in Pentostatin and Rituximab refractory hairy leukaemia (Jones et al., 2011).

1.9 Scope of this thesis (Aufgabenstellung)

Ribosome biogenesis is essential for growth and proliferation. It consumes an enormous amount of cellular energy and requires hundreds of different RNAs and proteins transcribed by all three RNA polymerases. Its functionality is a prerequisite for low levels of p53. Although the intimate connection between a functional nucleolus and its requirement to actively destabilize p53 is widely accepted, little effort has been carried out so far to investigate the molecular details of mammalian

ribosome biogenesis regulation and its therapeutic relevance. Ribosome biogenesis is often deregulated in cancer and its inhibition triggers a p53 stress response, cell cycle arrest in G_1-phase, and apoptosis. Novel drugs that target ribosome biogenesis may be a promising approach for selective inhibition of growth and proliferation in cancer cells. In this context, non-genotoxic inhibition of 47S rRNA processing by FL treatment is of particular interest. How is 47S rRNA processing regulated? Which kinase(s) regulate(s) 47S rRNA processing? Which factors are rate limiting for 47S rRNA processing? This thesis aims to (i) shed light on mechanistic details of 47S rRNA processing upon FL treatment, (ii) identify the kinase(s) involved in the regulation of 47S rRNA processing, (iii) investigate the nucleolus as a sensor of stress, and (iv) present an approach for non-genotoxic inhibition of ribosome biogenesis.

2. RESULTS

2.1 47S rRNA processing requires Cdk9

In previous work, I tested a comprehensive panel of classical chemotherapeutic drugs and small molecule inhibitors for their impact on rRNA synthesis. Building on that, FL-mediated inhibition of 47S rRNA processing was unravelled in human U2OS cells and mouse embryonic fibroblast (MEF) cells. A panel of additional Cdk-inhibitors was tested on their ability to interfere with 47S rRNA processing (Fig. 8). rRNA synthesis was studied by metabolic labelling of rRNA with [^{32}P]-ortho-phosphate. While 47S rRNA processing was functional after treatment with Compound 3 (Cdk2-inhibitor), or TG003 (Cdc2-like-kinase-inhibitor), treatment with Actinomycin D (ActD) or CGP74514A (Cdk1-inhibitor) strongly impaired the production of the 47S rRNA primary transcript (phenotype 1). Strikingly, five different Cdk9-inhibitors blocked 47S rRNA processing, but allowed its production (phenotype 2). Thus, chemical inhibition of Cdk9 causes a 47S rRNA processing defect.

Small molecule Cdk-inhibitors allow inhibitions of Cdks, but have drawbacks with specificity and toxicity. To investigate the requirement of individual kinases for 47S rRNA processing, I applied an RNAi approach for the selective knockdown of a panel of 30 candidate kinases (Table 2). A candidate list was assembled by three criteria: (i) it includes major Cdks; (ii) it includes all known kinases of the nucleolar proteome (Leung et al., 2006); and (iii) it includes kinases with a substrate phosphorylation motif identical to the Cdk9 substrate (SP-motif). U2OS cells were transiently transfected with short interfering (si)RNA targeting mRNAs of candidate kinases or with control siRNAs for mRNAs of luciferase, nucleolar CALM/AF10-interacting protein CATS (Archangelo et al., 2008) and the rRNA processing factor Pescadillo 1 (Pes1) (Hölzel et al., 2005). Knockdowns of the majority of kinases impaired neither rRNA transcription nor processing. In contrast, the knockdown of Cdk5, Cdk6, and Cdk9 displayed a strong rRNA synthesis phenotype

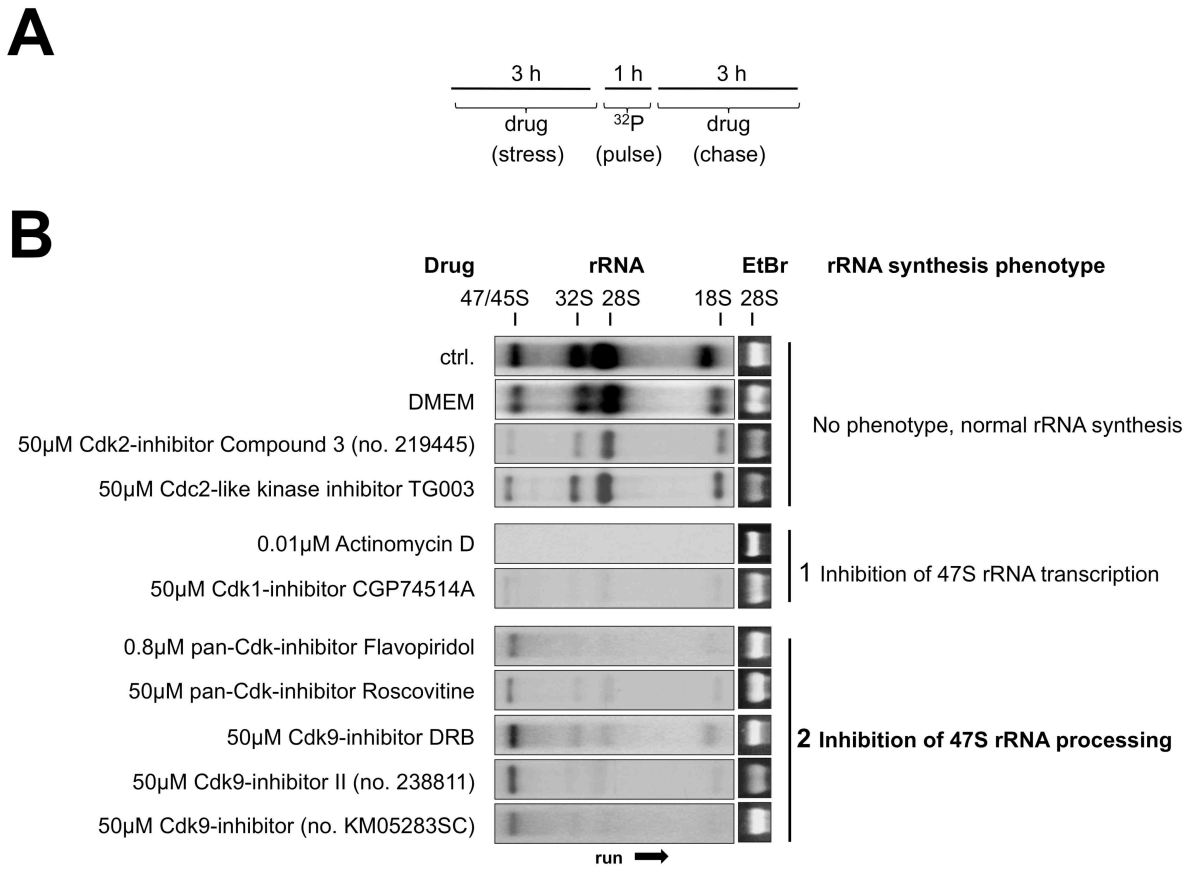

Figure 8. **Cdk9-inhibitors block 47S rRNA processing. (A)** Scheme of metabolic [^{32}P]-ortho-phosphate labelling. **(B)** Impact of Cdk-inhibitors on 47S rRNA processing. U2OS cells were treated with drugs for six hours and labelled. rRNA signals were analyzed by autoradiography. Compound 3 was used as Cdk2-inhibitor, TG003 as Cdk13-inhibitor, Actinomycin D as transcription inhibitor, CGP74514A as Cdk1-inhibitor, Flavopiridol, DRB, Roscovitine, Cdk9-inhibitor II, and KM05283SC as Cdk9-inhibitors. 28S EtBr: loading control in this and subsequent experiments. ctrl.: 0.1 % DMSO.

(Fig. 9A, only a selected number of knockdown experiments is displayed). Knockdown of Cdk5 diminished the levels of the 47S primary transcript, suggesting that Cdk5 is crucial for rRNA transcription. Consequently, the levels of 32S, 28S, and 18S rRNA were also reduced upon Cdk5 knockdown. In contrast, Cdk6 and Cdk9 knockdown specifically blocked rRNA processing. Cdk9 knockdown mimicked the impact of Cdk-inhibitors on rRNA synthesis best. While the production of the 47S

ATM (n)	*Ataxia Telangiectasia Rad3 Rel. Kinase*	**CDK9 (n)**	*Cyclin-Dependent Kinase 9*	**NEK6**	*(Never In Mitosis Gene A)-Rel. Kinase 6*
ATR (n)	*Ataxia Telangiectasia Mutated Kinase*	**CDK12**	*Cyclin-Dependent Kinase 12*	**PNK (n)**	*Polynucleotide Kinase 3'-Phosphatase*
AurB (n)	*Aurora Kinase B*	**CDK13 (n)**	*Cyclin-Dependent Kinase 13*	**PRK1 (n)**	*Protein Kinase, dsRNA-Dependent*
CDK1 (n)	*Cyclin-Dependent Kinase 1*	**CDK16**	*Cyclin-Dependent Kinase 16*	**hRio2**	*Human Rio Kinase 2*
CDK2 (n)	*Cyclin-Dependent Kinase 2*	**CHK1**	*Checkpoint Kinase 1*	**SNK1**	*Polo-Like Kinase Snk1*
CDK3	*Cyclin-Dependent Kinase 3*	**CK1 (n)**	*Casein Kinase 1*	**SRPK1 (n)**	*SFRS Protein Kinase 1*
CDK4	*Cyclin-Dependent Kinase 4*	**CK2 (n)**	*Casein Kinase 2*	**TTBK1 (n)**	*Tau-Tubulin Kinase 1*
CDK5	*Cyclin-Dependent Kinase 5*	**DNA-PK (n)**	*DNA-Activated Protein Pinase*	**VRK1 (n)**	*Vaccinia-Related Kinase 1*
CDK6	*Cyclin-Dependent Kinase 6*	**GSK3ß**	*Glycogen Synthase Kinase 3ß*		
CDK7 (n)	*Cyclin-Dependent Kinase 7*	**JNK3**	*C-Jun N-Terminal Kinase 3*	**Pes1 (n)**	*Pescadillo1*
CDK8	*Cyclin-Dependent Kinase 8*	**NLK**	*Nemo-Like Kinase*	**CATS (n)**	*CATS*

Table 2. **Candidate kinases for RNAi screen.** 30 kinases were chosen. (n): nucleolar kinase; Pescadillo1 (Pes1) is a ribosomal processing factor (positive control), CALM/AF-10 interactor CATS is a nucleolar proliferation marker (negative control).

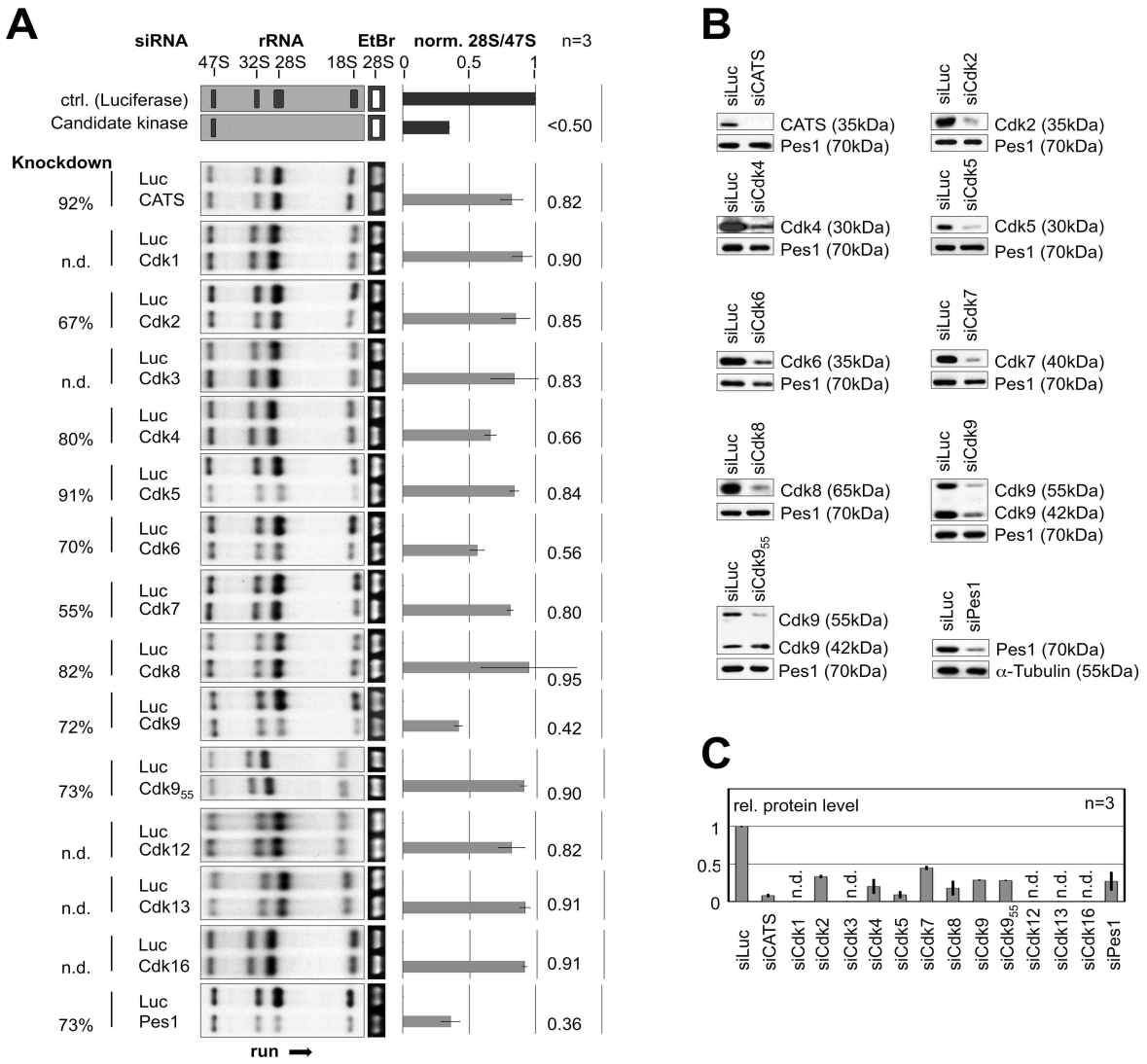

Figure 9. **Cdk9 knockdown inhibits rRNA processing. (A)** rRNA synthesis after Cdk9 knockdown. U2OS cells were seeded and cultured over night. Cells were transfected twice with siRNA (100 nM) for six hours at two consecutive days. Analysis of labelled rRNA was performed as described in *Materials and Methods*. Inhibition of rRNA processing was measured by autoradiography. Knockdown efficacy of Cdks in are displayed in %. Labelled rRNAs were quantified by a PhosphorImager and AIDA software. rRNA ratios were calculated and normalized to control cells (siLuc). Pes1 and CATS knockdown served as positive and negative controls, respectively. ctrl.: untreated cells. **(B)** Cdk knockdown controls. Knockdown efficacy was monitored by Western blot analysis using specific antibodies. Pes1 and α-Tubulin served as loading controls. **(C)** Knockdown efficacy was quantified by AIDA software. Cdk levels were calculated relative to control cells (siLuc) set as 1. n.d.; not determined.

primary transcript appeared to be unchanged, a strong reduction was observed for intermediate 32S rRNA and mature 28S and 18S rRNAs. In fact, 28S rRNA maturation was reduced by > 50 % as quantified by PhosphorImager analysis. Cdk6 knockdown also blocked 47S rRNA processing. However, inhibition of 47S rRNA processing was not as pronounced as after Cdk9 knockdown. Strong defects in 47S rRNA processing could not be observed after knockdown of other Cdks. Knockdown efficacies were monitored by Western blot analysis (Fig. 9B). Most siRNAs were

highly efficient and depleted 75-95 % of the endogenous proteins (Fig. 9C). Thus, Cdk9 knockdown induces a 47S rRNA processing defect similar to the processing defect observed upon FL treatment, suggesting that Cdk9 is the prime candidate to regulate mammalian 47S rRNA processing.

2.1.1 The nucleolar isoform of Cdk9 has no impact on rRNA processing

Two forms of Cdk9 with a molecular weight of 42 kDa and 55 kDa have been described in mammalian cells. The $Cdk9_{55}$ isoform carries an N-terminal extension of 117 amino acids and is generated from an mRNA that originates from a second promoter located upstream of the start point of transcription used to generate mRNA encoding $Cdk9_{42}$ (Shore et al., 2003; Shore et al., 2005). The relative abundance of $Cdk9_{55}$ and $Cdk9_{42}$ changes in various cell types upon differentiation. Interestingly, epitope-tagged $Cdk9_{42}$ localizes diffusely in the nucleoplasm, while $Cdk9_{55}$ accumulates in the nucleolus (Liu and Herrmann, 2005). I asked therefore, if knockdown of the nucleolus specific $Cdk9_{55}$ alters processing of rRNA. Transfection of U2OS cells with a siRNA for the first exon of $Cdk9_{55}$ reduced steady state levels of nucleolar form of Cdk9 by > 70 %, while expression of $Cdk9_{42}$ remained unaffected (Fig. 9B). Surprisingly, an rRNA processing defect could not be detected after $Cdk9_{55}$ knockdown (Fig. 9A), indicating that the large form of Cdk9 has apparently no specific function in rRNA processing and that nucleoplasmic $Cdk9_{42}$ is critical for processing of rRNA.

2.1.2 Cdk9-mediated rRNA processing is conserved in yeast

Having established that Cdk9 regulates 47S rRNA processing in human cells, I next asked, whether Cdk9 is also required for rRNA processing in the baker's yeast *S. cerevisiae* (Fig. 10). Two complexes functionally reconstitute the mammalian Cdk9 activity in yeast. On the one hand, the CTK kinase complex, which consists of Ctk1 kinase, Ctk2 cyclin, and a Ctk3 subunit, on the other hand the Cdk/cyclin complex Bur1/2 (Wood and Shilatifard, 2006). Conditional knockout strains were created to measure rRNA synthesis after depletion of Ctk1 or Bur1/2. In brief, the endogenous *CTK1* and *BUR2* genes were deleted and replaced by stably transformed, recombinant, TAP-tagged versions under control of a Galactose-inducible promoter (GAL::CTK1-TAP; GAL::BUR2-TAP). Note that Bur2 is the stabilizing cyclin of the Bur1 kinase. Bur2 cyclin depletion destabilizes the Bur1 kinase quantitatively (Wood et al., 2005). Ctk1-TAP and Bur2-TAP were expressed at comparable amounts in presence of Galactose-containing YPG medium. Medium switch to Glucose-

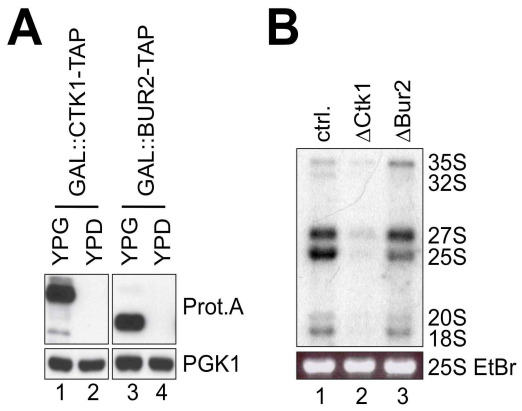

Figure 10. **Impact of yeast Ctk1 or Bur1/2 depletions on rRNA synthesis. (A)** Depletion of Ctk1 and Bur1/2. Conditional knockout yeast strains were created as described in *Materials and Methods*. Knockout was monitored by Western blot analysis with a protein-A antibody, specifically recognizing TAP-tagged proteins. Phosphoglycerate kinase 1 (PGK1): loading control. Dr. Britta Coordes performed Western blot analysis. **(B)** Ctk1 is required for rRNA transcription and processing, Bur1/2 for rRNA processing. Yeast ^{32}P metabolic labelling was performed as described in *Material and Methods.* ctrl.: wild type yeast W303.

containing YPD medium represses the expression of Ctk1-TAP and Bur2-TAP. Note that yeast cell growth is slightly reduced after Ctk1 depletion, but remains unchanged after Bur1/2 depletion (Röther and Strässer, 2007). Depletion of Ctk1-TAP and Bur2-TAP was monitored by Western blot analysis (Fig. 10A) and rRNA synthesis was measured by ^{32}P metabolic labelling (Fig. 10B). Ctk1 depletion reduced rRNA synthesis globally. Production of 35S rRNA, as well as maturation of 25S and 18S rRNA was reduced. In contrast, Bur1/2 depletion selectively blocked 27S rRNA processing compared to wild type cells, but had no inhibitory effect on production and processing of the 35S rRNA primary transcript. Instead, a slight accumulation of the 35S primary transcript could be detected upon Bur1/2 depletion. Thus, Bur1/2 depletion causes an rRNA processing phenotype, which, although not identical, is similar to Cdk9 knockdown. I conclude that Ctk1 and Bur1 have crucial functions in rRNA synthesis, although both knockout phenotypes differ from Cdk9 knockdown in mammalian cells.

2.2 Defective 47S rRNA processing feeds back on RNAP I transcription

To understand the dynamics of rRNA synthesis upon Cdk9 inactivation, I measured mammalian 47S rRNA processing in time kinetic experiments (Fig. 11). Confirming previous results, treatment of cells with FL inhibited 47S rRNA processing. However, when applying ^{32}P labelling experiments with various chase times, I noticed that FL not only blocked 47S rRNA processing, but also altered the kinetics of 47S rRNA accumulation (Fig. 11A). Control cells showed a rapid

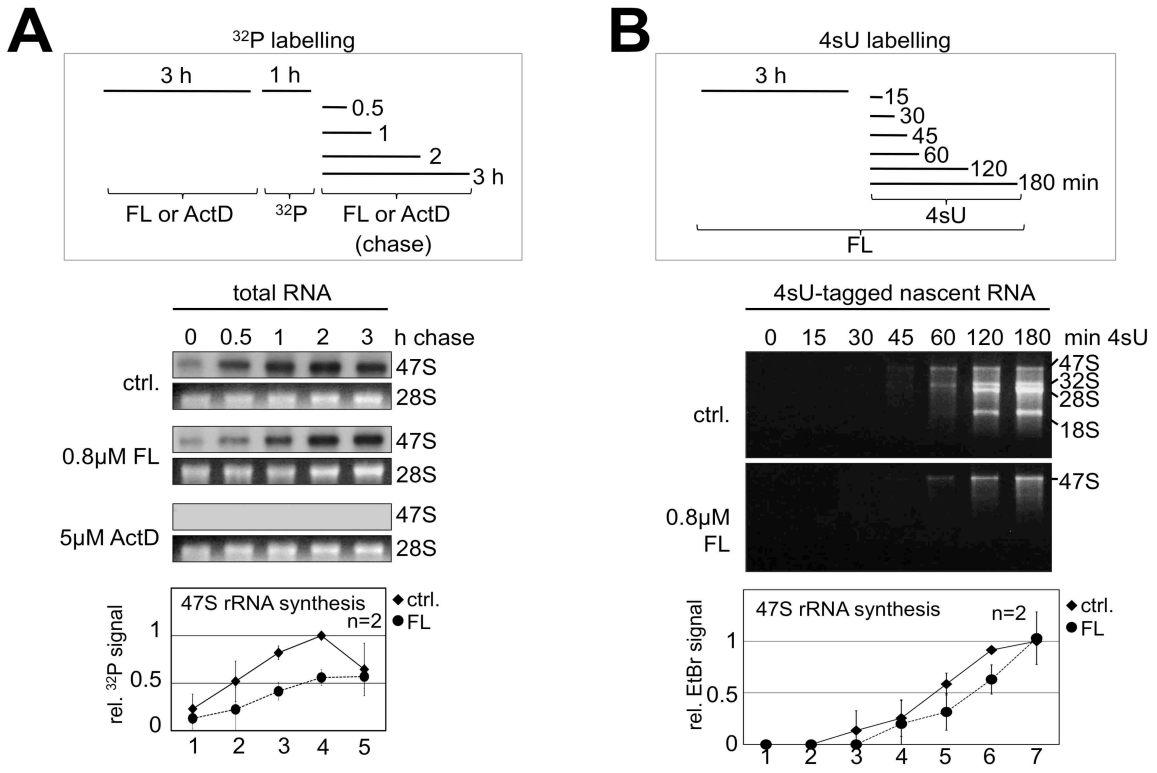

Figure 11. **Inhibition of 47S rRNA processing feeds back on RNAP I transcription. (A)** Flavopiridol diminishes 47S rRNA transcription. U2OS cells were treated with FL or ActD for six hours and labelled with [^{32}P]-ortho-phosphate as indicated. Relative ^{32}P signals for 47S rRNA were plotted. The 47S rRNA signal intensity for control cells (0.1 % DMSO) after two hours (lane 4) was set as 1. **(B)** Analysis of 47S rRNA transcription after Flavopiridol measured by 4sU-tagging. Cells were treated with FL and 4sU as indicated. 4sU-tagged RNA was purified from total RNA by biotin-streptavidin, separated by gel-electrophoresis, and stained with EtBr (see *Materials and Methods* for details). Relative EtBr signals for 47S rRNA were plotted and the 47S signal in control cells (0.1 % DMSO) after 180 minutes (lane 7) was set as 1.

incorporation of ^{32}P label in 47S rRNA already after 30 minutes of chase, with a maximal incorporation of label after two hours, and a decline after three hours. In contrast, incorporation of label in FL-treated cells was low after 30 minutes, but steadily increased, and reached a value similar to control cells after three hours. A diminished incorporation of ^{32}P label in 47S rRNA would be consistent with a reduced transcription rate of RNAP I in FL-treated cells.

To confirm this assumption, a second rRNA labelling experiment was conducted, using 4-thiouridine (4sU), a thiol-group containing, photo-reactive nucleoside with high cell permeability that can be introduced into eukaryotic cells and allow non-disruptive metabolic labelling of nascent RNA within minutes (Melvin et al., 1978). A 4sU-tagged RNA fraction can be analyzed by thiol-specific, covalent biotinylation, and subsequent purification on streptavidin-coated beads (Cleary et al., 2005). The 4sU-tagged, nascent RNA fraction was separated in a

denaturing agarose gel and stained with ethidium bromide (Fig. 11B). A faint band corresponding to the 47S rRNA became visible in control and FL-treated cells already 45 minutes after labelling, increased in intensity after 60 minutes, and reached steady state levels after 120 to 180 minutes in control and FL-treated cells. Notably, FL causes a slight, but significant reduction in nascent 47S rRNA production. A 32S rRNA processing intermediate became visible in control cells after 60 minutes, followed by the appearance of mature 18S and 28S rRNAs after two and three hours. In control cells, > 90 % of 4sU-labelled rRNA was processed to 32S, 28S, and 18S rRNA after three hours. In contrast, processing of the 47S rRNA precursor into immature 32S rRNA, or mature 28S rRNA and 18S rRNA did not occur in presence of FL. I conclude that the synthesis and turnover rates of 47S rRNA in FL-treated cells could be significantly reduced compared to control cells.

2.3 Cdk9 is required for correct 3'ETS processing

While 47S rRNA is produced normally in FL-treated cells (albeit at reduced rates), its processing into intermediate and mature rRNA forms is blocked entirely. However, the basis for this blockage is unclear. Therefore, I analyzed whether 47S rRNA displays structural abnormalities in FL-treated cells, which might be causative for the processing defect. 3' end formation downstream of the 3' ETS is the first processing step for the primary ribosomal transcript (Richard and Manley, 2009). Removal of 3'ETS as well as the 5' leader sequence of the 5'ETS have been described as subsequent processing steps, which lead to the formation of the 45S rRNA processing intermediate (Mullineux and Lafontaine, 2012). Unfortunately, 47S and 45S are large molecules with similar sizes and cannot be separated in agarose gel electrophoresis. Therefore, the signal marked as 47S rRNA in Northern blot hybridizations represents always a mixture of both 45S and 47S rRNAs, if hybridizations are performed with 45S-specific probes. The sequence composition of the rRNA primary transcript was analyzed with the help of 14 hybridization probes, which are distributed along the 47S rRNA sequence (probes 1 to 9) and downstream thereof (probes 10 to 14) (Fig. 12, upper panel). U2OS cells were treated with FL for six hours, total RNA was isolated, and analyzed by Northern blot hybridization. I could detect rRNA signals of a size of 47S with hybridization probes 1 to 9 in the control situation. The signal normally is indicative for 47S rRNA properly processed/terminated at the termination site (T). As expected, signals for probes 10 to 14 downstream of the T-site were not detected in control cells. The signals

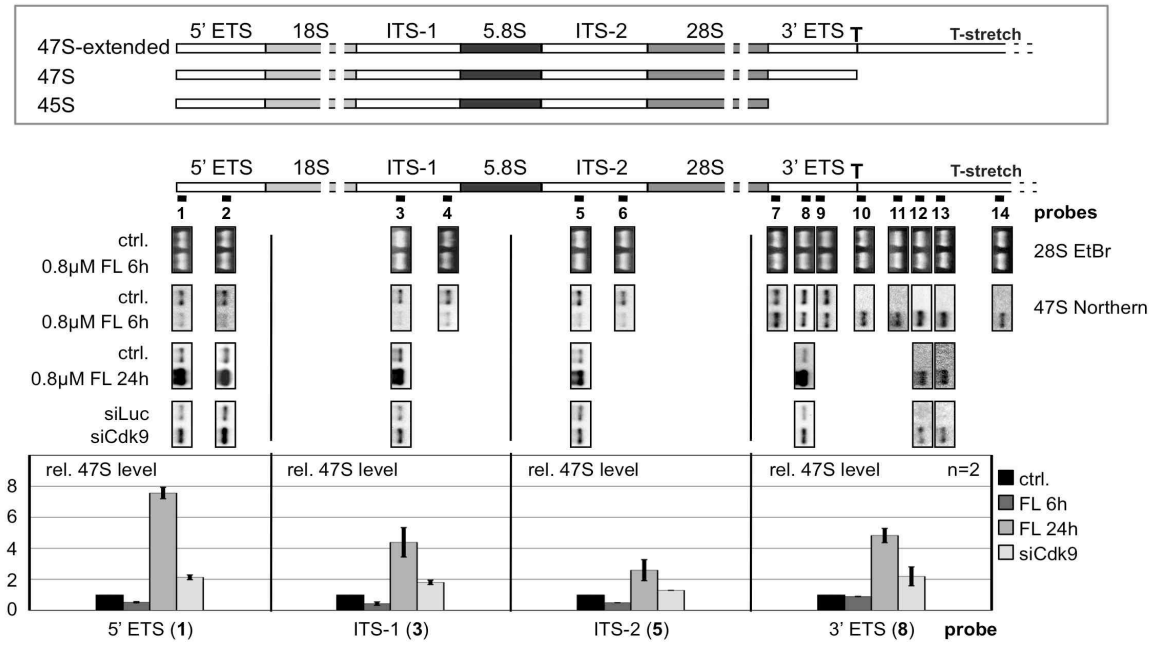

Figure 12. **Cdk9 inactivation causes defective 3'ETS processing**. U2OS cells were treated with FL or siRNA against Cdk9. Total RNA was isolated and analyzed by Northern blot hybridization with probes for 5'ETS, ITS-1, ITS-2, 3'ETS, or sequences downstream of 3'ETS (probes 1-14). Signals were analyzed by autoradiography and quantified by a PhosphorImager. 47S rRNA signals were plotted relative to signals from 0.1 % DMSO-treated or siLuc-transfected cells (ctrl.) set as 1. T: termination site, T-stretch: tymidine stretch. Grey box: rRNA forms with a size of 47S (47S-extended, 47S, and 45S rRNA).

obtained for 47S rRNA from FL-treated cells differed from control cells in several aspects. Each of the 47S signals for probes 1 to 6 was reduced by a factor of two in rRNA derived from FL-treated cells. Since FL-treated cell have a 47S rRNA processing defect, the observed reduction may be explained by a lack of properly produced 45S rRNA in the fraction of rRNA running at the size of 47S. In line with that, FL stabilized 47S rRNA (probes 7 to 9). I conclude that FL significantly changed the ratio of 45S/47S rRNA. The second remarkable difference for 47S rRNA in FL-treated cells is the appearance of hybridization signals for probes 10 to 14, indicating that FL causes a specific defect of 47S rRNA processing in the 3'ETS region, which leads to the stabilization of 3'-extended 47S rRNA. After 24 hours FL treatment, 47S-extended rRNA further accumulated by a factor of three to eight (probes 1 to 13). Elevated levels of 47S-extended rRNAs could also be detected after Cdk9 knockdown. Taken together, the data show that 47S rRNA can no longer be processed properly upon Cdk9 inactivation, due to a processing defect in the 3'ETS region at or downstream of the T-site. The processing defect stabilizes an extended 47S rRNA, which accumulates over time.

2.4 Processing of 47S rRNA is dependent on RNAP II transcription

Having established that Cdk9 is required for 47S rRNA processing, I next investigated the molecular details of this requirement. Cdk9 is the active component of pTEFb, which phosphorylates the RNAP II CTD to enable transcription elongation (Peterlin and Price, 2006). I assumed that Cdk9 inactivation might prevent RNAP II transcription and thereby interferes with 47S rRNA processing. To test this hypothesis, contribution of RNAP II transcription to 47S rRNA processing was analyzed directly by using α-amanitin (α-Am), a highly specific inhibitor of RNAP II transcription (Kedinger et al., 1971). α-Am binds the largest subunit of RNAP II (Rpb1) with high affinity and traps RNAP II in an inactive conformation that prevents nucleotide incorporation, translocation, and triggers RNAP II degradation *in vivo* (Bushnell et al., 2002; Kaplan et al., 2008; Brueckner and Cramer, 2008; Nguyen et al., 1996). I used ActD, FL, and α-Am to inhibit RNAP II transcription in U2OS cells and measured 47S rRNA processing in ^{32}P labelling experiments (Fig. 13A). ActD blocked RNAP I activity entirely and no signals for 47S rRNA or downstream forms could be detected. Remarkably, both FL and α-Am did not prevent production of the 47S rRNA precursor, but impaired its processing. 32S, 28S, and 18S rRNA levels were strongly reduced. 47S rRNA processing was also measured in HeLa cells, which express an α-Am-resistant version of RNAP II. Strikingly, 47S rRNA processing is completely functional in these cells in presence of α-Am (Fig. 13B). Importantly, α-Am induced the same 3'ETS processing defect in U2OS cells, as seen after Cdk9 inactivation (Fig. 13C), and stabilized a 3'-extended 47S rRNA. This indicates that RNAP II transcription is required for correct processing of the 47S rRNA primary transcript at the 3' end. The data strongly suggest that the Cdk9-dependent rRNA processing phenotype is caused by abrogation of RNAP II transcription.

RNAP II synthesizes different RNA species, which can be divided in two categories: protein-coding messenger (m)RNA and non-coding RNAs such as micro (mi)RNA, short-interfering (si)RNA, small nuclear (sn)RNA, or small nucleolar (sno)RNA involved in posttranscriptional gene regulation and RNA metabolism (Fig. 14A). Although RNAP II regulates expression of many genes encoding ribosomal proteins and processing factors, it is unclear to what extend the different RNAP II-dependent RNAs contribute to 47S rRNA processing. A number of experiments were designed to dissect the specific requirement of mRNA synthesis, small RNA biogenesis, and snoRNA synthesis for 47S rRNA processing.

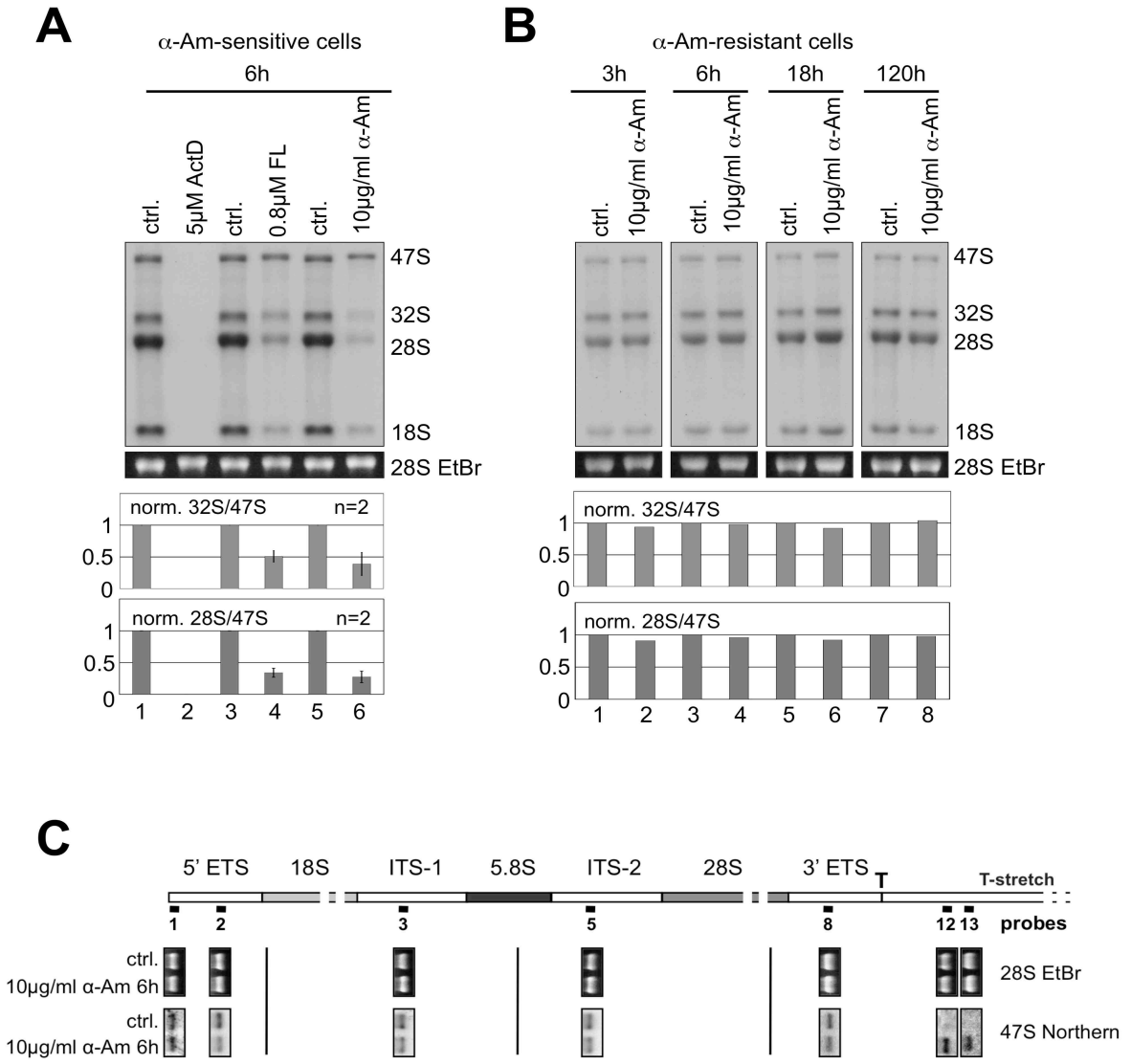

Figure 13. **Processing of 47S rRNA is dependent on RNAP II transcription. (A)** Inhibition of 47S rRNA processing in U2OS cells after treatment with α-amanitin (α-Am). Cells were treated with ActD, FL, or α-Am for six hours, followed by labelling with ^{32}P. rRNA ratios were calculated and normalized to rRNA ratios derived from control cells (0.1 % DMSO). **(B)** Normal processing of 47S rRNA in α-amanitin-resistant cells. HeLa cells were treated with α-Am for various time points. 47S rRNA processing was measured as described and normalized to rRNA ratios derived from control cells (0.1 % DMSO). **(C)** Inhibition of RNAP II transcription stabilizes a 3'-extended form of 47S rRNA. Cells were treated with α-Am (10 μg/ml). Total RNA was isolated an analyzed by Northern blot hybridization with indicated probes. T: termination site, T-stretch: tymidine stretch.

2.4.1 Inhibition of translation does not induce defective 3'ETS processing

mRNAs are the most prominent RNAP II-dependent transcripts. Cdk9 is required for coordinated transcription, processing, and surveillance of mRNAs (Chao and Price, 2001). mRNAs by themselves are not functional. They need to be translated. If an affected mRNA for a highly labile protein was required for 3'ETS processing of 47S rRNA, inhibition of translation should deplete such a factor rapidly

and trigger an rRNA processing defect. To test this hypothesis, U2OS cells were treated with two translational inhibitors, Cycloheximide (CHX) and Homoharringtonine (HHT), and rRNA processing was analyzed in ^{32}P labelling experiments (Fig. 14B). Effective inhibition of translation was monitored for the short-lived c-Myc protein (Fig. 14C). Both CHX and HHT displayed a strong rRNA processing defect, yet the phenotype of this defect differs markedly from the phenotype observed in cells treated with FL. While both drugs reduced 28S rRNA maturation (28S/47S ratio), the 32S/47S ratio was reduced solely in FL-treated cells,

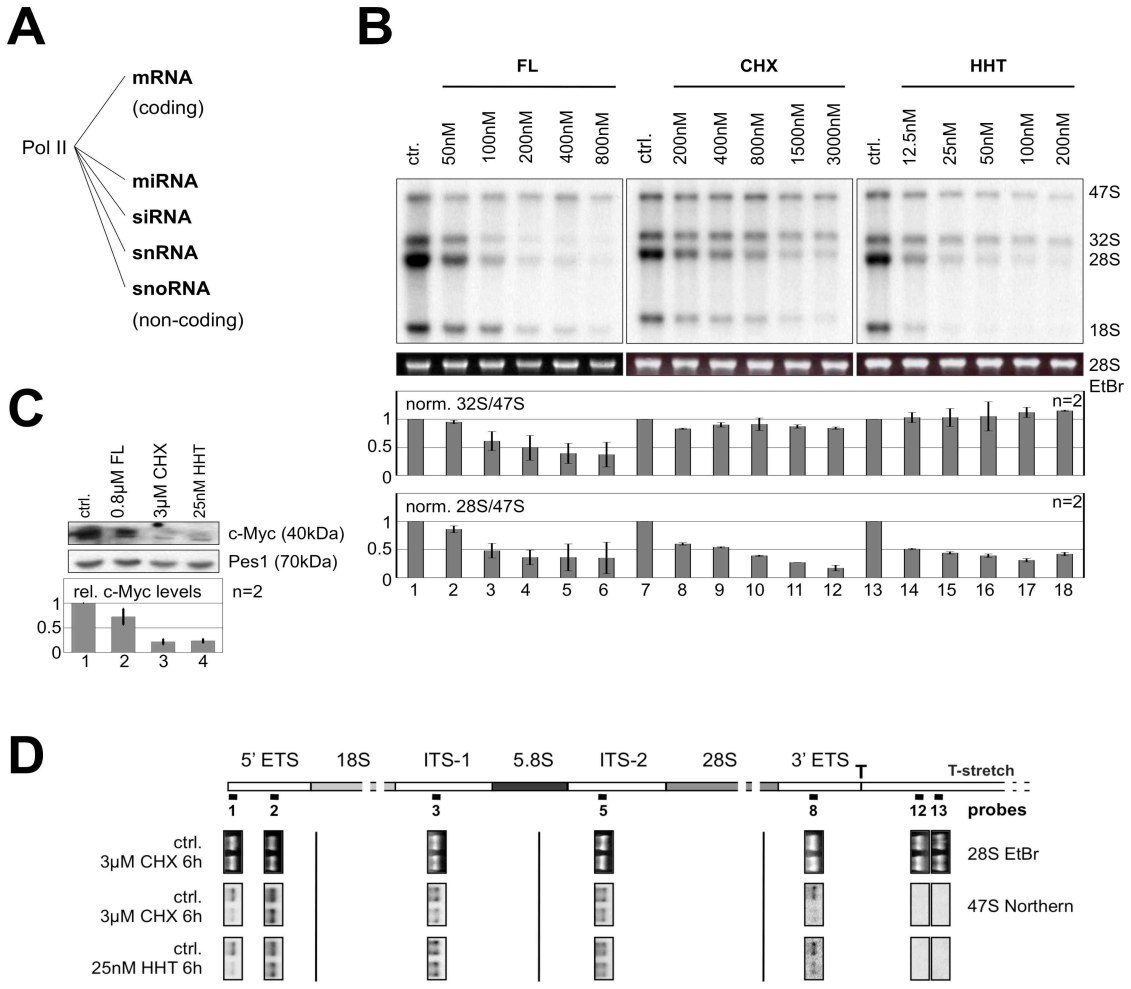

Figure 14. **Inhibition of translation does not induce a 3'ETS 47S rRNA processing defect. (A)** Scheme of major RNAP II-dependent transcripts: messenger RNAs (mRNAs), micro RNAs (miRNAs), short interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs). **(B)** Impact of translation inhibitors on rRNA processing. U2OS cells were treated with FL or translation inhibitors Cycloheximide (CHX), or Homoharringtonine (HHT) for six hours. Metabolic labelling was performed and rRNA signals were measured by autoradiography. Normalized ratios were calculated relative to control cells (0.1 % DMSO). **(C)** Efficient depletion of short-lived c-Myc protein after treatment with translation inhibitors. U2OS cells were treated with FL, CHX, or HHT for six hours. Whole cell lysates were prepared, separated by SDS-PAGE and analyzed by Western blot with specific antibodies. Protein levels were quantified by AIDA software and compared to control levels (0.1 % DMSO) set as 1. **(D)** rRNA 3' processing occurs in the presence of translational inhibitors. Cells were treated with CHX or HHT for six hours. RNA was isolated and analyzed by Northern blot hybridization with probes for 5'ETS, ITS-1, ITS-2, or 3'ETS sequences. T: termination site, T-stretch: tymidine stretch. ctrl.: 0.1 % DMSO.

but remained unchanged in cells treated with CHX or HHT. Importantly, translational inhibitors could also not induce a 3'-extended form of 47S rRNA, as observed in FL-treated cells (Fig. 14D). I conclude that translational inhibitors display an inhibitory effect on rRNA processing, but the phenotype is different from that observed in cells after Cdk9 knockdown or treatment with FL or α-Am. 47S rRNA processing is highly robust for inhibition of translation, indicating that defective mRNA synthesis is not the primary cause of defective 47S rRNA processing.

2.4.2 Dicer1 knockout does not inhibit 47S rRNA processing

Small non-coding RNAs such as miRNAs, siRNAs, or snRNAs are transcribed by RNAP II and processed by key processing enzymes Drosha, Dicer, and Argonaute in the RISC pathway. Dicer is a crucial exonuclease that processes non-coding RNA precursors into small double stranded 21-25 nucleotide long RNA fragments in the cytoplasm. I next asked whether 47S rRNA processing requires the function of small RNAs and is impaired in Dicer knockout cells? Conditional mouse embryonic fibroblast (MEF) Dicer1 knockout cells were used to answer this question. Cre-dependent depletion of Dicer1 was monitored by Western blot analysis and 47S rRNA processing was measured by ^{32}P metabolic labelling experiments after depletion (Fig. 15A). Interestingly, Dicer1 knockout strongly impaired maturation of 28S and 18S rRNA forms by a factor of two to three relative to Dicer1$^{+/+}$ control cells (Fig. 15B). Impaired 28S and 18S rRNA maturation is in line with the observed proliferation defect in Dicer1$^{-/-}$ cells (data not shown). Importantly, however, transcription of the 47S rRNA precursor and its processing efficacy into the 32S rRNA intermediate was fully functional in Dicer1$^{-/-}$ cells. The 32S/47S ratio, which is strongly reduced in FL-treated cells, remained unchanged in knockout cells. Consequently, no accumulation of the 47S rRNA could be observed with 3'ETS probes (Fig. 15C), as seen upon Cdk9 knockdown or FL treatment (Fig. 12). In summary, Dicer1 knockout strongly inhibits later rRNA processing steps and the formation of 18S and 28S rRNA, but does not affect the generation of 47S rRNA, or its proper 3' processing, and processing to 32S rRNA. Thus, small RNAs produced in a Dicer1-dependent manner are probably not crucial for 3' processing of 47S rRNA.

2.4.3 Downregulation of snoRNAs after Cdk9 inactivation

The synthesis of almost all small nucleolar RNAs (snoRNAs) is dependent on RNAP II transcription. The human genome encodes approximately 400 annotated snoRNAs (*www-snorna.biotoul.fr/*). Most snoRNAs are transcribed as intronic

sequences within pre-mRNAs, while some are transcribed form their own promoters. SnoRNAs regulate various modification and processing steps of rRNA and a subset of them are essential in yeast and mammals. Processing of snoRNAs occurs in the nucleus and therefore happens largely independently of Dicer activity. I asked whether Cdk9 inactivation could alter the steady state levels of snoRNAs and thus has an impact on rRNA processing. A subset of 12 representative snoRNAs was selected, including those, which are essential for growth in yeast or have been described as essential for processing of rRNA before (Table 3).

I first tested how steady state levels of these snoRNAs are affected, when cells were treated with ActD or FL (Fig. 16A). Strong differences were seen for the stability of snoRNAs. While some snoRNAs (SNORD24, SNORD14B, SNORA8, and SNORD76) showed high stability in the presence of ActD and FL, levels of other

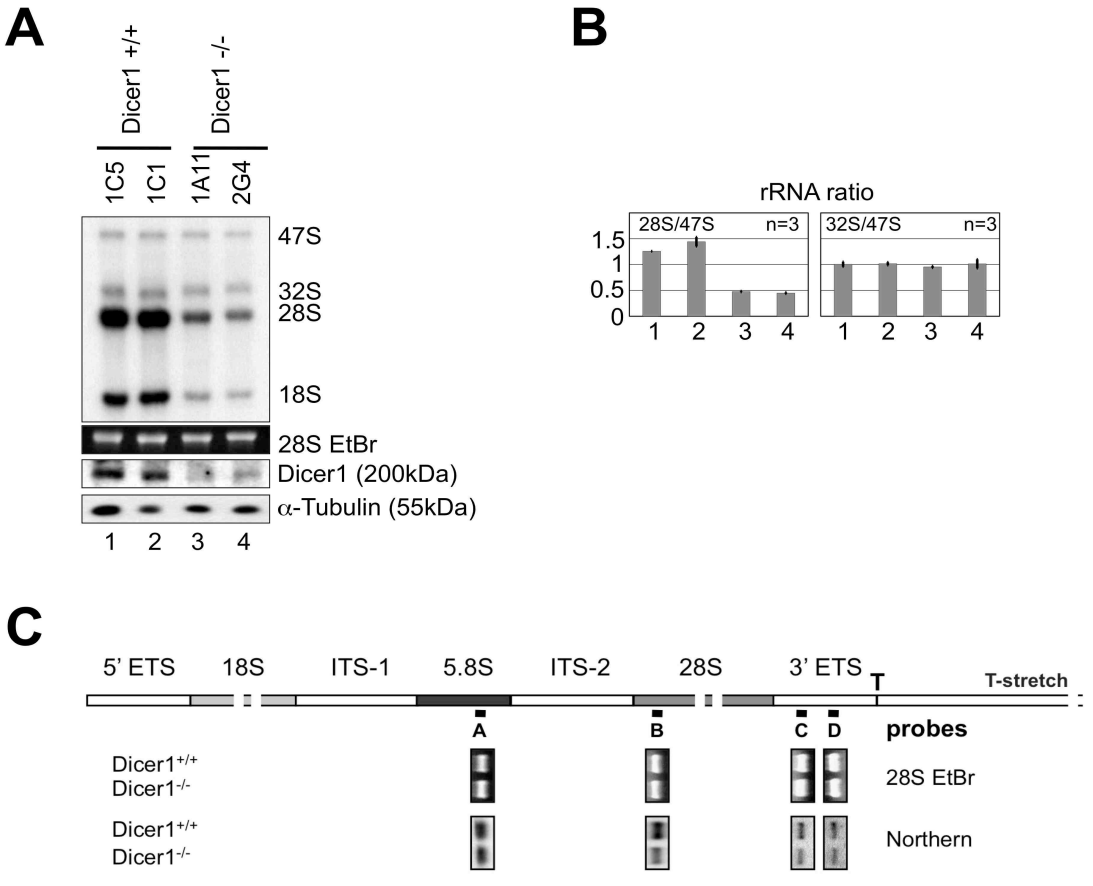

Figure 15. **Dicer1 knockout does not inhibit 47S rRNA processing.** Mouse embryonic fibroblasts (MEFs) with floxed Dicer1 alleles were incubated with Cre-recombinase to create a Dicer1 knockout cell line. Two wild type (1C5, 1C1) and two knockout (1A11, 2G4) cell clones were single cell cloned and whole cell extracts were prepared. **(A)** Dicer1 knockout was monitored by Western blot analysis with Dicer1-specific antibodies. Dicer1 knockout blocks the maturation of 28S and 18S rRNA, but not the production of the 47S rRNA precursor or 32S rRNA intermediate. rRNA labelling was performed as described in *Materials and Methods*. **(B)** 32S rRNA levels are normal in Dicer1 knockout cells. **(C)** Dicer1 depletion does not affect correct 3'ETS processing. MEF cell lines 1C1 (Dicer1+/+) or 1A11 (Dicer1-/-) were used, RNA was isolated an analyzed by Northern blot hybridization with probes for 5.8S (A), 28S (B), or 3'ETS (C, D). T: termination site, T-stretch: tymidine stretch.

Candidate snoRNA	Organization	Human locus	Target	Modification	Function in rRNA cleavage	rRNA synthesis phenotype in yeast	Viability in yeast
SNORD24 (U24)	Intronic	*RPL7A*	28S	Me	-	60S subunit accumulation	+
SNORD14B (U14B)	Intronic	*RPS13*	18S	Me	+	defective 5'ETS cleavage	-
SNORA8 (ACA8)	Intronic	*JOSD3*	18S, 28S	Ψ	-	35S rRNA accumulation	+
SNORD76 (U76)	Intronic	*GAS5*	28S	Me	-	60S subunit accumulation	+
SNORD117 (U83)	Intronic	*BAT1*	?	?	-	absent	absent
SNORA73A (U17a, E1)	Intronic	*RCC1*	?	?	+	defective 5'ETS cleavage	-
SNORA21 (ACA21)	Intronic	*RPL23*	28S	Ψ	-	rRNA intermediates accumulation	+
SNORA38B (ACA38B)	Intronic	*NOL11*	?	?	-	absent	absent
SNORD3 (U3)	Mono	*Indep. transcriptional unit*	5'ETS	-	+	defective 5'ETS cleavage	-
SNORD22 (U22)	Intronic	*SNHG1*	?	?	+	absent	absent
SNORD14A (U14A)	Intronic	*RPS13*	18S	Me	+	defective 5'ETS cleavage	-
SNORD118 (U8)	Mono	*Indep. transcriptional unit*	5.8S, 28S	-	+	absent	absent

Table 3. **SnoRNA list.** SnoRNAs are listed together with their host genes, target RNAs, and RNA modifications they are guiding. Six snoRNAs are required for rRNA cleavage. Eight snoRNA homologs have an rRNA synthesis phenotype in yeast. Me: Methylation; Ψ: Pseudouridylation.

snoRNAs (SNOD117, SNOR73A, SNORA21, SNORA38A, SNORD3, SNORD22, SNORD14A, and SNORD118) diminished to a greater or lesser extend. ActD and FL treatment for six hours had no impact on steady state mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In contrast, levels of short-lived c-myc mRNA were strongly reduced. I conclude that steady state levels of specific groups of snoRNAs are differently sensitive to inhibition of RNAP II transcription. I next asked, whether a decrease in snoRNA levels is also seen after Cdk9 knockdown. U2OS cells were treated with Cdk9-specific siRNA on two consecutive days and snoRNA levels were analyzed after additional 24 hours. Cdk9

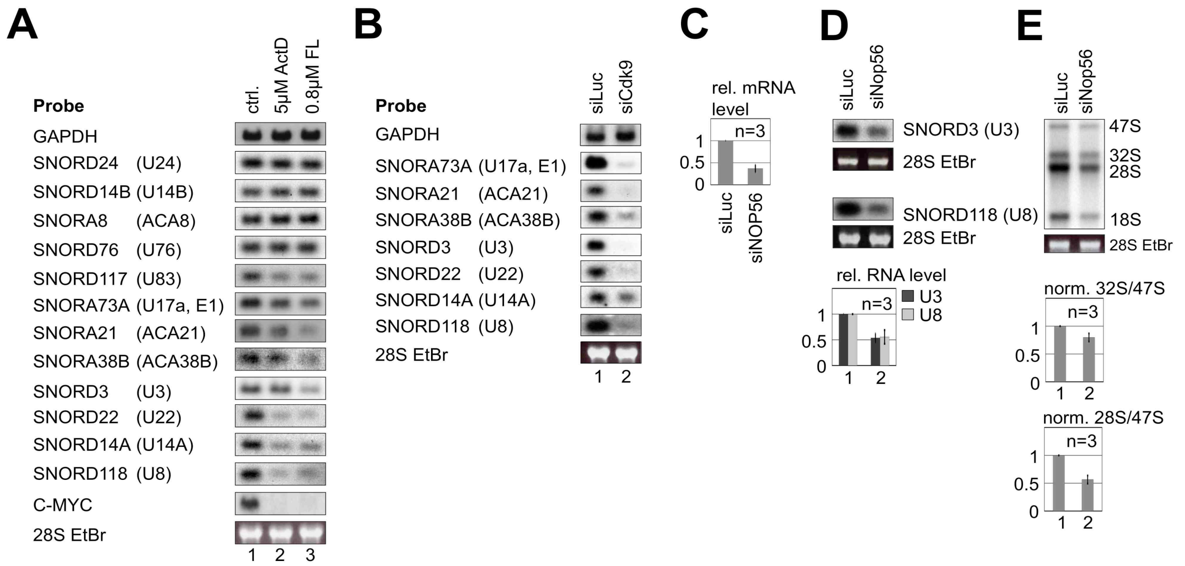

Figure 16. **Downregulation of snoRNAs after Cdk9 inactivation or Nop56 knockdown. (A)** SnoRNA levels in U2OS cells after treatment with ActD or FL for six hours. Northern blot analysis of RNA was performed with probes recognizing snoRNAs, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or c-myc RNA. ctrl.: 0.1 % DMSO. **(B)** SnoRNA levels after Cdk9 knockdown. Cells were transfected with Cdk9-specific siRNA, RNA was isolated 42 hours post transfection and analyzed as in (A). **(C)** Control of Nop56 mRNA depletion after knockdown. Quantitative real time PCR (qPCR) with specific primers was used to monitor knockdown efficacy (for details see *Material and Methods). **(D)*** Nop56 knockdown reduces SNORD3 (U3) and SNORD118 (U8) snoRNA levels. **(E)** Nop56 knockdown blocks 47S rRNA processing. Knockdown, Northern blot analysis, and quantitation were performed as described above. ctrl. knockdown: siLuc.

knockdown strongly reduced the same set of snoRNAs as treatment of cells with ActD or FL (Fig. 16B).

SnoRNAs are bound and stabilized by nucleolar proteins to form snoRNPs. In particular, box C/D snoRNAs like U3 and U8 require Nop56 for stabilization (Watkins and Bohnsack, 2011). Consequently, it was tested, whether Nop56 knockdown destabilizes U3 and U8 and inhibits 47S rRNA processing. Nop56 knockdown was monitored by qPCR analysis, since no antibody was available (Fig. 16C). Nop56 knockdown reduced U3 and U8 snoRNA levels (Fig. 16D) and blocked 28S rRNA maturation by about 50 % (Fig. 16E). In summary, the results suggest that snoRNAs are critical Cdk9-dependent transcripts and highly relevant for 47S rRNA processing. Defective 3'ETS processing of 47S rRNA after Cdk9 inactivation is most likely caused by a specific defect in snoRNA biogenesis.

2.5 Cdk9 inactivation triggers a nucleolar stress phenotype

Various kinds of stress can trigger nucleolar disintegration, which is accompanied by p53 stabilization and induction of cell cycle arrest and/or apoptosis. Having established that Cdk9 inactivation blocks 47S rRNA processing, the induction of nucleolar stress after Cdk9 knockdown was assessed (Fig. 17). A couple of nucleolar proteins can serve as marker for the structural integrity of the nucleolus. Nucleophosmin (NPM1), for example, is a ubiquitously expressed phosphoprotein, which belongs to the nucleoplasmin family of chaperones. NPM1 has multiple functions in ribosome biogenesis, centrosome duplication, DNA repair, and stress response. Under normal conditions, NPM1 localizes predominantly in interphase nucleoli. After stress, the nucleolus disrupts and NPM1 translocates into the nucleoplasm (Colombo et al., 2011; Chan et al., 1996). Approximately 50 % of the cells comprised a disintegrated nucleolar structure after Cdk9 knockdown, charaterized by nucleoplasmatic NPM1 staining (Fig. 17A). p53 induction is a direct consequence of nucleolar disintegration and was analyzed in U2OS cells after Cdk9 knockdown (Fig. 17B). p53 was stabilized by a factor of three after Cdk9 knockdown, comparable to the induction seen after knockdown of the rRNA processing factor Pes 1 (Grimm et al., 2006). Nucleolar stress not only stabilizes, but also activates p53 (Hölzel et al., 2010b). p53 activation is commonly associated with a cell cycle arrest and/or induction of apoptosis. Therefore, proliferation of U2OS cells was measured after Cdk9 knockdown (Fig. 17C). While Luciferase siRNA transfected cells continuously increased the cell index, transfection with siRNA specific for Cdk9 or Pes1 mRNA

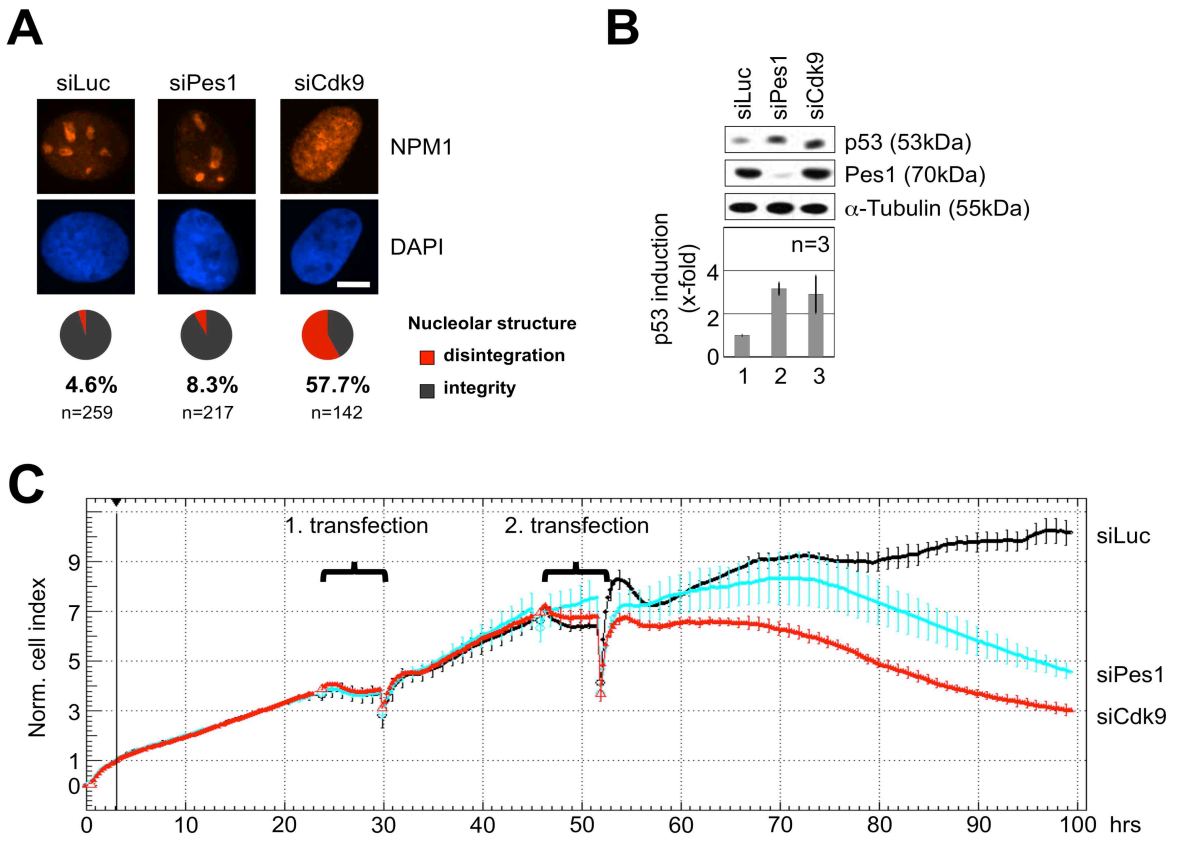

Figure 17. **Cdk9 knockdown causes nucleolar stress.** NPM1 translocation, p53 stabilization, and reduced proliferation after Cdk9 knockdown. U2OS cells were transfected with siRNA (100 nM) on two consecutive days targeting the mRNA of Pes1 or Cdk9. **(A)** Cells were fixed with 2 % para-formaldehyde and permeabilized with PBS containing 0.04 % Triton X-100 42 hours post transfection. Endogenous NPM1 was stained with a specific primary antibody and detected with a Cy3-conjugated secondary antibody. Cell nuclei were stained with DAPI (100 ng/ml). The rate of NPM1 translocation after siRNA transfection is shown together with the number of cells analyzed. The nucleolus was classified as disintegrated, if NPM1 was not predominantly localized to the nucleolus. Scale bar: 5 µm. **(B)** Whole cell extracts were prepared and separated by SDS-PAGE. p53 levels were detected by Western blot analysis with a specific antibody. Pes1 and α-Tubulin served as loading controls. p53 induction was plotted as x-fold induction relative to control p53 levels (siLuc) set as 1. **(C)** Cells were cultured over night. Culture medium was replaced by transfection medium containing oligofectamine and siRNA (100 nM). Medium was replaced again with culture medium after six hours (1. transfection). Cells were cultured over night and siRNA transfection was repeated (2. transfection). Normalized cell number was measured in real time and compared to control cells (siLuc). The cell number correlates to changes in impedance, which is termed 'cell index' (see details in *Material and Methods*). ctrl. knockdown: siLuc.

reduced the cell index three-fold after 100 hours measurement. Thus, Cdk9 inactivation impairs cell proliferation. In summary, Cdk9 inactivation disturbs nucleolar structure and function. It blocks 47S rRNA processing, thereby causing NPM1 translocation, p53 induction, and proliferation inhibition.

2.6 Application of 4-thiouridine in rRNA labelling

Metabolic *in vivo* labelling is a powerful approach to measure transcription and processing of nascent rRNA. Classical radioactive tracers like phosphate (^{32}P) are

directly incorporated by RNAP I and allow convenient autoradiographic detection of major rRNA forms, as shown extensively in previous experiments. As an alternative, non-radioactive approach to measure rRNA synthesis, I applied the pyrimidine analog 4-thiouridine (4sU) for labelling and detetion of nascent rRNA (4sU-tagging) (Fig. 11B). 4sU-tagging with 10 μM 4sU for up to three hours rendered rRNA synthesis normal. However, standard 4sU-tagging protocols to analyze nascent mRNA synthesis typically use a 10- to 50-fold higher 4sU concentration for one hour (Cleary et al., 2005; Rabani et al., 2011; Schwanhäusser et al., 2011). Interestingly, a number of purine and pyrimidine analogs, such as 5-Fluorouracil (5-FU), 6-Thioguanine (6-TG), or 6-Mercaptopurine (6-MP) have been shown to interfere with various cellular processes and induce cytotoxicity (Berger et al., 2002). 5-FU treatment, for example, strongly inhibits the RNA metabolism and ribosome biogenesis (Sun et al., 2007; Ghoshal and Jacob, 1994). Given that 4sU is a modified uridine analog that comprises high structural similarity to 5-FU, I next assessed whether 4sU could be a biological active compound that interferes with ribosome biogenesis at concentrations typically used in 4sU-tagging experiments.

2.6.1 4sU inhibits rRNA synthesis

U2OS cells were incubated with 100 μM 4sU, a concentration typically used to label nascent RNAs, and rRNA synthesis was assessed by ^{32}P metabolic labelling experiments (Fig. 18). Remarkably, 4sU globally inhibited rRNA synthesis both at the

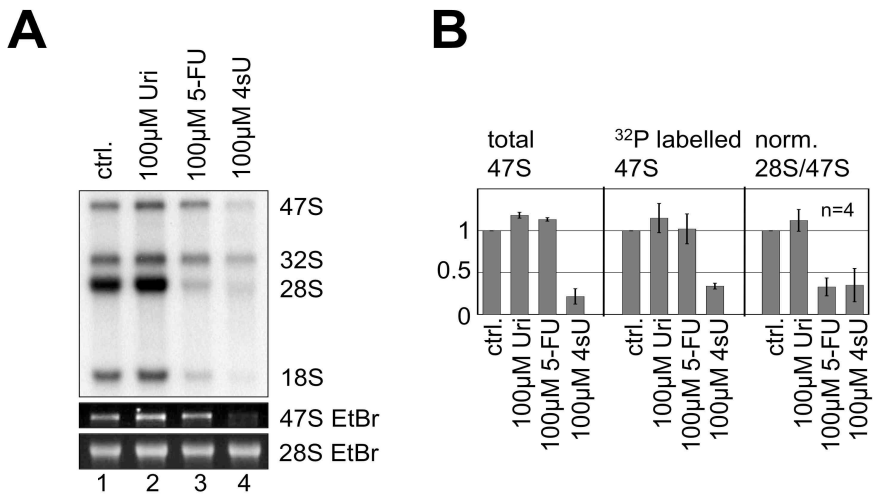

Figure 18. **4sU inhibits rRNA synthesis. (A)** 4sU inhibits both rRNA transcription and processing. U2OS cells were treated wit Uridine (Uri), 5-FU, or 4sU for six hours. Metabolic labelling was performed as described in *Materials and Methods*. Total 47S rRNA was visualized by EtBr staining under UV-light. ctrl.: 0.1 % DMSO. **(B)** Quantitation of rRNA synthesis after 4sU treatment. Total 47S rRNA levels were measured by quantitation of 47S EtBr UV-staining with ImageJ software, ^{32}P-labelled 47S rRNA was quantified by a PhosphorImager and AIDA software. rRNA signals and normalized ratios were calculated and compared to control signals and ratios (0.1 % DMSO).

level of rRNA transcription and processing (Fig. 18A). Treatment with 4sU reduced both total, unlabelled and nascent, ^{32}P-labelled 47S rRNA levels by about 75 % (Fig. 18B). Processing of the remaining 25 % nascent, ^{32}P-labelled 47S rRNA, which has been synthesized in presence of 4sU, was impaired by about 60 % compared to control cells (see normalized 28S/47S ratios). Treatment with 5-FU caused a profound inhibition of 28S rRNA maturation, but no significant inhibition of 47S rRNA production.

Recent 4sU-tagging protocols try to avoid prolonged incubation times. Therefore, I analyzed the impact of short-term 4sU treatment on rRNA synthesis in time course experiments (Fig. 19A). 100 μM 4sU impaired rRNA synthesis after one to two hours of incubation (Fig. 19B). A concomitant decrease of 47S, 32S, 28S, and 18S rRNA signals was detected with increasing 4sU incubation time (Fig. 19C).

The dynamics of rRNA transcription and processing in presence of 4sU can be analyzed in chase kinetic experiments in more detail. U2OS cells were incubated with 5-FU or 4sU for six hours, labelled with ^{32}P, and analyzed as indicated (Fig. 20A). In control cells, 47S rRNA signals peaked after two hours of chase and decreased to 50 % after six hours of chase. 28S rRNA signals were not detectable

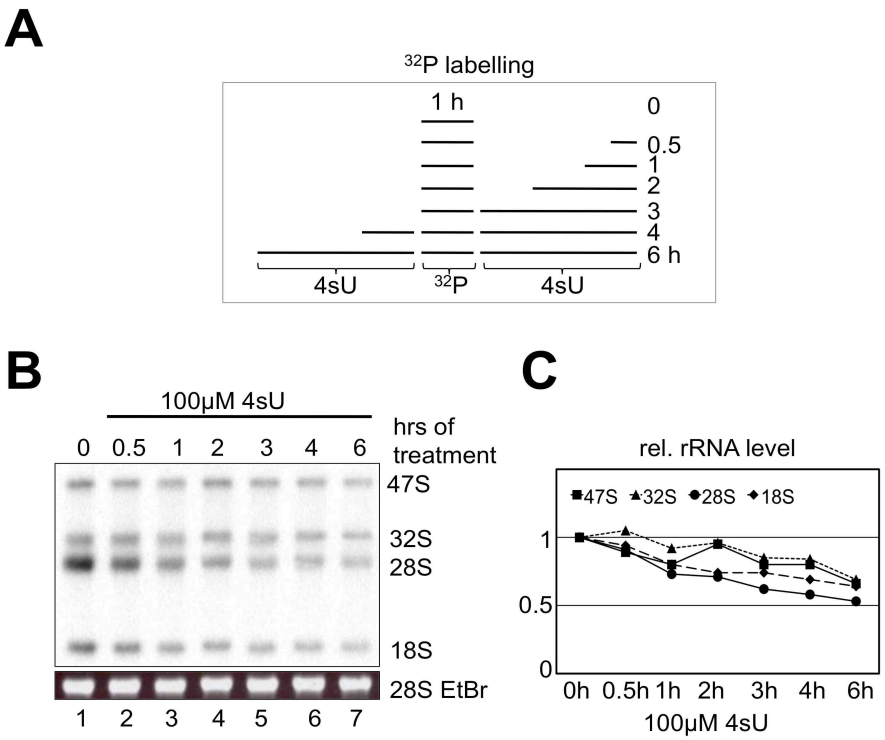

Figure 19. **Kinetics of 4sU-mediated rRNA synthesis inhibition. (A)** Scheme of ^{32}P labelling kinetics. rRNA synthesis was measured after different 4sU incubation times from zero to six hours. **(B)** 4sU impairs rRNA synthesis after one to two hours. U2OS rRNA was labelled *in vivo* with ^{32}P, RNA was isolated, separated, and signals were detected by autoradiography. **(C)** Quantitation of 4sU time kinetics. 47S, 32S, 28S, and 18S rRNA signals were analyzed by a PhosphorImager and AIDA software. rRNA levels were plotted relative to untreated cells (lane 1) set as 1.

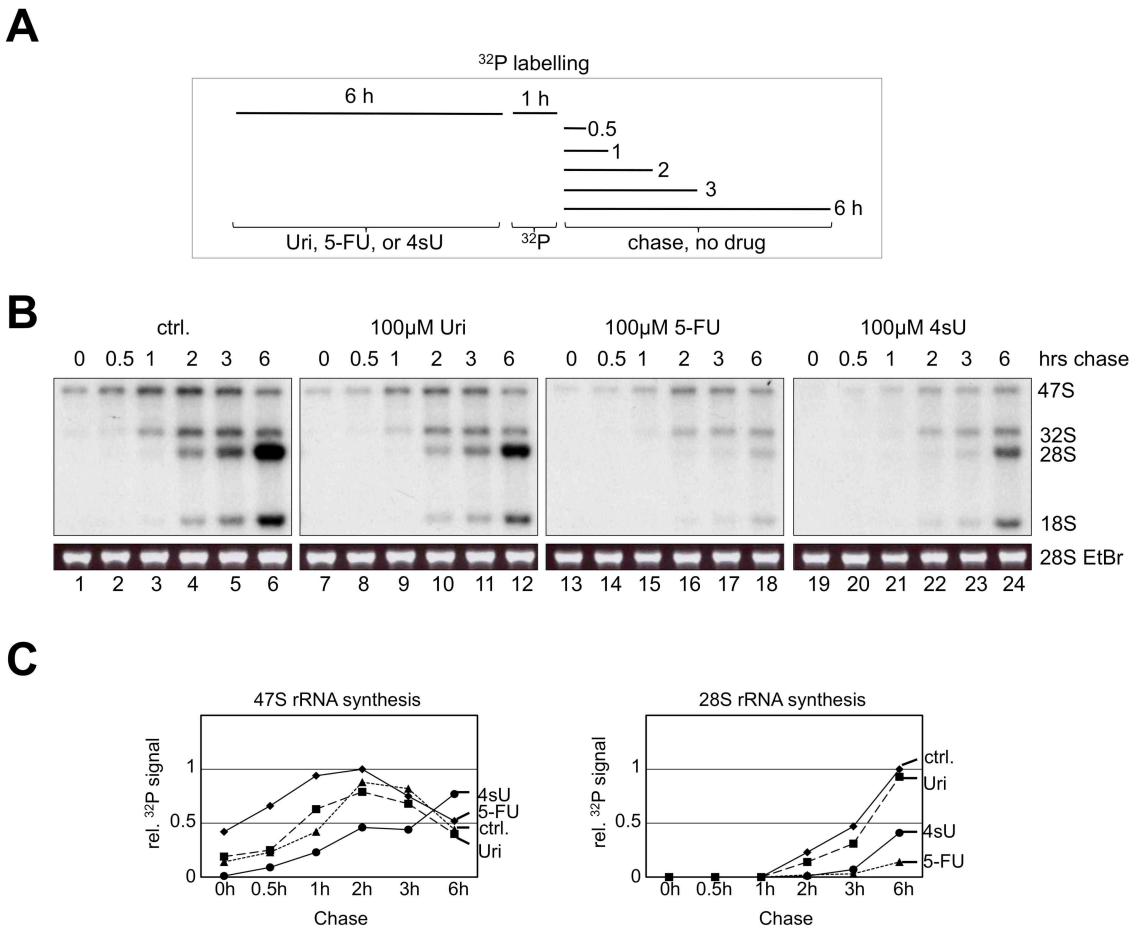

Figure 20. **4sU diminishes rRNA transcription and processing. (A)** Scheme of ^{32}P *in vivo* metabolic labelling workflow. U2OS cells were incubated with Uridine (Uri), 5-FU, or 4sU for six hours. ^{32}P was added for one hour (pulse) and cells were incubated for different chase times as indicated. **(B)** 4sU diminishes 47S rRNA production and 28S rRNA maturation. rRNA synthesis was analyzed as described in *Materials and Methods*. ctrl.: 0.1 % DMSO. **(C)** Quantitation of rRNA signals from (B). Signals were measured by a PhosphorImager and AIDA software and plotted as rRNA signals relative to 47S (lane 4) or 28S (lane 6) rRNA signals from control cells (0.1 % DMSO) set as 1.

within the first hour and peaked after six hours of chase (Fig. 20B). In line with previous results, 5-FU treatment reduced 28S rRNA maturation, but slightly diminished 47S rRNA accumulation. While 28S rRNA was barely detectable after six hours of chase (lane 18), the 47S rRNA signal peaked after three hours of chase (lane 11). In contrast, 4sU strongly reduced 47S rRNA accumulation (Fig. 20C). 47S rRNA signals peaked after six hours of chase (lane 24) and comprised less than 50 % after two hours of chase (lane 22), the time point when 47S rRNA peaked in control cells. 28S rRNA signals were barely detectable after one to three hours of chase in presence of 4sU (lanes 21-23), indicating that 4sU inhibits rRNA processing as well. Taken together, the data show that 4sU is a biologically active compound that globally inhibits rRNA synthesis when applied at high concentrations.

2.6.2 4sU triggers nucleolar stress

The potential of 4sU to induce key elements of nucleolar stress was assessed next (Fig. 21). Treatment with increasing concentrations of 4sU caused NPM1 translocation with up to 40 % of the cells comprising disintegrated nucleoli (Fig. 21A) and stabilized p53 by a factor of 15 (Fig. 21B). I conlude that highly concentrated 4sU is sensed by the nucleolus and triggers a nucleolar stress response.

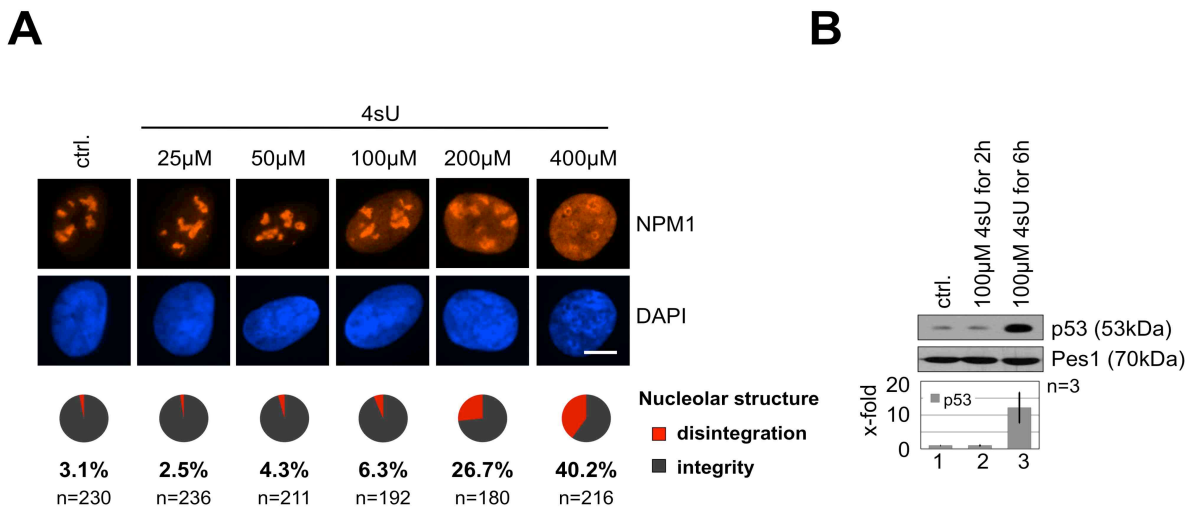

Figure 21. **4sU causes nucleoplasmic translocation of nucleolar NPM1 and induces p53. (A)** NPM1 translocation. U2OS cells were treated with various concentrations of 4sU for six hours. Cells were fixed with 2 % para-formaldehyde and permeabilized with PBS containing 0.04 % Triton X-100. Endogenous NPM1 was stained with a specific primary antibody and detected with a Cy3-conjugated secondary antibody. Cell nuclei were stained with DAPI (100 ng/ml). Scale bar: 5 µm. ctrl.: 0.1 % DMSO. **(B)** p53 stabilization. Cells were treated with 4sU as indicated. Whole cell extracts were prepared and separated by SDS-PAGE. p53 levels were detected by Western blot analysis. Pes1 serves as loading control. p53 induction was plotted as x-fold induction relative to p53 levels from control cells (0.1 % DMSO) set as 1.

3. DISCUSSION

This thesis suggests an intimate link between Cdk9-mediated RNAP II transcription, snoRNA biogenesis, and rRNA processing. The discussion part contains a critical evaluation of the results and proposes a model for their explanation and integration into the current view. The clinical relevance of ribosome biogenesis will be discussed, too.

3.1 Kinases at the interface between RNAP II transcription and rRNA processing

Kinases are key regulatory players. Although a large body of knowledge exists for many kinase-mediated cellular processes, evidence for kinases involved in rRNA processing is limited. So far, four kinases have been shown to be required for mammalian rRNA processing. Depletion of human (h)Rio kinase family members hRio1, hRio2, or hRio3 specifically inhibits 18S rRNA maturation (Widmann et al., 2012; Zemp et al., 2009; Baumas et al., 2012). The Nol9 kinase regulates processing of 32S rRNA into mature 28S and 5.8S rRNA by an unknown mechanism (Heindl and Martinez, 2010). Inactivation of the hRio kinase family and Nol9 blocks later rRNA processing steps. In contrast, studies with Cdk-inhibitors from others and our laboratory suggest a role for Cdks in early processing of the rRNA precursor. This work is based particularly on the observation that Flavopiridol (FL), a pan-specific Cdk-inhibitor, blocks 47S rRNA processing. To define the target of FL with relevance in 47S rRNA processing, a combination of genetic depletions and chemical inhibitors was applied. Both approaches displayed Cdk9 as critical regulator of 47S rRNA processing. Notably, the rRNA processing phenotype after Cdk9 knockdown was similar, but not identical to FL treatment. Complete inactivation of Cdk9 by RNAi is difficult to achieve. A stable pool of inactive Cdk9 is associated with the small

nuclear RNA 7SK and the HEXIM1 protein. On demand, the cell can gain additional active Cdk9 by dissociating it from 7SK RNA and HEXIM1 (Nguyen et al., 2001; Yang et al., 2001; Michels et al., 2003). In contrast, FL is a direct modulator of Cdk9 activity. It binds the ATP-binding pocket of Cdk9 quantetatively with its benzopyran part (De Azevedo et al., 1997; Baumli et al., 2008). This may explain why 47S rRNA processing is less potently inhibited after knockdown than after chemical inhibition of Cdk9.

Previous reports described a specific nucleolar form of Cdk9, $Cdk9_{55}$, generated from an mRNA that originates from a promoter upstream of the regular $Cdk9_{42}$ promoter. Surprisingly, the specific knockdown of $Cdk9_{55}$ did not inhibit rRNA synthesis, suggesting that the reason for the 47S rRNA processing defect rather lies in the nucleoplasm and is caused by $Cdk9_{42}$. Cdk9 is the catalytic subunit of the positive transcription elongation factor b (pTEFb), which augments RNAP II transcription, but also phosphorylates negative regulators of RNAP II like Spt4/5 and NELF. I therefore wondered, if RNAP II could be the cellular target that is responsible for defective 47S rRNA processing. Various kinases have been described to modulate RNAP II transcription by changing the phosphorylation patterns of the RNAP II CTD in concert with Cdk9. For example, the Brd4 kinase binds the CTD and regulates RNAP II transcription either directly by phosphorylation of the CTD or indirectly by Cdk9 recruitment (Devaiah et al., 2012; Wu and Chiang, 2007). The DNA-activated protein kinase (DNA-PK) can phosphorylate the CTD at serines and threonines with comparable efficacy (Lu et al., 1992; Dvir et al., 1993). Brd4- and DNA-PK-mediated CTD phosphorylations include the classical Cdk9 residue serine-2. However, Brd4 and DNA-PK were not regarded as FL-sensitive candidate kinases, since they modify the CTD only *in vitro* and are both atypical kinases. In fact, Brd4 is insensitive to FL-treatment (Devaiah et al., 2012). Cdk7 and Cdk8 are *bona fide* CTD-kinases, which introduce CTD phosphorylations critical for RNAP II initiation and co-transcriptional processing. Recent work defined Cdk12 and Cdk13 as CTD-kinases, too. Depletion of Cdk12 alters the CTD phosphorylation status, suggesting a functional interplay with Cdk9 in the regulation of RNAP II transcription (Bartkowiak et al., 2010). Surprisingly, neither inactivation of Cdk7, Cdk8, Cdk12, nor Cdk13 significantly changed rRNA processing. Cdk7 and Cdk8 are classical CTD-kinases for serines-5 and -7 required for initiation, but do not phosphorylate serine-2 required for elongation. I conculde that RNAP II elongation, rather than initiation, is crucial for 47S rRNA processing. However, Cdk redundancy could mask possible rRNA processing phenotypes, as shown for Cdk2, Cdk3, and Cdk4, which

are dispensable for cell cycle progression (Tetsu and McCormick, 2003; Ortega et al., 2003). One should also regard possible issues with knockdown efficacy and specificity. Although the requirement for Cdk9 in 47S rRNA processing was confirmed by knockdowns with two independent siRNAs, knockdown efficacy has been monitored only with a subset of kinases and in case an rRNA synthesis phenotype was visible. Importantly, the RNAP II-specific inhibitor α-amanitin phenocopied the Cdk9-dependent 47S rRNA processing phenotype by inducing the same phenotype as FL. This result unambiguously proves that maintenance of RNAP II transcription is required for 47S rRNA processing. I conclude that Cdk9 inactivation affects the synthesis of RNAP II-dependent transcripts critical for 47S rRNA processing. Whether Cdks different from Cdk9 definitely have no function rRNA processing or may assist Cdk9 in rRNA processing, requires further investigations.

3.2 Adaption of RNAP I transcription to 47S rRNA processing

Inhibition of RNAP II activity by α-amanitin or Cdk9 inactivation induced a specific 47S rRNA processing defect in the 3'ETS region. The defect is characterized by the occurrence of additional sequences downstream of the termination site (T) in 47S rRNA. In principle, this phenotype could be caused either by defective termination of RNAP I at the T-site or by an rRNA processing defect. In the first case, RNAP I would terminate immediately downstream of the T-site without transcribing sequences further downstream. Alternatively, RNAP I transcription is not affected, but the processing of rRNA at the T-site is impaired. Several pieces of evidence argue for the second scenario. Inhibition of RNAP II activity or Cdk9 inactivation prevented termination of RNAP I at the T-site. Sequences further downstream were transcribed, stabilized, and accumulated as unprocessed 47S-extened rRNA. Termination probably occurred at the first tymidine-stretch downstream. Not only sequences downstream of the T-site remained associated with the precursor, but also the 3'ETS itself, which usually is rapidly removed from 47S rRNA. There is also no indication for the removal of the leader sequence of the 5'ETS from the primary transcript, and no evidence for processing of ITS-1 and ITS-2 sequences. This suggests that all sites for endonucleolytic processing in the primary transcript are not targeted by the respective nuclease, if RNAP II is inhibited.

Inhibition of RNA processing is commonly accompanied by the accumulation of an unprocessed precursor RNA. Indeed, 47S rRNA accumulation after FL- or α-

Am treatment could be detected in Northern blot hybridizations, but not in ^{32}P metabolic labelling experiments, even when displaying strong inhibition of 47S rRNA processing. The reason for this discrepancy was unclear. ^{32}P metabolic labelling experiments are based on end-point analysis. However, end-point analysis is suboptimal to determine nascent 47S rRNA levels, since steady state could be reached within this period. Two labelling kinetics, with ^{32}P and 4sU, assisted to solve this problem. Kinetic data revealed that FL or α-Am treatment reduced RNAP I transcription rate. The kinetic alterations of RNAP I transcription were masked in ^{32}P metabolic labelling experiments, since defective 3'ETS processing is sensed by RNAP I transcription in a negative feedback loop. Thus, RNAP I transcription adapts to defective 3'ETS processing to harmonize rRNA synthesis. But how can a 3' processing defect in 47S rRNA feed back on RNAP I transcription? It has been shown that the RNAP I termination region of active rDNA genes is physically and functionally linked to the promoter of the subsequent rDNA repeat by the SL1 complex (Németh et al., 2008; Denissov et al., 2011). If RNAP I fails to terminate at the end of the gene due to a 3' processing block, this failure may have direct impact on initiation of the next RNAP I molecule at the adjacent promoter. Consequently, the transcription rate is slowed down.

Adaption of RNAP I transcription to reduced 47S rRNA processing raises the question, whether RNAP I and early rRNA processing are coupled events that mutually respond to each other. Interestingly, previous studies from our group showed that RNAP I occupancy is constantly high and remains unchanged upon induction of rRNA processing by overexpression of c-Myc or serum stimulation or upon starvation (Schlosser et al., 2003). The data suggest that high amounts of stalled RNAP I are constantly present on rDNA. Serum stimulation induces RNAP I elongation to harmonize 47S rRNA production with enhanced rRNA processing kinetics. Along the same lines, an elongation defective RNAP I mutant causes faulty rRNA processing and ribosome assembly, indicating that RNAP I elongation rates directly influence rRNA processing (Schneider et al., 2007). The creation of RNAP I gain of function mutants with enhanced elongation rates will help to further elucidate connections between RNAP I transcription and rRNA processing.

3.3 SnoRNAs are rate limiting for 47S rRNA processing

Searching for the subclass of RNAP II transcribed RNAs responsible for the observed rRNA processing defect in 3'ETS, a specific role of mRNAs and of small, Dicer-

dependently processed RNAs was excluded first. Although prevention of mRNA translation and inhibition of small RNA synthesis (miRNAs) interfered with specific rRNA processing steps, it did not affect proper 3' processing of the 47S rRNA. A number of studies suggest that Dicer and other key components of the human RISC pathway are implicated in rRNA processing. Knockdown of Dicer or Drosha in human cells blocks 5.8S rRNA maturation and leads to an accumulation of pre-5.8S rRNA species of various sizes (Liang and Crooke, 2011). Depletion of Drosha or Dicer may trigger changes in pre-rRNA secondary structures, which are unfavourable for processing (Wu et al., 2000; Fukuda et al., 2007; Liang and Crooke, 2011). It has also been reported that Dicer and other RISC components are involved in snoRNA biogenesis. For example, ACA45 snoRNA maturation is blocked after Dicer knockout in mammalian cells and *Giardia lamblia* (Ender et al., 2008; Saraiya and Wang, 2008). Drosha-associated DGCR8 nuclease was found to degrade snoRNAs U17a, U16, and U92. (Macias et al., 2012). However, a DGCR8 knockout displays no rRNA processing phenotype (Wang et al., 2007). Taken together, my observation that Dicer1 is required for later rRNA processing steps, but not for nucleolar 3'ETS processing, is largely confirmed by the literature.

Instead, a panel of snoRNAs was found as crucial transcripts for Cdk9-mediated rRNA processing. Almost all snoRNAs are transcribed by RNAP II, either as part of intronic sequences of coding genes, or as genes with their own promoter. While the majority of snoRNAs are not essential and mainly guide site-specific rRNA-modifications, a small group of snoRNAs is essential and, in addition, is involved in the proper endonucleolytic processing of pre-rRNA (Venema and Tollervey, 1999). In this context, SNORD118 (U8) deserves a particular consideration. U8 snoRNA is present in higher eukaryotes, but absent in yeast. U8 binds the 47S rRNA precursor both at the 5.8S rRNA sequence and the 28S rRNA sequence, guiding cleavage in the ITS-2 and, importantly, in the 3'ETS region. Depletion of U8 in *Xenopus laevis* causes an inhibition of 47S rRNA processing and an accumulation of abnormal precursors. U8 deletion mutants indicate that the 5' domain of U8 RNA is essential for its function. U8 has been suggested to function as a chaperone in long-range folding of pre-rRNA (Peculis and Steitz, 1993; Peculis et al., 1994; Peculis, 1997; Michot et al., 1999). Recent studies showed that the removal of U8 snoRNA from pre-rRNA by the helicase Ddx51 is a crucial step for the successful processing of 47S rRNA 3' sequences (Srivastava et al., 2010). Ddx51 knockdown blocks 3'ETS processing, since U8 remains associated with unprocessed pre-rRNA. Thus, the reduction of U8 levels after Cdk9 inactivation may explain the observed 47S rRNA

processing defect. U8 is the only known snoRNA required for proper processing of 3'ETS sequences, so far.

In addition, Cdk9 inactivation reduced the levels of snoRNAs U17a, U3, U22, and U14A. These snoRNAs are also involved in early nucleolytic cleavages of the rRNA precursor. U17a is required for 18S rRNA maturation (Atzorn et al., 2004). U3 is part of a large ribonucleoprotein complex, the small subunit (SSU) processome. Depletion of U3 proteins prevents 47S rRNA processing and 18S rRNA maturation (Beltrame and Tollervey, 1995; Dragon et al., 2002). U3 binds the 47S rRNA precursor at two distinct sites and cleaves in the 5'ETS region and in the ITS-1 region of the precursor (Beltrame and Tollervey, 1992; Kiss et al., 1991). U3 is also required for 5.8S rRNA maturation (Savino and Gerbi, 1990). U22 functions in 18S rRNA maturation (Tycowski et al., 1994). U14A complementary base pairs with the 18S rRNA and is required for its maturation (Dragon et al., 2002; Li et al., 1990; Hughes and Ares, 1991; Liang and Fournier, 1995). In total, depletion of a subset of critical snoRNAs explains 47S rRNA stabilization after Cdk9 inactivation. In particular, depletion of U8 makes 3'ETS processing impossible. Lack of U17a, U3, U22, and U14A prevents 47S rRNA cleavages in 5'ETS and ITS-1, leading to an artificial 47S rRNA precursor within a non-functional 90S pre-ribosome.

3.4 A model for the regulation of ribosome biogenesis by Cdk9

From the data discussed above I propose the following model for the function of Cdk9 in ribosome biogenesis. In unstressed cells, RNAP I transcription produces correctly processed, modified, and folded 47S rRNAs, which scaffold the assembly of 90S pre-ribosomes (Fig. 22A). Critical snoRNAs like U3, U14A, and U8 promote the correct formation of rRNA secondary structures, which enable correct processing steps. After transcribing the termination site (T), RNAP I is paused by the transcription termination factor 1 (TTF-1), which is bound to an evolutionary conserved, tymidine-rich TTF-1 binding sequence, the Sal box (Kuhn et al., 1990; Jansa et al., 1998; Pfleiderer et al., 1990; Braglia et al., 2011). An unknown, but essential RNAse 3-like enzyme (Rnt1 in yeast) cleaves the 47S rRNA primary transcript in its cognate 3' processing site, to produce the 3' end of the 47S rRNA and a free 5' end of the nascent transcript. RNAse 3-like cleavage provides an entry site for a 5'-3' exonuclease (Rat1 in yeast), which degrades the RNAP I associated transcript, destabilizes paused RNAP I and terminates RNAP I transcription by a torpedo mechanism (Kufel et al., 1999; Henras et al., 2004; El Hage et al., 2008;

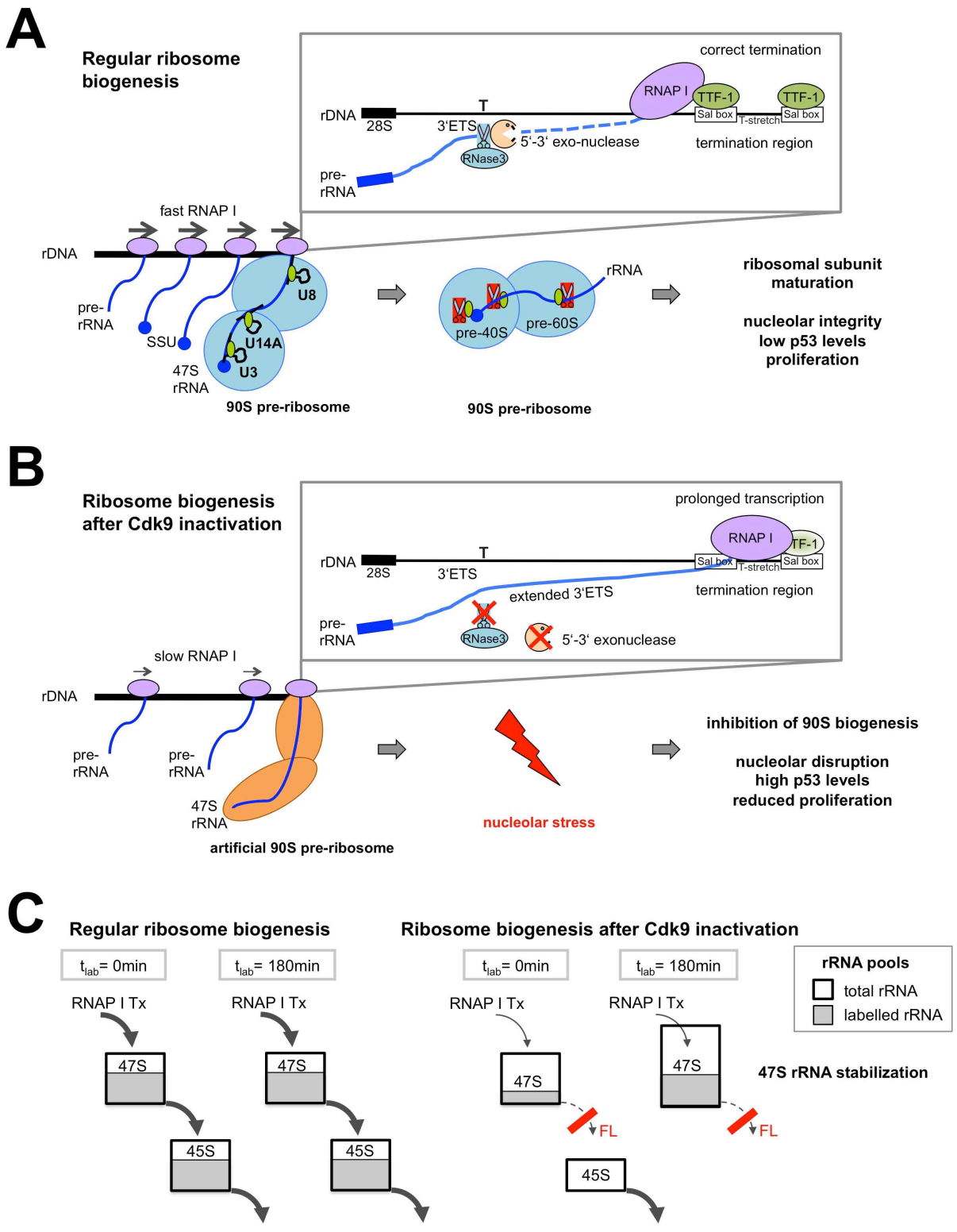

Figure 22. **A model for impaired ribosome biogenesis after Cdk9 inactivation. (A)** Regular ribosome biogenesis. Functional, Cdk9-mediated RNAP II transcription produces sufficient levels of critical snoRNAs like U3, U14A, and U8 for correct processing of the 47S rRNA primary transcript, which includes the 3'ETS formation by human homologues of yeast RNAse3 Rnt1 and 5'-3' exonuclease Rat1 at the termination site T. RNAP I transcription is paused by termination factor TTF-1 at the tymidine-rich Sal box region (T-stretch). Normal 90S ribosomes are processed to mature ribosomal subunits, which prevents nuclolar stress. Green dots: rRNA cleavage sites; scissors: rRNA cleavage factors.

Figure 22. (continued) **(B)** Ribosome biogenesis after Cdk9 inactivation. Insufficient levels of critical snoRNAs impair rRNA cleavages and reduce rRNA modifications and stem-loop formation. 47S rRNA is less efficiently processed and misfolded. This may impact on incorrect secondary structures in the 3'ETS region, which prevent an RNAse3-like enzyme from binding 47S rRNA, leading to a 3'-extended 47S rRNA primary transcript. RNAP I may read about 1 kb through the termination region. The 47S rRNA processing defect is sensed by transcribing RNAP I in a negative feedback loop, which slows RNAP I transcription and results in a non-functional, artificial 90S pre-ribosome. Defective ribosome biogenesis triggers a nucleolar stress response. **(C)** Flavopiridol inhibits 47S rRNA transcription and processing, but keeps 47S rRNA levels at steady state. The combination of reduced transcription and almost completely blocked processing allows the initially depleted 47S rRNA pool (0 min of labelling) to recover to normal levels and to accumulate over time (180 min of labelling), which results in 47S rRNA processing inhibition as net phenotype. FL: Flavopiridol; T_{lab}: labelling time.

Kawauchi et al., 2008). Correct 3' end formation and RNAP I release also requires the polymerase and transcript release factor PTRF, which is associated with TTF-1 and disassembles RNAP I from rDNA (Jansa and Grummt, 1999). The 3' end of the 47S rRNA is now capable to initiate 47S rRNA processing. Sufficient amounts of U8 snoRNA may be of particular importance for 3'ETS processing. U8 might facilitate initial 3'ETS processing by the correct formation of stem-loops, or the recruitment of downstream processing factors like endonucleases or the exosome (Peculis, 1997). Upon Cdk9 inactivation, RNAP II transcription is strongly impaired and critical snoRNAs, including U8, are no longer produced (Fig. 22B). Consequently, the 47S rRNA precursor lacks critical cleavage factors and secondary structures, which causes defective 47S rRNA processing in the 3'ETS region. Thus, the RNAse 3-like enzyme is no longer capable of recognizing the cleavage site and a 3'-extended version of 47S rRNA is stabilized. Whether the 3'-extended transcript remains associated with RNAP I and thereby is protected from exosomal degradation remains unclear. More likely, depletion of U8 causes severe structural deformations of the 3'ETS sequence, which prevents it from being processed. Consequently, the nucleolus produces misfolded, artificial 90S pre-ribosomes, which no longer can mature to pre-60S and pre-40S particles .

In yeast, pre-rRNA processing occurs co-transcriptionally (Osheim et al., 2004). To test, if co-transcriptional rRNA processing is present in human cells, I applied kinetic labelling experiments with 4sU. No rRNA forms were detected prior to the occurrence of 47S rRNA. Thus, I propose that processing of the 3'ETS sequence is the initial step in human rRNA processing, which is crucial for functional ribosome biogenesis. Defective 47S rRNA processing causes low turnover of 47S rRNA and is sensed by RNAP I in a negative feedback loop (Fig. 22C). Slow, but ongoing RNAP I transcription refills the 47S rRNA pool to regain steady state by adapting the influx rate to the reduced efflux rate. Accumulation of a 3'-extended 47S rRNA can be detected over time.

3.5 RNAP II CTD is required for snoRNA biogenesis

Cdk9 is a major kinase of the RNAP II CTD and phosphorylates serine-2 and serine-5 residues. CTD phosphorylation is a prerequisite for the recruitment of various cellular factors to the transcription machinery, which enables transcription of chromatin templates, but also correct capping, splicing, termination, and export of mRNAs. Recent evidence suggests that specific CTD modifications are also required for the controlled synthesis of snoRNAs. RNAP II CTD is methylated at a single arginine residue (R1810) by the arginine methyltransferase 1 (CARM1). Mutation of R1810 specifically enhances expression of a variety of small nuclear (sn)RNAs and snoRNAs, an effect that is also observed in CARM1$^{-/-}$ cells (Sims et al., 2011). The result suggests that snoRNA production is well controlled and that not each snoRNA-containing transcript will be processed into a mature snoRNA. Further evidence for a tightly regulated production of snoRNAs comes from recently published 4sU labelling experiments, which describe key characteristics of RNA processing at nucleotide resolution. The authors show that processing of most, but not all snoRNA-containing introns is remarkably inefficient, with the majority of introns being spliced and degraded rather than processed into mature snoRNAs (Windhager et al., 2012). But what could be the specific role of CTD phosphorylation by Cdk9 for snoRNA production. Although I cannot rule out a general transcription block of certain genes after Cdk9 knockdown, several reports described a specific RNA processing defect upon inhibition of Cdk9 activity. While transcription of U2 snRNA genes and histone H2b genes does not depend on Cdk9, the activity of the kinase is required for recognition of the 3′ RNA processing signal of U2 snRNA by processing factors. Mutation of the CTD serine-2 residue causes RNAP II read-through at the U2 snRNA 3′ end (Medlin et al., 2005). Likewise, Flavopiridol has been reported to reduce the accumulation of hsp70 mRNAs in Drosophila cells without affecting RNAP II density on the gene. Instead, a major defect was observed at the level of 3′ end processing (Ni et al., 2004). Similar results have been obtained from experiments in *Xenopus laevis* with the Cdk9-inhibitor DRB (Bird et al., 2004). Therefore, I speculate that Cdk9 regulates snoRNAs levels by post-transcriptional processing.

Cdk9 inactivation impairs the expression of snoRNAs, which are transcribed as separate transcription units (U3 and U8) and those, which are processed from intron sequences of pre-mRNAs (U22, U14A, and U17a). In yeast, snoRNAs of the

first category terminate transcription through a pathway directed by the Nrd1 complex, which contains the nascent RNA-binding factors Nrd1 and Nab3 as well as the RNA helicase Sen1 (Steinmetz et al., 2001; Thiebaut et al., 2006; Carroll et al., 2004) (Fig. 23). Nrd1 comprises a CTD interaction domain (CID) that preferentially binds CTD phosphorylated at serine-5 (Gudipathi et al., 2008). Nrd1 recruitment is specifically enhanced at the 3′ end of genes, which is in agreement with the model that the Nrd1-Nab3-Sen1 termination complex acts specifically at short RNAP II transcribed genes (Jamonnak et al., 2011; Vasiljeva 2008). After termination, the Nrd1 complex dissociates from the CTD to recruit the TRAMP complex and the exosome. TRAMP adds a short oligo-A-tail to the cleaved 3′ end, which primes for subsequent exosomal 3′-5′ processing (Thiebaut et al., 2006; Wlotzka et al., 2011). Nrd1-dependent termination of non-coding RNAs is also dependent on the activity

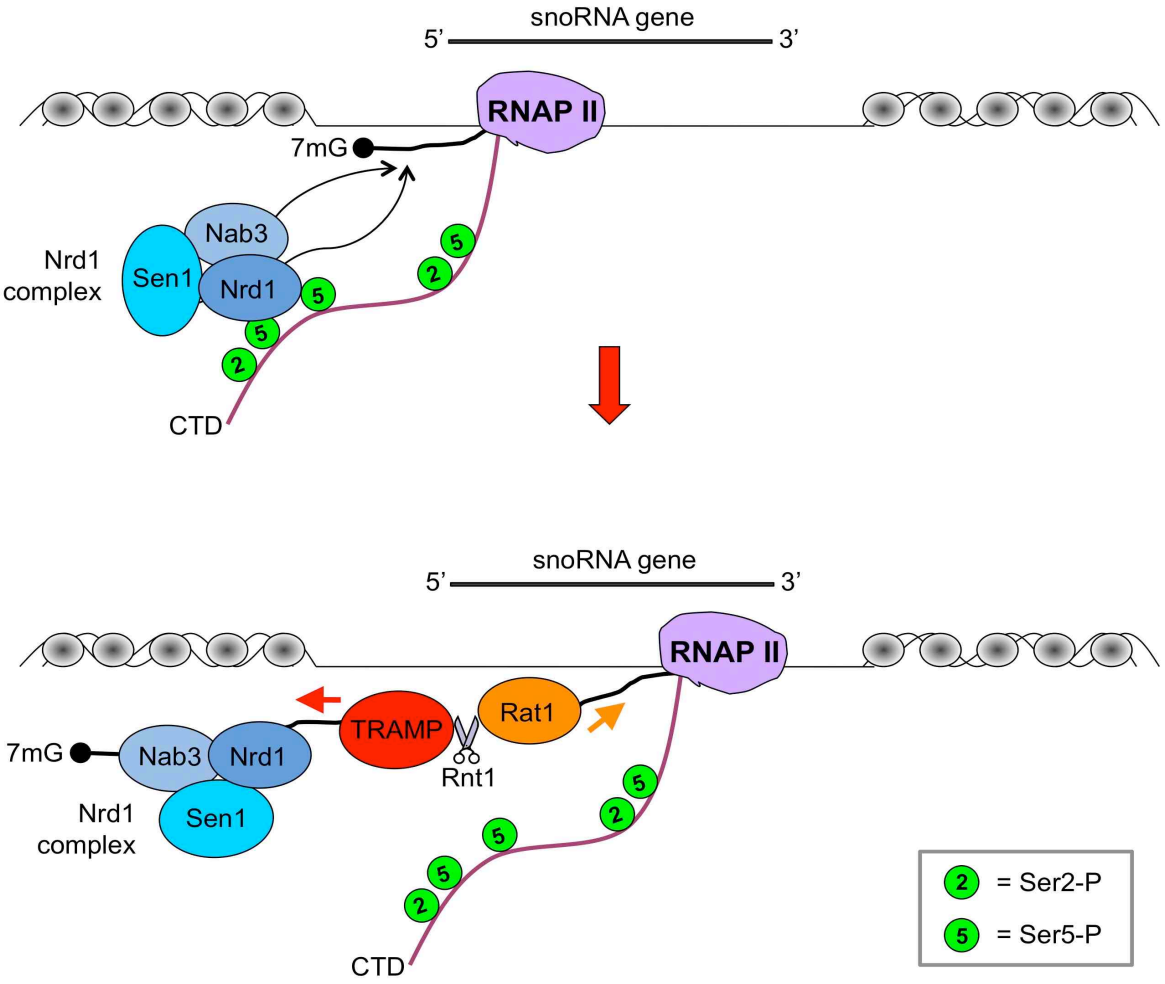

Figure 23. **Nrd1-dependent RNAP II termination of snoRNA genes in yeast.** The Nrd1-Nab3-Sen1 complex is recruited via direct interaction between Nrd1 and the RNAP II CTD, when phosphorylated at serine-5. During elongation, both Nrd1 and Nab3 scan the nascent RNA for their preferred termination sequence. After binding to their consensus sequence, the Nrd1 complex dissociates from the CTD and recruits the TRAMP (Trf4/5-Air1/2-Mtr4) complex for 3'-5' trimming of the transcript and Rat1 exonuclease for degradation of the remaining RNA exiting from RNAP II. Rnt1 endonulease may contribute to cleavage of nascent RNA (adapted from Zhang et al., 2012).

of the propyl isomerase Ess1. Ess1 binds the serine-2 and -5 phosphorylated CTD and regulates transcription by conformational isomerization of serine-proline bounds within the CTD. Defects in termination of snoRNAs and other small RNAs are observed in Ess1 mutants (Singh et al., 2009). Recent work demonstrated that CID point mutations of Nrd1 residues at the interface to serine-5 of the CTD diminish the processing of snoRNA snR13 (Kubicek et al., 2012). Together, the data suggest that cellular factors involved in snoRNA processing are specifically recruited to snoRNA genes by serine-2 and -5 dependent CTD phosphorylation marks. However, no human homologs of the Nrd1 complex have been identified, so far. Instead, it has been shown that the 3' processing of small nuclear (sn)RNAs in mammals is dependent on the Integrator complex, which is recruited to serine-7 phosphorylated RNAP II CTD. Mutation of serine-7 causes a defect in snRNA transcription (Egloff et al., 2007). A possible connection between Cdk9, the Integrator, and pre-snoRNA processing requires further investigations.

Correct transcription and processing of nascent pre-snoRNAs could also be a matter of speed. Cdk9 promotes RNAP II elongation not only via CTD phosphorylation, but also by phosphorylation of Spt4/5 and NELF. Cdk9 inactivation may reduce the capability of RNAP II to transcribe through chromatin, in case Spt4/5 and NELF lack critical modifications, remain with RNAP II, and fail to recruit elongation factors like the Paf1 complex. Reduced elongation may contribute to reduced co-transcriptional processing of pre-snoRNAs, as shown for alternative splicing that depends on the elongation rate (de la Mata et al., 2003). Maturation of pre-snoRNA sequences and their association with binding proteins to form snoRNP complexes requires functional pre-mRNA splicing and involves the Hsp90 co-chaperone complex R2TP and the nuclear assembly factor 1 (Naf1) (see Fig. 6 for details). Depletion of Hsp90 and R2TP components inhibits snoRNP maturation, reduces box C/D snoRNAs and blocks rRNA processing (Zhao et al., 2008; Boulon et al., 2008). Interestingly, Naf1 has been shown to bind phosphorylated RNAP II CTD (Richard et al., 2006; Fatica and Tollervey, 2002). Taken together, several additive mechanisms may contribute to the strong depletion of snoRNAs.

3.6 Differential regulation of rRNA synthesis by Ctk1 and Bur1/2

Depletion of the yeast Cdk9 homolog Bur1/2 specifically inhibited later rRNA processing, but had no impact on production of 35S rRNA. In contrast, Ctk1 blocked 35S rRNA transcription and its maturation to 25S and 18S rRNA. How can these

phenotypes be explained and why do they differ? Bur1/2 and Ctk1 have well-known functions in RNAP II transcription. Both kinases are critical for RNAP II elongation. Both kinases phosphorylate the RNAP II CTD at position serine-2, Bur1/2 also phosphorylates the negative elongation factor Spt4/5 (Cho et al., 2001; Zhou et al., 2009; Keogh et al., 2003). Spt4/5 is highly conserved and can close the DNA clamp of RNAP II, enabling processive elongation (Martinez-Rucobo et al., 2011; Hartzog et al., 1998). Recent studies suggest that Ctk1 and Spt4/5 functions also impact on RNAP I transcription and rRNA processing. Dual functions make interpretation of phenotypes difficult, since an effect via RNAP II transcription is difficult to exclude. Ctk1 partially localizes in the nucleolus and interacts directly with RNAP I to foster rRNA transcription initiation. Depletion of Ctk1 inhibits RNAP I, decreases the number of rDNA repeats and causes nucleolar disruption (Bouchoux et al., 2004; Grenetier et al., 2006). Consequently, I find rRNA transcription reduced after depletion of Ctk1, but fully functional after Bur1/2 depletion, which, in turn, inhibits rRNA processing. Spt4/5 function may explain this phenotype. Mutations in Spt4/5 decrease rDNA copy numbers and lead to an rRNA processing defect, which closely resembles inhibition of 27S rRNA processing after Bur1/2 depletion (Schneider et al., 2006). Spt4/5 has been shown to associate with rDNA *in vivo* and interact with RNAP I (Leporé and Lafontaine, 2011). Non-phosphorylated Spt4/5 pauses RNAP I before elongation (Schneider et al., 2006; Anderson et al., 2011). Upon phosphorylation, Spt4/5 recruits the Paf1 complex, which is crucial for efficient transcription elongation through chromatin (Zhang et al., 2009; Zhang et al., 2010). I assume that both Spt4/5 mutation and Bur1/2 depletion impair Paf1 recruitment to RNAP I and thereby impair elongation. Based on similarities to RNAP II elongation, Bur1/2 might modulate Spt4/5 activity to regulate RNAP I elongation and co-transcriptional rRNA processing.

One could also speculate that Ctk1 and Bur1/2 orchestrate the expression of different subsets of genes, which are, for example, required for different steps in ribosome biogenesis. While Ctk1 primarily supports transcription of rRNA, Bur1/2 may preferentially promote the expression of factors required for rRNA processing. Bur1/2 depletion may cause an imbalance in the synthesis of non-coding RNAs, required for rRNA processing, but not transcription, which might at least partially explain the Bur1/2-mediated inhibition of 27S rRNA processing. Bur1/2 depletion could also interfere with premature cleavage (PMC). It has been shown that co-transcriptional premature cleavage in the ITS-1 produces up to 70 % of immature 27S rRNA in yeast under growth favourable conditions (Kos and Tollervey, 2010).

Reduced PMC could explain the observed accumulation of 35S rRNA, which could no longer be processed adequately into 25S rRNA upon depletion of Bur1/2-dependent early rRNA processing factors. The different contributions of Ctk1 and Bur1/2 to the regulation of gene expression are subject of current research (Wood and Shilatifard, 2006).

After having shed light on the molecular mechanism of Cdk9-mediated rRNA processing, the focus will now be on the nucleolar stress response, which is induced upon rRNA processing inhibition and connects ribosome biogenesis to cell cycle control.

3.7 Defective rRNA processing triggers nucleolar stress

The existence of an interphase nucleolus is dependent on the constant production of pre-rRNA, to balance the loss of rRNA from the nucleolus by maturation of pre-rRNA. Nucleolar stress abrogates pre-rRNA synthesis. This imbalance causes translocation of nucleolar factors like NPM1 into the nucleoplasm. Consequently, the nucleolar structure collapses and disintegrates into substructures (Hernandez-Verdun et al., 2010). Nucleoplasmatic NPM1 localization was used as a marker for nucleolar disruption after Cdk9 knockdown. NPM1 relocates to the nucleolus upon stress relief (Chan et al., 1996). NPM1 translocation to the nucleoplasm is accompanied by p53 stabilization. Importantly, p53 induction differs from the classical DNA damage response, which involves Ataxia telangiectasia mutated kinase (ATM), checkpoint kinase 1 (CHK1), and phosphorylation of p53 at position serine-15. Active p53 induces cell cycle inhibitors like $p21^{Cip}$ and $p27^{Cip}$, which negatively regulate Cdk2 activity and arrest the cell cycle in G_1-phase (Lane, 1992; Levine, 1997; Sakaguchi et al., 1998; Ljungman, 2000; Harper et al., 1995; Vogelstein et al., 2000). Remarkably, p53 serine-15 phosphorylation is absent after nucleolar stress, suggesting alternative pathways to active p53 (Hölzel et al., 2010a). Recent studies revealed that the levels rather than modifications of p53 are crucial for its activity. p53 modifications rather fine-tune its activity (Blattner et al., 1999; Efeyan et al., 2006). The nucleolar stress-mediated p53 response is superior to DNA-damage signalling, since it induces p53 by all means and independently of functional kinases. Consequently, Cdk9 knockdown reduced cell proliferation. Ongoing proliferation directly after onset of nucleolar stress indicates that NPM1 translocation, and p53 induction precedes inhibition of proliferation. Taken together, the data support the

current view that inhibition of ribosome biogenesis is the cause and not a consequence of p53-mediated cell cycle arrest.

High concentrations of 4sU globally inhibited rRNA synthesis and induced nucleolar stress. The molecular details of the nucleolar stress phenotype have been discussed above. Interestingly, the inhibition of rRNA synthesis by 4sU differed from 5-FU, which specifically blocks 32S rRNA processing (Ghoshal and Jacob, 1994; Burger et al., 2010). How can this difference be explained? Both drugs are uridine analogs, but are modified at different positions, resulting in different structures. Both 4sU and 5-FU can be incorporated at potential target sites for pseudouridylation. Incorporation of 5-FU at such target site has been reported. Incorporation of 5-FU into U2 small nuclear (sn)RNA prevents pseudouridylation and formation of functional snRNPs for pre-mRNA splicing (Zhao and Yu, 2007). In analogy, inhibition of 32S rRNA processing by 5-FU could be explained by its incorporation into rRNA and/or snoRNAs, and the lack of critical pseudouridylations. Taken together, 4sU impairs rRNA synthesis and potentially changes the secondary structure and function of mature 28S, 5.8S and 18S rRNA within the ribosome. It is tempting to speculate, if 4sU allows correct formation of fully functional ribosomes.

4sU-tagging of nascent RNA is a sophisticated approach to measure transcription, processing, and turnover of newly transcribed RNA (Dölken et al., 2008; Friedel et al., 2009; Rabani et al., 2011; Windhager et al., 2012). Data from this thesis underscore that metabolic labelling of RNA with 4sU is a feasible approach. However, labelling time and 4sU concentration should be kept as short as possible, with regard to the severe nucleolar stress 4sU can cause.

3.8 Defects in ribosome biogenesis are the origin of multiple diseases

Mutations of single ribosomal proteins and processing factors cause a number of haematological diseases, so called ribosomopathies. Mutations in ribosomal proteins S19, L5, L11, S24, L35a, S17, or S7 cause Diamond-Blackfan anemia (DBA) and an increased susceptibility to haematological malignancies (Narla and Ebert, 2010). S19 is the most prominent driver of DBA. Mutations in S19 and S24 impair 18S rRNA maturation and 40S subunit formation (Draptchinskaia et al., 1999; Hölzel et al., 2010a; Choesmel et al., 2007, Flygare et al., 2007). A point mutation in ribosomal protein S14 causes the $5q^-$-syndrome, an independent subtype of the myelodysplastic syndrome, which includes macrocytic anaemia. S14 haploinsufficiency impairs 18S rRNA maturation and 40S subunit formation (Ebert et al., 2008). p53 triggers a $5q^-$-

syndrome phenotype in a S14 knockout mouse (Barlow et al., 2010). Mutations in the *SBDS* gene cause the Schwachman-Diamond syndrome (SDS), which includes ineffective haematopoiesis and an increased risk to develop leukaemia. SBDS is required for rRNA processing and 60S subunit formation. SBDS also catalyzes translation initiation by stimulating ribosomal subunit joining (Boocock et al., 2003; Ganapathi et al., 2007; Finch et al., 2011). Dyskerin (DKC1) is the pseudouridine synthase, which complexes in box H/ACA snoRNPs and catalyzes the pseudouridine rRNA modification. Mutations in DKC1 are associated with *Dyskeratosis congenita*, reduce rRNA pseudouridylation, induce defective rRNA processing in mice, and cause bone marrow failure (Lafontaine et al., 1998; Dokal, 2000; Ruggero et al., 2003). A number of additional diseases are caused by dysfunctional ribosomes (Narla and Ebert, 2010).

It is not entirely clear, how mutations in ribosome biogenesis factors, present in each cell, can cause specific haematological diseases or malignancies restricted to particular tissues. p53 stabilization and induction of cell cycle arrest and apoptosis are common features of ribosomopathies (McGowan et al., 2008; Fumagalli et al., 2009). Apoptosis might shatter the erythroid/myeoloid lineage more severe than the lymphoid lineage, which may cause an imbalance in the haematological system.

Breast cancer cells not only produce higher amounts of ribosomes, but also ribosomes of a different quality. Ribosomes in breast cancer are synthesized by alternative rRNA processing pathways, which leads to the accumulation of unusual pre-rRNA forms. These so called 'cancer ribosomes' have different rRNA modification patterns and altered translational capacities. In fact, rRNA in 'cancer ribosomes' carries additional and novel methylation sites (Belin et al., 2009). Changes in the rRNA modification pattern are thought to decrease translational fidelity, induce misfolding of rRNA and reduce internal ribosome entry site (IRES)-dependent translation. Given that the expression of many tumor suppressors like p27 or p58 relies on IRES-dependent translation, 'cancer ribosomes' may be of direct relevance for transformation (Kullmann et al., 2002; Cornelis et al., 2000; Vagner et al., 2001; Silvera et al., 2010). One could speculate that snoRNA-guided rRNA modification patterns differ in 'cancer ribosomes' or among tissues. Distinct, tissue-specific rRNA modification signatures may exist, that regulate translation of key cell cycle regulating factors. Alternatively, defective ribosomal subunits could diminish translation of globin genes, which leads to a relative excess of free heme and could trigger erythroid specific apoptosis and anaemia (Keel et al., 2008). Other models rely on possible extraribosomal functions of several ribosomal proteins. Mutations in

ribosomal proteins may alter DNA replication or DNA repair, too (Warner and McIntosh, 2009).

A number of options to interfere with snoRNA biogenesis and rRNA synthesis have been defined, recently. For example, the Hsp90 chaperone inhibitor Geldanamycin prevents snoRNP assembly on nascent snoRNAs by impairing the correct folding of snoRNP stabilizing proteins like Nop56, Nop58, or Fibrillarin. Unprotected snoRNAs are rapidly degraded and rRNA processing is inhibited (Boulon et al., 2008). Since Hsp90 is overexpressed in many tumors, Geldanamycin is a promising drug for treatment of cancer. However, inhibition of protein folding may have global impact on many cellular processes (Pearl and Prodromou, 2006; Caplan et al., 2007). Inhibition of pre-mRNA splicing is an alternative approach to interfere with snoRNA biogenesis. The biogenesis of snoRNAs is actively coupled with splicing. The natural product Isoginkgetin, for example, is a general inhibitor of splicing; it prevents the recruitment of spliceosomal snRNP subunits U4/U5/U6 (O'Brien et al., 2008). Isoginkgetin is a known anti-tumor drug (Yoon et al., 2006). Similarly, Spliceostatin A and Pladienolide B also impair pre-mRNA splicing. Both substances bind and inactivate SF3b, a subcomplex of the U2 snRNP to prevent spliceosome assembly (Kaida et al., 2007). Unfortunately, inhibition of splicing may also globally interfere with pre-mRNA synthesis. In 2011, Drygin and colleagues have reported a more direct approach to inhibit rRNA synthesis. They found that the small molecule CX-5461 is a selective inhibitor of RNAP I transcription initiation that specifically blocks recruitment of the SL1 complex and impairs PIC formation. The anti-tumor activity is conferred by p53 stabilization. CX-5461 is effective in human solid tumors; its therapeutic potential is currently tested in clinical trials. Taken together, these examples show that ribosome biogenesis is an emerging field in cancer research. Exciting recent findings led to a number of models and hypothesises, to elucidate the intimate connection between ribosome biogenesis regulation and its deregulation in cancer.

Cdk9 is no classical proto-oncogene. However, Cdk9 mRNA levels are specifically elevated in Acute Myeloid Leukaemia (AML) and in Acute Lymphoid Leukemia (ALL) (*www.genesapiens.org*). Tumor growth is often driven by hyperactivation of kinases. Cdk9 mutations could hind to alterations in RNAP II transcription, snoRNA biogenesis, or ribosome biogenesis. Specific Cdk9-inhibitors might be of particular interest in the therapy of haematological diseases. However, no mutations in Cdk9 have been found in the AML genome, so far (Mardis et al., 2009; Greif et al., 2011). Why is Cdk9 unregulated in AML/ALL? Are AML/ALL

cells hyposensitive to FL treatment? Further investigations are necessary to clarify, whether elevated Cdk9 expression could be directly connected to rRNA processing and may contribute to the onset of haematologic diseases.

3.9 Outlook

This thesis contributes to the establishment of ribosome biogenesis as a Cdk9-dependent process with possible therapeutic relevance. Cdk9 inactivation is a powerful approach that abrogates snoRNA biogenesis, causes defective 47S rRNA processing, and induces nucleolar stress. Given that pre-mRNA splicing and snoRNA synthesis are coupled events and depend on Cdk9-mediated RNAP II transcription elongation, one could imagine that mutations in the CTD or alterations of its modification pattern may influence snoRNP biogenesis by the abrogation of proper Nrd1-mediated 3' processing, but also by preventing the function of CTD-associated snoRNP assembly factors, like R2TP or Naf1. It will be interesting to find out, whether the association of Naf1 or R2TP to the CTD is altered after changes of the CTD modification pattern, as demonstrated for Nrd1 termination factor (Kubicek et al., 2012). This might further point towards a direct regulation of snoRNA biogenesis by RNAP II. Deregulation of snoRNA biogenesis alters the rRNA modification status quantitatively and qualitatively. Alterations in Cdk9-mediated RNAP II transcription may lead to changes of rRNA modification patterns, cause misfolding of the ribosome, and reduce its translational fidelity. It will be exciting to investigate the contribution of RNAP II transcription and snoRNA biogenesis to the formation of rRNA modifications patterns and translation in 'cancer ribosomes' or among tissues and may help to further understand the connection between defective ribosome biogenesis and the onset of ribosomopathies.

Apart from modulating RNAP II transcription, the possibility that Cdk9 phosphorylates additional nucleolar substrate(s) cannot be excluded. The knockout of the yeast Cdk9 homolog Ctk1 alters the phosphorylation patterns of several nucleolar proteins, most prominently of the yeast pseudouridine synthase Cbf5 (SILAC screen by Dr. Katja Strässer, unpublished results). Although Cbf5 could not be confirmed as a direct substrate of Ctk1 in an *in vitro* kinase assay, it will be interesting to analyze, whether Ctk1 or Bur1/2 can phosphorylate components of the snoRNA biogenesis machinery such as Nrd1, Naf1, R2TP complex, or snoRNP proteins.

In the course of investigating Cdk9-dependent 47S rRNA processing, I found that Cdk9 is specifically required for the processing of the 3'ETS sequence within the 47S rRNA. Cdk9 inactivation stabilizes a 3'-extended 47S primary transcript accompanied by reduced RNAP I transcription. Does the 3'-extended primary transcript remain associated with RNAP I and is thereby protected from polyadenylation and exosomal degradation? Is Cdk9 involved in RNAP I termination? Is there a failsafe termination mechanism existing as demonstrated in yeast? Unanswerd questions leave room for further investigations.

4. MATERIALS

4.1 Reagents

(4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES)	MP Biomedicals Inc., Eschwege
[^{32}P]-ortho-phosphate (^{32}P)	Hartmann-Analytik, Braunschweig
[γ-^{32}P]-Desoxyadenosine Triphosphate (γ-^{32}P-dATP), 6000 μCi/mmol	Hartmann-Analytik, Braunschweig
1,4-Dithiothreitol (DTT)	Carl Roth GmbH&CoKG, Karlsruhe
1 kb DNA ladder	Invitrogen, Karlsruhe
3-(N-Morpholino)-propanesulfonic Acid (MOPS)	Sigma-Aldrich Chemie, GmbH, Deisenhofen
4'-6-Diamino-2-phenylindole (DAPI)	Sigma-Aldrich Chemie, GmbH, Deisenhofen
4-Thiouridine (4sU)	Sigma-Aldrich Chemie GmbH, Deisenhofen
5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB)	Sigma-Aldrich Chemie GmbH, Deisenhofen
5-Fluorouracil (5-FU)	Sigma-Aldrich Chemie GmbH, Deisenhofen
5 x First Strand Buffer	Invitrogen, Karlsruhe
Acetate (Ac)	Carl Roth GmbH&CoKG, Karlsruhe
Actinomycin D (ActD)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Agarose, Ultra Pure	Invitrogen, Karlsruhe
Albumin Fraction V (BSA)	Carl Roth GmbH&CoKG, Karlsruhe
α-Amanitin (α-Am)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Ammonium Hydroxide (NH_4OH)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Ammonium Persulfate (APS)	Carl Roth GmbH&CoKG, Karlsruhe
Amplify Solution	GE-Healthcare, München
Anisomycin (AIM)	Sigma-Aldrich Chemie GmbH, Deisenhofen
β-Mercaptoethanol (β-ME)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Biotin-HPDP	Pierce, Rockford, USA
Bromophenol Blue (BPB)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Cdk1-inhibitor CGP74514A	Merck, Darmstadt
Cdk2-inhibitor Compound 3	CalBioChem
Cdk9-inhibitor II	CalBioChem
Cdk9-inhibitor KM05283SC	Maybridge, Cornwall
Cdk13-inhibitor TG003	Tocris Bioscience, Ellisville
Chloroform	Carl Roth GmbH&CoKG, Karlsruhe
Cycloheximide (CHX)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Desoxyribonucleosidetriphosphate mix (dNTPs)	Promega, Mannheim
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Dimethyl Formamide (DMF)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Disodium Hydrogen Phosphate ($Na_2H_2PO_4$)	Merck KGaA, Darmstadt
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, Karlsruhe
Dulbecco's Modified Eagle Medium, Phosphate-free (DMEM-P)	Invitrogen, Karlsruhe
Ethanol (EtOH), absolut	Merck, Darmstadt
Ethidium Bromide (EtBr)	Fluka Chemie GmbH, Buchs
Ethylenediaminetetraacetic Acid (EDTA)	Carl Roth GmbH&CoKG, Karlsruhe
Fetal Bovine Serum (FBS)	PAA Laboratories, Pasching, Österreich
Fetal Bovine Serum, dialyzed, Phosphate-free (FBS-P)	Invitrogen, Karlsruhe
Flavopiridol (FL)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Fluorescent Mounting Medium, Vecta Shield	Dako GmbH, Hamburg
Formaldehyde (37 %)	Carl Roth GmbH&CoKG, Karlsruhe

Formamide	Sigma-Aldrich Chemie GmbH, Deisenhofen
Galactose	Merck, Darmstadt
Glucose	Merck, Darmstadt
Glycerol 86 %	Carl Roth GmbH&CoKG, Karlsruhe
Glycine	Carl Roth GmbH&CoKG, Karlsruhe
Homoharringtonine (HHT)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Hydrogen Chloride (HCl)	Merck, Darmstadt
Hygromycin B (Hyg)	Invitrogen, Karlsruhe
Immersol 518F	Carl Zeiss AG, Oberkochem
Isopropanol, absolut	Carl Roth, Karlsruhe
Kalium Acetate (KAc)	Merck KGaA, Darmstadt
Kalium Chloride (KCl)	Merck KGaA, Darmstadt
Kalium Dihydrogen Phosphate (KaH_2PO_4)	Merck KGaA, Darmstadt
Kalium Hydroxide (KOH)	Merck KGaA, Darmstadt
L-Glutamine 200mM (100 x)	Gibco BRL Life Technologies, Eggenstein
Magnesium Chloride ($MgCl_2$)	Merck KGaA, Darmstadt
Magnesium Sulfate ($MgSO_4$)	Merck KGaA, Darmstadt
Methanol (MeOH), absolut	Merck KGaA, Darmstadt
Methotrexate (MTX)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Oligofectamine	Invitrogen, Karlsruhe
OptiMEM	Invitrogen, Karlsruhe
Orange G	Sigma-Aldrich Chemie GmbH, Deisenhofen
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Peniciline/Streptomycin (100x)	Invitrogen, Karlsruhe
Phenylmethanesulfonyl Fluoride (PMSF)	ICN Biomedicals Inc., Fountain Pkwy, USA
Piperazine-N-N'-bis(2-ethanesulfonic Acide (PIPES)	ICN Biomedicals Inc., Fountain Pkwy, USA
Polyacrylamide 30 % (PAA)	Carl Roth GmbH&CoKG, Karlsruhe
Powdered Milk, blotting grade	Carl Roth GmbH&CoKG, Karlsruhe
Prestained Protein Ladder Plus	Fermentas, St. Leon-Rot
Puromycin (PM)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Random Hexamer Primer (0.2 $\mu g/\mu l$)	Fermentas, St. Leon-Rot
RNAsin Rnase-inhibitor	Promega, Mannheim
Roscovitine	Sigma-Aldrich Chemie GmbH, Deisenhofen
Roswell Park Memorial Institute Medium (RPMI) 1640	Invitrogen, Karlsruhe
Select Peptone	Merck, Darmstadt
Sodium Acetate (NaAc)	Merck KGaA, Darmstadt
Sodium Azide (NaN_3)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Sodium Chloride (NaCl)	Merck KGaA, Darmstadt
Sodium Citrate	Merck KGaA, Darmstadt
Sodium Dodecyl Sulfate (SDS)	Carl Roth, Karlsruhe
Sodium Hydrogen Phosphate (NaH_3PO_4)	Merck KGaA, Darmstadt
Sodium Hydroxide (NaOH)	Merck KGaA, Darmstadt
Sorbitol	Merck KGaA, Darmstadt
Sucrose	ICN, Northeim
Superscript Reverse Transcriptase II	Invitrogen, Karlsruhe
T4-Polynucleotide Kinase (PNK)	New England Biolabs, Ipswich
Tetramethylethylenediamine (TEMED)	Carl Roth GmbH&CoKG, Karlsruhe
Tris(hydroxymethyl)aminoethane (TRIS)	Carl Roth GmbH&CoKG, Karlsruhe
Triton-X-100	Sigma-Aldrich Chemie GmbH, Deisenhofen
Tryphan Blue	Invitrogen, Karlsruhe
Trypsin-EDTA (0.5 %)	Gibco BRL Life Technologies, Eggenstein
Tween-20	Carl Roth GmbH&CoKG, Karlsruhe
Uridine (Uri)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Xylene Cyanole	Sigma-Aldrich Chemie GmbH, Deisenhofen
Yeast Extract	Merck, Darmstadt
Zymolyase 20T	MP Biomedicals Inc., Eschwege

4.2 Consumables and kits

Amersham hyperfilm ECL	GE Healthcare, München
Amersham hyperfilm MP	GE Healthcare, München
Cell Scraper	Sarsted, Newton
Cover Slides	Menzel, Braunschweig

Cyrovials 1.5 ml	Nunc GmbH, Wiesbaden
ECL kit	GE Healthcare, München
Gel Blotting Paper GB003	Schleicher & Schuell, Deutschland
Hybond N+ Nylon Membrane	GE Healthcare, München
Hybond-ECL Nitrocellulose Membrane	GE Healthcare, München
Laboratory Glassware	Duran Productions GmbH & Co. KG, Mainz
Light Cycler Capillaries	Roche Chemicals, Mannheim
Light Cycler FastStart DNA Master SYBR Green I	Roche Chemicals, Mannheim
Micro Slides	Karl Hecht KG, Sondheim
MicroSpin S-200HR Columns	GE-Healthcare, München
μMACS Streptavidin kit	Miltenyi, Bergisch-Gladbach
Nail Polish	Manhatten Base Coat
Nitrile Gloves	Kimberly-Clark, Koblenz
Pasteur Pipettes	Hirschmann Laborgeräte, Eberstadt
Pipette Tips ART 10, 20, 200, 1000)	MolecularBio-Products, San Diego
Plastic ware for cell culture	Greiner Bio-One,Frickenhausen
Phase Trap Gel Heavy Tubes	PeqLab Biotechnologie GmbH, Erlangen
PVDC Saran Foil	Dow, Midland
Reaction Tubes 1.5 ml, 2 ml	Eppendorf, Hamburg
Reaction Tubes 15 ml, 50 ml	Becton Dickinson Biosiences, Heidelberg
Scalpel	Braun, Tuttlingen
Total RNA kit PeqGOLD	PeqLab Biotechnologie GmbH, Erlangen
Whatman Paper	Whatman, London

4.3 Equipment

Axiovert 200M Fluorescence Microscope	Carl Zeiss Jena GmbH, Göttingen
BAS Imaging Plate	Fujifilm, Düsseldorf
Biofuge 13	Heraeus Sepatech GmbH, Osterode
BioPhotometer	Eppendorf-Netheler-Hinz GmbH, Hamburg
Blotting chamber	Bio-Rad Laboratories GmbH, München
Branson Sonifier 250	Heinemann Ultraschall- und Labortechnik
Cawomat 2000 IR developer	CAWO GmbH, Schrobenhausen
Combi-printer PIXMA MP510	Canon GmbH, Krefeld
Electrophoresis equipment	Bio-Rad Laboratories GmbH, München
Eppendorf Centrifuge 5417R	Eppendorf, Hamburg
Eraser	Raytest GmbH, Straubenhardt
FLA-5100 PhosphorImager	Fujifilm, Düsseldorf
Fridge KU 171	Liebherr, Biberach
Freezer	Siemens, München
Fuchs-Rosentahl chamber	GLW Gesellschaft für Laborbedarf GmbH
Gel dryer 543	Bio-Rad Laboratories GmbH, München
Gilson Pipettes 2,10,20,200,1000	Gilson, Bad Camberg
GS Gene Linker UV Chamber	Bio-Rad Laboratories GmbH, München
Hybaid hybridising machine	Binder, Tuttlingen
Hypercassette	GE Healthcare, München
Inkubator Heraeus 6000	Heraeus Sepatech GmbbH, Osterode
Kyowaglass	Kurary Co., Japan
Label counter, scintillation detector, QC-4000	Bioscan, Washington, DC
Laminar Flow Hood	BDK GmbH, Sonnenbühl
LB122 Berthold (β-, γ-detection)	Berthold Technologies, Bad Wildbad
Light cycler 2.0	Rcohe Diagnostics, Penzberg
Magnet stirer M23	GLW, Würzburg
Magnetic Stand OctoMACS Seperator	Miltenyi, Bergisch-Gladbach
Megafuge 2.0	Heraeus Sepatech GmbH, Osterode
Microwave	Panasonic, Hamburg
Multi-calimatic pH-meter	Knick GmbH & Co. KG, Berlin
Multichannel pipette 50M	PeqLab Biotechnologie GmbH, Erlangen
Nanodrop 1000	Thermo Scientific, Braunschweig
PipetMan P	Gilson, Bad Camberg
PowerPack	Bio-Rad Laboratories GmbH, München
Rollermixer SRT 2	Dunn GmbH, Augsburg
SDS-PAGE gel tank	Amersham Pharmacia Biotech, Freiburg

Telaval 31 microscope	Carl Zeiss Jena GmbH, Göttingen
Thermal printer P91	Mitsubishi, Japan
Thermomixer 5436	Eppendorf-Netheler-Hinz GmbH, Hamburg
UV lamp VL-4. LC	PeqLab Biotechnologie GmbH, Erlangen
Vacuum pump	Vacuumbrand GmBH & CoKG, Tuttlingen
Vortex Genie II	GLW, Würzburg
Wet chamber	self-made
xCELLigence E-Plate 16	Roche Diagnostics, Penzberg
xCELLigence Real-Time Cell Analyzer (RTCA) DP	Roche Diagnostics, Penzberg

4.4 Software

AIDA Version 3.20.116	Raytest GmbH, Straubenhardt
Canon MP Navigator 3.0.0	Canon GmbH, Krefeld
ImageJ 1.34s	Wayne Rasband, Maryland
Microsoft Office 2005, 2008, 2011	Microsoft, Washington, DC
Openlab demo 3.0.8	Improvision, Coventry

4.5 Buffers and solutions

1 % Agarose-Formaldehyde-Gel for RNA	4 g Agarose 300 ml ddH_2O boil in microwave cool to 65°C 40 ml MOPS-Buffer (10 x) 67 ml Formaldehyde (37 %)
2 % Agarose-TAE-Gel for DNA	4 g Agarose 400 ml 1x TAE boil in microwave cool to 65°C add EtBr (375 ng/µl)
Antibody Stock Solution	5 % BSA in TBST add NaN_3
10 x Biotinylation Buffer	100 mM Tris pH 7.4 10 mM EDTA
Church Buffer (1 L)	400 ml 1 M Na_2HPO_4 100 ml 1 M NaH_2PO_4 adjust pH to 7.1 350 ml 20 % SDS 2 ml 0.5 M EDTA ad 1 L H2O
DEPC-H_2O	0.1 % DEPC in H_2O incubate at 37°C over night autoclave
10 x DNA Loading Dye	20 g Sucrose 100 mg Orange G ad 50 ml H_2O
DAPI Stain Solution	0.025 % (m/v) DAPI in 1 x PBS
FBS Antibody Solution	0.15 % FBS in 1 x PBS
FBS Blocking Solution	10 % FBS in 1 x PBS

2 x Lämmli Buffer	2 % SDS 100 mM DTT 10 mM EDTA 10 % Glycerol 60 mM Tris/HCl pH 6.8 0.01 % BPB 1 mM PMSF
Milk powder solution	5 % powdered milk in 1 x TBST
1 x µMACS Washing Buffer	100 mM Tris pH 7.5 10 mM EDTA 1 M NaCl 0.1 % Tween-20
10 x MOPS-Buffer pH 7.0	20 mM MOPS 50 mM NaAc (H_2O-free) 1 mM EDTA
1 x MOPS Running Buffer	300 ml 10 x MOPS Buffer ad 3 L H_2O
1 x PBS (pH 7.2-7.4)	137 mM NaCl 2.7 mM KCl 4.3 mM Na_2HPO_4*6H_2O 1.4 mM KH_2PO_4
0.04 % Triton-X-100 (PBST)	0.04 % (v/v) Trition-X-100 in 1 x PBS
2 x RNA Loading Dye	5 ml Formamide 1.5 ml Formaldehyde 1 ml 10 x MOPS Buffer 1 ml BPB (1 %) 10 µl EtBr (10 mg/ml) 1.5 ml DEPC-H_2O
S-Buffer	1 M Sorbitol 10 mM PIPES adjust pH to 6.5
10 x SDS Running Buffer	30,24 g Tris Base 144 g Glycine 50 ml 20 % SDS ad 1 L H_2O
SDS-PAGE Stacking Gel (4 %)	2 ml 30 % PAA 7.5 ml 2 x Tris/SDS pH 6.8 5.4 ml H_2O 90 µl APS 20 µl TEMED
SDS-PAGE Separating Gel (10.5 %)	7 ml 30 % PAA 10 ml 2 x Tris/SDS pH 8.8 0.5 ml H_2O 167 µl APS 17 µl TEMED
20 x SSC Buffer	3 M NaCl 0.3 M Sodium Citrate pH 7.0
0.1 x SSC Wash Buffer (500 ml)	2.5 ml 20 x SSC (0.1 x) 12.5 ml 20 % SDS (0.5 %) ad 500 ml H_2O
2 x SSC Wash Buffer (500 ml)	50 ml 20 x SSC (2 x)

	25 ml 20 % SDS (1 %) ad 500 ml H_2O
1 x TAE Buffer	40 mM Tris Acetate 1 mM EDTA adjust pH to 8.0
10 x TBS Buffer	100 mM NaCl 10 mM Tris/HCl pH 7.5
10 x TBS-Tween (TBST)	1 % Tween-20 in TBS
10 x Transfer Buffer	30,24 g Tris Base 144 g Glycine 50 ml 20 % SDS 200 ml MeOH ad 1 L H_2O
1 x Tris/EDTA (TE) pH 8.0	10 mM Tris 1 mM EDTA adjust pH to 8.0
2 x Tris/SDS pH 6.8	30.24 g Tris Base 10 ml 20 % SDS ad 1 L H_2O adjust pH to 6.8
2 x Tris/SDS pH 8.8	90,72 g Tris Base 10 ml 20 % SDS ad 1 L H_2O adjust pH to 8.8

4.6 Media

Depletion Medium	10 % FBS, dialyzed in DMEM-P
DMEM complete	89 % DMEM 9 % FBS 1 % L-Glutamine (200 mM) (100 x) 1 % Peniciline/Streptomycin (100 x)
Freeze Medium	90 % FBS 10 % DMSO
Pulse Medium	10 % FBS, dialyzed in DMEM-P 15 μCi/ml [^{32}P]-ortho-phosphate
RPMI complete	89 % RPMI 9 % FBS 1 % L-Glutamine (200 mM) (100 x) 1 % Peniciline/Streptomycin (100 x)
Thaw Medium	80 % DMEM 20 % FBS
Yeast Complete Galactose Medium (YPG)	20 g Select Peptone 10 g Yeast Extract 935 ml H_20 autoclave add 2 % Galactose
Yeast Complete Glucose Medium (YPD)	20 g Select Peptone 10 g Yeast Extract

	935 ml H_20 autoclave add 2 % Glucose
Phosphate-free Yeast Depletion Medium	20 g Select Peptone 10 g Yeast Extract 935 ml H_20 10 ml 1 M $MgSO_4$ 10 ml aqueous NH_4OH remove precipitated phosphate by filtration adjust to pH 5.8 autoclave add 2 % Glucose
Phosphate-free Yeast Pulse Medium	20 g Select Peptone 10 g Yeast Extract 935 ml H_20 10 ml 1 M $MgSO_4$ 10 ml aqueous NH_4OH remove precipitated phosphate by filtration adjust to pH 5.8 autoclave add 2 % Glucose add 15 μCi/ml [^{32}P]-ortho-phosphate

4.7 Cell lines

U2OS	Human Osteosarcoma cell line
2fTGH	Human Fibrosarcoma cell line
HeLa RPB1-α-Am (Rec-WT)	with mutated RPB1 α-Am binding site
Dicer1 +/+ MEF conditional knockout cells	MEF clone 1C5
Dicer1 +/+ MEF conditional knockout cells	MEF clone 1C1
Dicer1 -/- MEF conditional knockout cells	MEF clone 1A11
Dicer1 -/- MEF conditional knockout cells	MEF clone 2G4
	All MEF cells comprise floxed Dicer1 alleles, conditionally depleted by Cre-recombinase, and kindly provided by Dr. Vigo Heissmeyer

4.8 Yeast strains

Conditional knockout strains	
CTK1 +/+ Strain	GAL1::CTK1-TAP
CTK1 -/- Strain	Δctk1::HIS3
BUR2 +/+ Strain	GAL1::BUR2-TAP
BUR2 -/- Strain	Δbur2::HIS3
	All strains are derived from W303 wt (Mata/a; ura3-1; trp1-1; his3-11,15; leu2-3,112; ade2-1; can1-100; GAL+) kindly provided by Dr. Katja Strässer

4.9 Oligonucleotides

Name	Sequence (5′- to -3′)
siATM (Zhu et al., 2004)	UAGAGCUACAGAACGAAAGdTdT
siATR (Zhu et al., 2004)	AACCUCCGUGAUGUUGCUUGAdTdT
siAurB (this thesis)	GGAGAAUAGCAGUGGGACAdTdT

siCATS (this thesis)	CUUGACCUUGAGCCUUCUAUUdTdT
siCdk1 (L'Italien et al. 2006)	GAUCAACUCUUCAGGAUUUdTdT
siCdk2 (Zhang et al., 2004)	AAGGUGGUGGCGCUUAAGAAAdTdT
siCdk3 (this thesis)	GAGCAAAGCACUAAGGAAUdTdT
siCdk4 (Molenaar et al., 2008)	AACCCACACAAGCGAAUCUCUdTdT
siCdk5 (Meuer et al., 2007)	GAGGAUCUUUCGACUGCUAdTdT
siCdk6 (Molenaar et al., 2008)	GUGAUUGGACUCCCAGGAGAAdTdT
siCdk7 (Wohlbold et al., 2006)	GCCUACAUGUUGAUGACUCdTdT
siCdk8 (Tsutsui et al., 2008)	CUACAAAGCCAAGAGGAAAdTdT
siCdk9 (Pirngruber et al., 2009)	GCAAGGGUAGUAUAUACCUGGUGUUdTdT
siCdk9-55 (5'UTR/E1) (this thesis)	CCGUCGGUUGCCAUGUCAAdTdT
siCdk12 (Bartkowiak et al., 2010)	*Qiagen FlexiTube siRNA (SI00288442)*
siCdk13 (Bartkowiak et al., 2010)	*Qiagen FlexiTube siRNA (SI02621969)*
siCdk16 (this thesis)	UGAGAUUGGCUUUGGGAAAdTdT
siCHK1 (Shiromizu et al., 2006)	AAGCGUGCCGUAGACUGUCCAdTdT
siCK I (Chen et al., 2005)	AAUCUCAGAAGGCCAGGCAUCdTdT
siCK II (Rebholz et al., 2009)	GUCCAUGAAAUAUUCCACCdTdT
siDNA-PK (this thesis)	CUGAAACACUAAAGAGAAAdTdT
siGSK3 (this thesis)	AGAAAGUAUUGCAGGACAAdTdT
siJNK3 (this thesis)	GCAAGAAACUGUUGAAUUAdTdT
siLuc (GL2) (Eurofins/MWG)	CGUACGCGGAAUACUUCGAdTdT
siNLK (this thesis)	GAGUGAUGGUGGAAGAUAAdTdT
siNEK6 (this thesis)	CCACGGAAGUCGAGAAUUAdTdT
siNop56 (Knox et al., 2011)	CAAUAUGAUCAUCCAGUCCAUdTdT
siPes1 (Rohrmoser et al., 2007)	AGGUCUUCCUGUCCAUCAAdTdT
siPNK (Rasouli-Nia et al., 2004)	AGAGAUGACGGACUCCUCUdTdT
siPRK1 (Metzger et al., 2008)	GAAAGUCCUAGAUCCACACGCAAUdTdT
sihRIO2 (Zemp et al., 2009)	GGAUCUUGGAUAUGUUUAAdTdT
siSNK (Burns et al., 2003)	AAGCGCUACUGCCGGGGCAAAdTdT
siSRPK1 (this thesis)	GGACAAAGCCCAAAGGAAAdTdT
siTTBK1 (this thesis)	GGUGAGAGGCAGUGGGCAAdTdT
siVRK1 (Vega et al., 2004)	GAAAGAGAGUCCAGAAGUAdTdT

4.10 Probes

Name		Sequence (5'- to -3')
mRNA (Homo sapiens)		
GAPDH		CCAGCAGTGAGGGTCTCTCTCTTCCTCTTG
c-Myc		GGAGGCTGCTGGTTTTCCACTACCCGAAAAAAATCCA
rRNA (*Homo sapiens*)		
5'ETS	(1)	CGGAGGCCCAACCTCTCCGACGACAGGTCGCCAGAGGACAGCGTGTCAGC
5'ETS	(2)	CGGTACCCCCAAGGCACGCCTCTCAGATCGCTAGAGAAGGCTTTTCTC
ITS-1	(3)	AGCGCGGACACCACCCCACAGGCGCCCGGGGGTTCC
ITS-1	(4)	TCCCGACGACGCACCGGGAGGAGGCCCTTCCTGGCGCGGCACGTCCCC
ITS-2	(5)	CTCTCTTTCCCTCTCCGTCTTCCGGCGGCGGCGCCGCCCTCCCCGTCT
ITS-2	(6)	TACGCGCGGGGAGGGCGAGGAGGACGG CGGGGCCTCGGAGGA
3'ETS	(7)	AACGCGCACGCCCGCCGGGCCCCCCGCACGCAC
3'ETS	(8)	CTCCCAAACCACGCTCCCCGGACCCCGTCCCGGCCCGGAG
3'ETS	(9)	ACG GGG AGG AGG CGG GAA CCGAAGAAGCGGGGCGGCCGACCGGGGTC
3'ETS	(10)	TCGACCCGTGCGGAGGAGCGAGGAGGAAGGACG
3'ETS	(11)	GCTAAGTCCGGAGCTCGCGGGCGGCAGCTGGTC
3'ETS	(12)	GAGAGGGAGTTCCGCGTGGTCCCAGCTCCACCGCG

3′ETS	(13)	CGCGGACGCAAACTCGCGGTGGGGCTGAA
3′ETS	(14)	GCGAGAGGGCGAGAGCGACAGAGAGAGAGAG

rRNA (*Mus musculus*)

5.8S	(A)	CTGCAATTCACATTAATTCTCGCAGCTAGCTGCGTTCTTCATCGA
28S	(B)	AGGTCGCGGTCAGAAAGGGGGGACACGCGCCCAGCCGCGGGAAGA
3′ETS	(C)	ACCGCCCCCGTGACGCCCGGGCGACGACACGGTCGCGTGCGAGCG
3′ETS	(D)	ACGGACGCGCCGAGGATGGGGATCCCACCGTCGGTCACCGGCCCG

snoRNA (*Homo sapiens*)

SNORA8 (ACA8)	CCTAGCAGGTGTAAACTGCTGAGTGCAGATACCATG
SNORA21 (ACA21)	CGATGTTTTCACTCTCCCCTTTCTTGACGGCTCAATAG
SNORA38B (ACA38B)	AAGCTGGCCCCAGATTTCTCCAAGTCCACATTTC
SNORA73A (U17A, E1)	TTCCAACGTTGTGGAAAGGGACTTGTACATCATGG
SNORD3 (U3)	CTTCACGCTCAGGAGAAAACGCTACCTCTCTTCCT
SNORD14A (U14A)	CTTTCTGGTGGAAACTGCGAATGTTGGAAAACCATCATCAC
SNORD14B (U14B)	ATTTCTGGTGGAAACTGCGAATGTCTGGCAACCAATCATCATAG
SNORD22 (U22)	ACAGGCTCTGGGACTAGGACAGAGAGTAAGACATGT
SNORD24 (U24)	GCATCAGCGATCTTGGTGGTTTAAAATGTCATCACCATCTC
SNORD76 (U76)	TCAGTTAAGATAATGGTGGTTAAGATCCTCATCATTCTAGCACTCAAGA
SNORD117 (U83)	GCCATCAGTCATGGGTGATAGATAAGAGTCGTCCT
SNORD118 (U8)	CAAGTCCTGATTACGCAGAGACGTTAATCACGTTTCATGC

4.11 Primers

Name	Sequence (5′- to -3′)	
mRNA (Homo sapiens)		
Nop56	FWD:	AATTCCACAGCATCGTTCG
	REV:	GCGGAGGTCCTCATGAAC

4.12 Antibodies

Primary Antibodies	**source**	**clone**	**species**	**usage**
Human anti-CATS	Dr. E. Kremmer	2C4	rat	WB 1:10
Human anti-Cdk2	Santa Cruz (sc-163)	M2	rabbit	WB 1:2000
Human anti-Cdk4	Santa Cruz (sc-260)	C22	rabbit	WB 1:5000
Human anti-Cdk5	Santa Cruz (sc-173)	C8	rabbit	WB 1:5000
Human anti-Cdk6	Santa Cruz (sc-177)	C21	rabbit	WB 1:2000
Human anti-Cdk7	Santa Cruz (sc-529)	C19	mouse	WB 1:10000
Human anti-Cdk8	Santa Cruz (sc-13155)	D-9	mouse	WB 1:1000
Human anti-Cdk9	Santa Cruz (sc-484)	C20	rabbit	WB/IF 1:2000
Human anti-c-Myc	Dr. E. Kremmer	9E10	mouse	WB 1:10
Human anti-NPM1 (B23)	Sigma (B0556)	FC82291	mouse	IF 1:2000
Human anti-p53	Santa Cruz (sc-126)	DO-1	mouse	WB 1:2000

Human anti-Pes1	Dr. E. Kremmer	8E9	rat	WB 1:10
Human anti-α-Tubulin	Sigma (TG199)	DM1A	mouse	WB 1:10000
Mouse anti-Dicer1	Dr. V. Heissmeyer	polyclonal	rabbit	WB 1:100

Secondary Antibodies	**source**	**species**	**usage**
Cy3-AffiniPure Goat	Jackson InnunoRes.	mouse	IF 1:300
Cy3-AffiniPure Goat	Jackson InnunoRes.	rabbit	IF 1:300
HRP-conjugated α-IgG	Promega	rabbit	WB 1:2500
HRP-conjugated α-IgG	Promega	mouse	WB 1:5000
HRP-conjugated α-IgG	Promega	rat	WB 1:5000

WB: Western blot
IF: Immunofluorescence

5. METHODS

5.1 Cell culture

5.1.1 Cell thawing

Deep frozen cell stock aliquots (-80°C) were thawed in a 37°C water bath, resuspended in pre-warmed thaw medium and centrifuged (4 min, 1200 rpm). The cell pellet was washed twice with 1 x PBS and centrifuged (4 min, 1200 rpm). The cell pellet was resuspended in thaw medium and cells were plated and cultured for 24 hours at 37°C, 8 % CO_2. Subsequently, thaw medium was replaced by DMEM complete (adherent cells) or RPMI complete (suspension cells).

5.1.2 Cell cultivation

Adherent cells were cultured at 37°C and 8 % CO_2 (suspension cells at 5 % CO_2). A running culture was split and diluted 1:10 every two to three days. Therefore, adherent cells were washed with 1 x PBS, trypsinated for 5 min, and resuspended in DMEM complete. Suspension cells were split 1:10 by discarding 90 % of the culture medium every two to three days.

5.1.3 Cell counting

Cell number was determined in a Fuchs-Rosenthal chamber. Trypsinated and resuspended adherent cells were mixed 1:1 with 0.4 % Tryphan Blue (suspension cells were directly mixed 1:1). Bright staining, living cells were counted under a microscope. The cell number in 1 ml medium is calculated by multiplication of the median number of cells from four squares with factor 10^4. This factor transfers the volume of one square (= 0.1 mm^3) into 1 ml.

5.1.4 Proliferation assay

Cell proliferation was measured indirectly by electric impedance detection using the Roche xCELLigence device. 3000 cells were plated on an electronic plate (E-plate), which is capable of measuring electric impedance in real time by electrodes in direct contact with adherent cells. Impedance increases with the area on electrodes, which is covered by proliferating cells and is termed 'cell index'. After plating, cells were cultured over night, transfected, and cultured for additional three days. In total, cells were measured for 100 hours in 15 min intervals.

5.2 Cell manipulation

5.2.1 Cell selection

Recombinant HeLa cells, harboring an α-amanitin (α-Am) resistant RNA polymerase II (RNAP II) were created as described elsewhere (Bartolomei and Corden 1987; Meininghaus et al., 2000). Pre-selected HeLa cells, which express an α-Am-resistant recombinant wild type RNAP II (Rec-WT HeLa), were enriched and protected from revertants by cultivating them in 2 μg/ml α-Am.

Recombinant Dicer1 conditional knockout mouse embryonic fibroblast (MEFs) cells were created as described elsewhere (Bernstein et al., 2003). Conditional Dicer1 double knockout was induced by addition of Cre-recombinase to MEF cells. A stable Dicer1 knockout cell line was obtained by single cell cloning. Conditional Dicer1 MEFs were a generous gift from Dr. Vigo Heissmeyer.

5.2.2 Cell transfection

Adherent cells were plated in six-well plates (90000 cells/well) and cultured for 24 hours in DMEM complete. Subsequently, cells were washed with 1 x PBS and pre-incubated with OptiMEM transfection medium. For transient transfection of 100 nM short interfering (si)RNA, both oligofectamine transfection reagent and siRNA (100 μM) were diluted in OptiMEM (3 μl Oligofectamine in 150 μl OptiMEM; 0.9 μl siRNA in 150 μl OptiMEM) and incubated separately at room temperature (RT) for 5 min. Both dilutions were united and incubated at RT for 20 min. Subsequently, 600 μl OptiMEM were added to the mixture, resulting in a 900 μl transfection sample. Pre-incubated cells were aspirated and incubated with the transfection sample for 6 hours. Cells were aspirated and cultured in DMEM complete. Transient transfection was repeated after 24 hours.

5.2.3 Drug treatment

Drugs were stored at -20°C as stock solutions dissolved in appropriate solvent (DMSO, H_2O, or EtOH). For treatment of cells, drugs stocks were freshly pre-diluted in 1 x PBS or DMSO. Pre-dilutions were added to culture medium for 6 hours or less.

5.2.4 [^{32}P]-ortho-phosphate metabolic *in vivo* labelling

200000 adherent cells were metabolically labelled with radioactive [^{32}P]-ortho-phosphate (^{32}P). Cells were stressed with 100 nM siRNA (2 x 6 hours) or drug treatment (2 hours) prior to labelling. Subsequently, DMEM was replaced by depletion medium for 1 hour to deplete endogenous phosphate pool. In case of drug treatment, depletion medium also was supplemented with the drug. Depletion medium was replaced by pulse medium containing 15 μCi/ml ^{32}P for 1 hour in absence of the drug (pulse). Pulse medium was replaced by DMEM complete for up to 3 hours in presence of the drug (chase). Culture medium was removed and cells were lyzed in 350 μl RLT lysis buffer. Lysates were shredded by loading to a DNA removal column and centrifugation (3 min, 13000 rpm). Lysates were frozen at -20°C. Medium change steps in metabolic labelling of suspension cells required pelleting (4 min, 1200 rpm) prior to medium change.

5.2.5 Cell freezing

A confluent adherent cell culture was washed with 1 x PBS, trypsinated, and resuspended in DMEM complete. A suspension culture aliquot was taken directly without trypsination. Cells were pelleted (4 min, 1200 rpm), washed in 1 x PBS, and pelleted again (4 min, 1200 rpm). Cell pellets were resuspended in freeze medium and deep frozen in Cryovails at -80°C.

5.3 Yeast culture

5.3.1 Recombinant yeast strains

Conditional yeast Ctk1 or Bur1/2 knockout strains were created as described elsewhere (Röther and Strässer, 2007). In brief, endogenous *CTK1* or *BUR2* genes were deleted in yeast wild type strain W303 (Mata/a; ura3-1; trp1-1; his3-11, 15; leu2-3, 112; ade2-1; can1-100; GAL+) and replaced by stable transformation with conditional expression plasmids coding for recombinant TAP-tagged Ctk1 or Bur2 (CTK1-TAP or BUR2-TAP). Expression of Ctk1-TAP and Bur2-TAP is under control

of a galactose inducible promoter. Conditional knockout was induced by medium switch from YPG to YPD. Yeast growth was monitored by optical density measurement at 600 nm. Conditional knockout strains were a generous gift from Dr. Katja Strässer.

5.3.2 [^{32}P]-ortho-phosphate metabolic yeast *in vivo* labelling

Yeast was cultured over night in YPD to induce knockout for 18 hours. Over night cultures were diluted to OD_{600}= 0.15 in YPD-P and cultured for additional 6 hours to deplete the endogenous phosphate pool. Phosphate-free YPD-P was obtained by alkalic phosphate precipitation with 10 mM $MgSO_4$ and 1 % aqueous NH_4OH (Rubin, 1974). Yeast optical density was kept below OD_{600}= 1.5. Exponentially growing yeast was metabolically labelled with 15 µCi/ml ^{32}P for 5 min (pulse). Subsequently, labelled yeast was harvested (30 min, 3000 rpm) and washed in 400 µl TE. Yeast was again harvested (30 sec, 3000 rpm) and resuspended in 200 µl S-buffer, containing 1 M Sorbitol. For spheroblasting, 250 µl S-buffer supplemented with 20 mg/ml Zymolyase 20T and 2 µl β-ME/ml was added to the yeast suspension. Yeast cell wall was digested at 30° for 3 min (spheroblasting). Spheroblasts were harvested (30 sec, 3000 rpm), and resuspended in S-Buffer. Yeast spheroblasts were centrifuged (1 min, 300 rpm), S-Buffer was completely removed, and the spheroblast pellet was resuspended in 350 µl RLT lysis buffer. Spheroblasts were vortexed in RLT lysis buffer for 5 min. Lysates were frozen at -20°C.

5.4 DNA analytics

5.4.1 Complementary DNA (cDNA) synthesis

Human U2OS cDNA was synthesized as a reverse transcribed, random primed library derived from 2 µg total RNA, which was purified with the PeqGOLD total RNA kit (see RNA analytics part for details).

20 µl reverse transcription reaction (one sample):

Random Hexamer Primer (0.2 µg/µl)		1 µl
Total RNA (2 µg)		x µl
dNTPs (10 mM each)		1 µl
ddH_2O	ad	12 µl

mix, incubate at 65°C, 5 min, add:

5 x First-Strand-Buffer	4 µl
0.1 M β-Mercaptoethanol	2 µl

RNAsin RNAse-inhibitor	1 µl

mix, incubate at RT, 2 min, add

Superscript Reverse Transcriptase (200 units)	1 µl

mix, incubate, at RT, 10 min
incubate at 42°C, 50 min (reverse transcription)
incubate at 70°C, 15 min (inactivation)
freeze at -20°C

5.4.2 Quantitative real-time polymerase chain reaction (qPCR)

Specific primers for qPCR of random primed human cDNA were designed with the Roche universal probe library. The cDNA levels were measured with the Roche Light Cycler FastStart DNA Master SYBR Green I kit and calculated by comparison of Cp-values. Relative cDNA levels were calculated by the ΔΔCp-method (e.g. ΔCp = 3.98, i.e. $1/2^{3.98} = 0.063 = 6$ % cDNA compared to control situation).

10 µl reaction mixture (one sample):

H_2O (PCR grade)	4.2 µl
DMSO	0.5 µl
$MgCl_2$	0.8 µl
Roche-Mix (a+b)	1 µl
cDNA (1:20 dilution)	2.5 µl
Primer-Mix (5 µM FWD+REV)	1 µl

PCR program:

Step	Cycles	Conditions
Denaturation	1	95°C, 10 min
Amplification		95°C, 1 sec
	35	62°C, 10 sec
		72°C, 8 sec
Melting	1	70° to 97°, 20°/sec ramp rate
Cooling	1	40°, ∞

Primer specificity was monitored in cDNA dilution series experiments by measuring Cp-values of three different cDNA dilutions. The melting temperature of the qPCR product was measured. The predicted qPCR product size was confirmed by agarose-gel electrophoresis.

5.4.3 DNA agarose-gel electrophoresis

For a 1.5 % DNA agarose-gel, 6 g agarose was boiled in 400 ml 1 x TAE buffer. 3.75 µl/100 ml Ethidium Bromide (EtBr, stock: 10 mg/µl) was added after cooling of the gel to 65°C. PCR products were mixed with 10 x DNA loading dye and loaded on the

gel. The gel was run with a 1 kilobase DNA ladder in 1 x TAE buffer (100 V, 3 hours). A gel photo was taken under UV light.

5.5 RNA analytics

5.5.1 RNA isolation and concentration detection

The PeqGOLD total RNA kit was used for total RNA isolation from human cells and yeast. Deep frozen lysates were thawed and RLT lysis buffer was mixed with an equal amount (350 µl) of 70 % EtOH. Lysates were loaded on an RNA binding column and centrifuged (30 sec, 13000 rpm). The column was washed once with 600 µl wash buffer I and twice with 500 µl wash buffer II (30 sec, 13000 rpm). The column was dried by empty centrifugation at 14000 rpm for 3 min. RNA was eluted in 35 µl DEPC-H_2O. RNA concentration was measured with the Nanodrop System.

5.5.2 RNA agarose-gel electrophoresis

A 1.25 % formaldehyde agarose-gel was used to analyze total RNA. 4.5 g agarose was boiled in 300 ml DEPC-H_2O. After cooling to 65°C, 40 ml 10 x MOPS buffer and 67 ml 37 % Formaldehyde were added. Equal amounts of total RNA samples (1 µg for detection of metabolic label, 8 µg for Northern blot experiments) were prepared by mixing RNA with a 2 x RNA loading dye (50 % v/v) and DEPC-H_2O to adjust the volume. RNA samples were incubated at 55°C for 12 min prior to loading. The gel was run in 1 x MOPS buffer at 100 V for 3-6 hours. A gel photo was taken under UV light.

5.5.3 Autoradiography

Metabolically labelled RNA was analyzed by autoradiography after separation by RNA agarose-gel electrophoresis. The gel was put in a gel dryer on top of a Whatman paper and covered with a PVDC foil. A vacuum pump was used to dry the gel for 5 hours at 80°C. The dried gel was put into a detection cassette and covered with an autoradiography detection film, which was exposed at -80°C for 4 hours. A developing machine was used to visualize signals. Dried gels were exposed to a pre-erased BAS Imaging Plate for 5 min for quantitation of metabolically labelled RNA. rRNA signals were read out with a PhosphorImager and quantified by AIDA software.

5.5.4 4sU-tagging

1500000 cells were cultured with 10 µM 4-thiouridine (4sU) for various time points. Cells were lyzed and total RNA was isolated as described above. 50 µg total RNA was incubated with 1 mg/ml Biotin-HPDP (2 µl/µg RNA) in 10 x biotinylation buffer (1 µl/µg RNA) for 1.5 hours with rotation at room temperature in a final volume of 500 µl. Biotin-HPDP is pyridyldithiol-activated and allows thiol-specific biotinylation of 4sU-tagged RNA by the formation of disulfide bonds with the -SH group of 4sU. An equal volume of chloroform was added, mixed, and incubated with biotinylated RNA for 3 min. The mixture was separated by centrifugation (5 min, 16000 rpm) in pre-spun Phase Trap Gel Heavy Tubes. For RNA precipitation and removal of unincorporated Biotin-HPDP, an 1/10 volume 5 M NaCl and an equal volume of absolute isopropanol were added to the aqueous phase and centrifuged (20 min, 16000 rpm). The pellet was washed in an equal volume of 75 % ethanol and centrifuged (10 min, 16000 rpm). RNA was resuspended in 100 µl RNAse-free H_2O. For separation of 4sU-tagged and untagged RNA, RNA was first heated to 65°C for 10 min and cooled on ice for 5 min. Subsequently, RNA was incubated with 75 µl Streptavidin-coated magnetic beads (Miltenyi) for 15 min with rotation. The reaction volume was applied to µMACs columns (Miltenyi), which were placed in an OctoMACS Seperator magnetic stand, and equilibrated with 900 µl 1 x µMACS washing buffer. The columns were washed three times with 800 µl 65°C warm 1 x µMACS washing buffer and two times with 800 µl cold 1 x µMACS washing buffer. Nascent 4sU-Biotin-Streptavidin-tagged RNA was eluted in 700 µl RLT lysis buffer with twice 100 µl DDT (100 mM) by reducing 4sU-S-S-Biotin disulfide bonds. Nascent, 4sU-tagged RNA was recovered with the PeqGOLD total RNA kit as described above. Untagged, unbound RNA was recovered from the flow through and first wash of µMACS columns by isopropanol/ethanol precipitation as described above. RNA fractions were analyzed by RNA agarose-gel electrophoresis.

5.5.5 Northern blot hybridization

Unlabelled RNA was analyzed by Northern blot hybridization after separation by RNA agarose-gel electrophoresis. RNA was transferred to a Hybond N+ nylon membrane by vertical transfer over night in 10 x SSC buffer and cross-linked to the membrane with UV light (120 mJoule, 30 sec). Specific probes were used for detection of unlabelled RNA. Probes were end-labelled with γ-^{32}P-Desoxyadenosinetriphosphate (γ-^{32}P-dATP) and T4 Polynucleotide Kinase (PNK) at 37°C for 1 hour.

20 µl reaction mixture (one sample):	
DEPC-H_2O	2 µl
DNA-probe (1 µM)	1 µl
PNK buffer (10 x)	1 µl
PNK (10000 units)	1 µl
γ-^{32}P-dATP (6000 µCi/mmol)	5 µl

End-labelled probes were mixed with 40 µl 1 x TE buffer, purified by Micro Spin columns (2 min, 3500 rpm), and denaturated at 90°C for 2 min. For hybridization, purified probes were added to hybridization tubes containing RNA cross-linked on membranes, which were pre-incubated with 15 ml 65°C warm Church buffer for 1 hour. The probes were hybridized to membranes at 65°C over night. Membranes were washed 10 min each with 20 ml 2 x SSC wash buffer and 0.5 x SSC wash buffer. Membranes were put into a detection cassette, covered with PVDC foil, and signals were detected and quantified as described in part *Autoradiography*.

5.6 Protein analytics

5.6.1 Immunofluorescence microscopy

80000 cells were plated on sterile glass slides. Cells were washed with 1 x PBS and fixed with 37°C pre-warmed 2 % para-formaldehyde (PFA) for 2 min. PFA was removed and inactivated by washing three times with 1 x PBS. Fixed cells were permeabilized with 0.04 % PBST for 7 min and incubated with FBS blocking solution for 2 hours. Cells were incubated with primary antibodies, diluted in FBS antibody solution, specifically recognizing endogenous proteins, in a wet chamber at 4°C over night. Primary antibodies were washed three times with 0.04 % PBST for 3 min. Cell were incubated with fluorochrome-conjugated secondary antibodies, diluted in FBS antibody solution, specifically recognizing primary antibodies, for 2 hours at RT. Afterwards, secondary antibodies were washed three times with 0.04 % PBST for 3 min. Cells were incubated with DAPI staining solution for 2 min and washed twice with 1 x PBS. Cells were then mounted in Vecta Shield medium and protected from drying with nail polish. A Zeiss Axiovert 200M IF microscope (63 x Ph3) was used for analysis. Pictures were taken at constant acquisition times with a CCD camera and enhanced for contrast and staining by Openlab Software. See also part *Antibodies* for details.

5.6.2 Western blot hybridization

200000 cells were plated for Western blot analysis. Cells were washed in 1 x PBS and lyzed in 2 x Lämmli buffer. Lysates were transferred to 1.5 ml reaction tubes and boiled at 95°C for 3 min. Lysates were sonicated (50 % duty cycle, output level: 5, shots: 4). 10 µl lysate samples were loaded and separated on a denaturating SDS-polyacrylamide gel with a 10.5 % separating gel and a 4 % stacking gel in 1 x SDS running buffer (SDS-PAGE, 30 mA for 1.5 hours). 3.5 µl of a pre-stained protein ladder was used as a running marker. The gel was transferred to a nitrocellulose membrane by semi-dry blotting in 1 x transfer buffer (450 mA, 1.5 hours). The membrane was blocked in milk powder solution (1 hour at RT) and incubated with specific primary antibodies, diluted in antibody stock solution, on a roller mixer at 4°C over night. Primary antibodies were removed and membranes were washed three times with 1 x TBST for 5 min. Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, specifically recognizing primary antibodies. Secondary antibodies were diluted in milk powder solution and hybridized to membranes for 1.5 hours at RT. Membranes were washed three times with 1 x TBST for 5 min and briefly poured in H_2O. An enhanced chemiluminescence (ECL) kit was used for signal detection. Detection reagents were mixed (1:1) and incubated with membranes for 2 min. Membranes were put into a detection cassette, a film was exposed and signals were detected with a developing machine. Signals were quantified by AIDA and ImageJ software. See also part *Antibodies* for details.

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7. APPENDIX

A *Curriculum vitae*

Kaspar Burger
Born 3rd of October, 1983 in Bad Tölz, Germany

Education

1990 - 1994	Grundschule Wackersberg, Bad Tölz
1994 - 2003	A-levels 1.5 (Hons) Gabriel-von-Seidl Gymnasium Bad Tölz
2003 - 2008	Diploma 1.0 (Hons) Faculty for Biology Ludwig Maximilian University (LMU) of Munich, Germany

Research Experience

2007	Working Student Institute for Clinical Molecular Biology and Tumor Genetics Deptartment of Molecular Epigenetics Helmholtz Zentrum München (HMGU), Germany
2007	Semester Abroad Leeds Institute of Molecular Medicine St. James's University Hospital University of Leeds, United Kingdom
2008 - 2012	Doctoral Thesis Institute for Clinical Molecular Biology and Tumor Genetics Deptartment of Molecular Epigenetics Helmholtz Zentrum München (HMGU), Germany "Cdk9 links RNA polymerase II transcription to processing of ribosomal RNA" Prof. Dr. Dirk Eick

Scholarships and Awards

2003	Book Award Gabriel-von-Seidl Gymnasium Bad Tölz
2009 - 2011	E-fellows Online Scholarship E-fellows.net Online Career Network, Germany
2009 - 2012	Research Fellowship German José Carreras Leukemia Foundation e.V. Project: DJCLS F 09/03

B Publications

[*]**Burger, K.**, [*]Mühl, B., Harasim, T., Rohrmoser, M., Malamoussi, A., Orban, M., Kellner, M., Gruber-Eber, A., Kremmer, E., Hölzel, M., Eick, D. (2010) Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J Biol Chem* 285, 12416-12425. (*shared first authors)

Hölzel, M., **Burger, K.**, Mühl, B., Orban, M., Kellner, M., Eick, D. (2010) The tumor suppressor p53 connects ribosome biogenesis to cell cycle control: a double-edged sword. *OncoTarget* 1, 43-47.

Windhager, L., Bonfert, T., **Burger, K.**, Ruzsics, Z., Krebs, S., Kaufmann, S., Malterer, G., Schilhabel, M., Schreiber, S., Rosenstiel, P., Zimmer, R., Koszinowski, U.H., Eick, D., Friedel, C.C., Dölken, L. (2012) Ultra short and progressive 4sU-tagging reveals key characteristics of RNA processing at nucleotide resolution. *Genome Res.* 10 2031-2042.

Burger, K., Mühl, B., Rohrmoser, M., Coordes, B., Kellner, M., Gruber-Eber, A., Heissmeyer, V., Eick, D. (2013) Cdk9 links Pol II transcription to processing of ribosomal RNA. *submitted.*

Coordes, B., Brünger, K.M., **Burger, K.**, Soufi, B., Horenk, J., Eick, D., Olsen, J.V., Sträßer, K. (2013) Ctk1 Function affects Translation Initiation in the Yeast *S. cerevisiae. submitted.*

Mühl, B., Malamoussi, A., **Burger, K.**, Rohrmoser, M., Keller, M., Harasim T., Orban, M., Gruber-Eber, A., Eick, D. (2013) Ppan is required for cell proliferation and ribosome biogenesis. *submitted.*

C Oral presentations and Workshops

2008	Talk: SFB 684 Symposium (Harvard-Munich Meeting) Garmisch-Partenkirchen, Germany "Ribosome biogenesis and Cytostatic Drugs. Effects of chemotherapeutic stress on nucleolar structure and function."
2009	Workshop: co-organization of the SFB 684 PhD retreat Invitation of Guest Speaker Prof. Dr. Robert Slany, University of Erlangen, Germany
2009 - 2012	Member of the Doktorandeninitiative (DINI) Munich Graduate Student Representative (Deputy)
2013	Seminar: Sir William Dunn School of Pathology, University of Oxford, UK "Cdk9 links RNA polymerase II transcription to processing of ribosomal RNA."

D Poster presentations

2007	RNA-Polymerase-I transcription Meeting, Regensburg, Germany
2009	SFB 684 Symposium (Molecular mechanisms of normal and malignant haematopoiesis), Munich, Germany
2009	8th International EMBO Conference on Ribosome Synthesis, Regensburg, Germany
2010	EMBO Workshop RNA Quality Control, Vienna, Austria
2011	RNA 2011, 16th Annual Meeting of the RNA Society, Kyoto, Japan
2012	9th International Conference on Ribosome Synthesis, Banff, Canada

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