Functional ribosome biogenesis is a prerequisite for p53 destabilization: impact of chemotherapy on nucleolar functions and RNA metabolism

Abstract: The production and processing of ribosomal RNA is a complex and well-coordinated nucleolar process for ribosome biogenesis. Progress in understanding nucleolar structure and function has led to the unexpected discovery of the nucleolus as a highly sensitive sensor of cellular stress and an important regulator of the tumor suppressor p53. Inhibition of ribosomal RNA metabolism has been shown to activate a signaling pathway for p53 induction. This review elucidates the potential of classical and recently developed chemotherapeutic drugs to stabilize p53 by inhibiting nucleolar functions.

Keywords: chemotherapy; kinase inhibitor; ribosome biogenesis; rRNA processing, tumor suppressor p53.

Ribosome biogenesis takes place in the nucleolus

Ribosomes in eukaryotes are synthesized and assembled in a specialized nuclear organelle, the nucleolus (Brown and Gurdon, 1964; Miller and Beatty, 1969). In human cells, nucleoli form around nucleolar organizer regions (NORs) on acrocentric chromosomes 13, 14, 15, 21, and 22 as membrane-free sub-cellular structures. In total, NORs contain about 400 tandemly repeated ribosomal (r)DNA genes coding for ribosomal (r)RNA (Perry, 1962; Ritossa and Spiegelman, 1965). Ribosome biogenesis emerged as one of the most complex and controlled cellular processes that consumes up to 80% of the cellular energy in proliferating cells (Thomas, 2000; Prieto and McStay, 2008). Mammalian nucleoli display a tripartite structure (Figure 1) consisting of the fibrillar centre (FC), the dense fibrillar component (DFC), and the granular component (GC). Distinct steps in ribosome biogenesis can be allocated to one of the three components of the tripartite structure. Transcription of the primary 47S rRNA by RNA polymerase (RNAP) I takes place at the interface of FC and DFC and requires several 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). These factors regulate start site selection, recruitment of RNAPI to and escape from the promoter (Schneider, 2012). The first processing steps of the 47S rRNA precursor occur in the pre-90S ribosome and involve a cleavage cascade and stepwise production of intermediate forms of rRNAs, which finally mature to 18S, 5.8S, and 28S rRNA (Hadjiolova et al., 1993) (Figure 2). Processing of rRNA is orchestrated by the interplay of >200 factors, which catalyse snoRNA-mediated rRNA modification, and cleavage and trimming of rRNA by endo- and exonucleases, respectively. Finally, 40S and 60S ribosomal subunits are exported to the cytoplasm to translate messenger (m)RNA. The ribosome maturation process is described in more detail in Figure 2. Recent findings suggest that ribosomes are more than just machines that translate mRNA. Protein composition of ribosomes and/or mutations in ribosomal proteins can alter gene regulation and tissue development. For example, a mutation in ribosomal protein Rpl38 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 (Xue and Barna, 2012).

Ribosome biogenesis in cancer cells

Ribosome biogenesis is intimately connected to cell growth and proliferation and up- or down-regulated
Ki Burger and D. Eick: Functional ribosome biogenesis is a prerequisite for p53 destabilization dependent on growth factor stimulation or nutrient availability. Changes in ribosome biogenesis rates also probably have major implications for tumor development (Ruggero and Pandolfi, 2003; White, 2005). Activation of proto-oncogenes and inactivation of tumor suppressor genes strongly up-regulate transcription and processing rates for rRNAs (Drygin et al., 2010; Hannan et al., 2011).

For example, the proto-oncogene c-Myc, the casein kinase II (CK II), the extracellular signal-regulated kinase (ERK), or the PI3K (phosphatidylinositol-3-kinase)-AKT-mTOR (mammalian target of rapamycin) axis promote transcription and/or processing of rRNA and are frequently activated in cancer cells (Boon et al., 2001; Schuhmacher et al., 2001; Schlosser et al., 2003). However, it is still unclear, whether up-regulation of ribosome biogenesis in cancer cells reflects the cause of tumorigenesis or the consequence of elevated growth and proliferation. Recent findings support the first theory.

Mutations in genes encoding ribosomal proteins have been described for various hematologic disorders with cancer predisposition (Narla and Ebert, 2010; van Riggelen et al., 2010). Ribosomal genes RPL5 and RPL11 are frequently mutated in T-cell lymphoblastic leukemia (De Keersmaecker et al., 2013). RNAPI transcription factors UBF and TIF-1A are already increased in pre-malignant cells, suggesting that up-regulation of ribosome biogenesis may drive tumor formation and is not simply a consequence of tumor progression (Ruggero, 2012).

The nucleolus as sensor and integrator of cellular stress

Damage of DNA and interference with gene expression are key characteristics of mutagens such as viruses, irradiation, cytotoxic drugs, and other environmental hazards like heat shock or hypoxia. How can cells integrate such a variety of different stresses? Stabilization of the tumor suppressor p53 and its ability to induce cell cycle arrest, senescence, or apoptosis has been known for a long time to be central for the cellular stress response (Levine, 1997; Vousden and Prives, 2009). However, a comprehensive model of how to integrate various stresses and funnel them to p53 has been lacking. A hallmark study defined the nucleolus as universal stress integrator (Rubbi and Milner, 2003). Using micropore UV-irradiation, Rubbi and Milner could show that damaging of nucleolar, but not nucleoplasmatic DNA is necessary and sufficient to induce p53. Interestingly, cells can tolerate high amounts of nucleoplasmatic irradiation and DNA damage as long as lesions are excluded from rDNA. Further experiments showed that inhibition of the RNAPI transcription machinery by anti-UBF antibodies triggered p53 stabilization in the absence of DNA damage. The knockout of RNAPI transcription factor TIF-1A results in retarded development of embryos and increased rates of apoptosis (Yuan et al., 2005). TIF-1A knockout destroys the integrity of nucleoli, stabilizes p53 and induces apoptosis. Similar results were obtained after depletion of rRNA processing factors Utp18, Bop1, Pes1, WDR12 or Nucleostemin (Pestov et al., 2001; Holzel et al., 2005; Romanova et al., 2009; Holzel et al., 2010b). In agreement with these observations, p53 levels show cell cycle-specific changes. The levels of p53 are low during interphase, but enhanced in mitosis and early G1-phase as long as the nucleoli are disassembled (David-Pfeuty, 1999). Taken together, several lines of evidence indicate that the integrity of the nucleolus is a prerequisite for low levels of p53. The high liability of the nucleolus to stress elevates p53 levels.

But how does the nucleolus respond to stress mechanistically? The E3-ubiquitin ligase Hdm2 is the key enzyme that regulates p53 levels. Hdm2 continuously binds and ubiquitinites p53, thereby targeting p53 for proteasomal degradation (Stommel et al., 1999; Ljungman, 2000; Xirodimas et al., 2001). For its ubiquitination and degradation, p53 requires the transit through a functionally intact nucleolus...
Figure 2  Mammalian ribosome biogenesis.

(A) Ribosome biogenesis consists of rRNA synthesis, rRNA processing and ribosome maturation. RNAPI transcribes a 14 kb-long, polycistronic 47S rRNA precursor that contains 18S, 5.8S, and 28S rRNAs. The fraction of rRNA within the transcriptome can be up to 90%, implying that transcriptional active rDNA genes are heavily transcribed (Rabani et al., 2011). RNAPII transcription facilitates the synthesis of 79 ribosomal L- and S-proteins, a large cohort of small nucleolar (sno)RNAs, and >200 mRNAs encoding non-ribosomal nucleolar factors, including kinases, helicases, nucleases, and GTPases. In addition, 5S rRNA is produced by RNAPIII. The orchestrated activity of all three RNA polymerases ensures the correct transcription, processing, and modification of rRNA and its assembly into 40S and 60S ribosomal subunits that compose functional 80S ribosomes in the cytoplasm (Eichler and Craig, 1994; Boisvert et al., 2007).

(B) Mammalian rRNA processing. After synthesis the 47S primary transcript is processed in mature 18S, 5.8S, and 28S rRNAs. Endo- and exonucleases cleave and trim external (ETS) and internal transcribed spacers (ITS) of 47S rRNA. Five critical snoRNAs are required for initial cleavages of the 47S rRNA precursor at the 5' end (snoRNAs U3, U14, U17, and U22) or 3' end (snoRNA U8), respectively. In addition, distinct sites of the 47S rRNA precursor are methylated at the 2' oxygen of the ribose (Me) and a subset of uridines is converted to pseudouridines ($\psi$). In total, several hundreds of modifications are introduced in rRNA, assisted by small nucleolar ribonucleoproteins (snoRNPs). Each snoRNP contains one modifying enzyme (fibrillarin methyltransferase for Me, dyskerin pseudouridine synthase for $\psi$), one snoRNA, and additional stabilizing proteins (Kiss, 2002). Mutations in the pseudouridine synthase dyskerin impair the correct formation of $\psi$ modification patterns and reduce translational fidelity of ribosomes (Ruggero et al., 2003). Extensive rRNA modification occurs at catalytical active rRNA regions within the decoding and peptidyl transferase centers of the ribosome (Bakin et al., 1994). Specific processing factors have been characterized in mammals. For example, the DEAD box helicase Ddx51 is crucial for processing of 47S rRNA 3' sequences (Srivastava et al., 2010). Ddx51 catalyses the removal of snoRNA U8 from the unprocessed 3' end, thereby facilitating exosomal degradation of the 3'ETS sequence. The rRNA processing factors Pes1, Bop1, and WDR12 form a trimeric complex (PeBoW) and are required for 32S rRNA processing and large 60S subunit formation (Lapik et al., 2004; Holzel et al., 2005; Grimm et al., 2006; Holzel et al., 2007; Rohrmoser et al., 2007). Human (h)Rio kinase family members hRio1, hRio2, and hRio3 are required for the cytoplasmatic processing of 21S rRNA to mature 18S rRNA and 40S subunit maturation (Zemp et al., 2009; Baumas et al., 2012; Widmann et al., 2012).
Inhibition of rRNA transcription and processing triggers the translocation of various nucleolar proteins into the nucleoplasm, most prominently of the ribosomal proteins Rpl5, Rpl11, and Rpl23, which bind and inactivate Hdm2 and thereby stabilize p53 (Lohrum et al., 2003; Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004; Hernandez-Verdun et al., 2010). It has recently been shown that Rpl5 and Rpl11 mutually stabilize each other and are continuously imported into nucleoli to colocalize with Hdm2 even after nucleolar disruption, suggesting that the main source of Rpl5 and Rpl11 that binds to Hdm2 is newly synthesized rather than pre-existing (Bursac et al., 2012). Interestingly, recent work identified Pict1 as a tethering factor for ribosomal proteins and as an important regulator of p53. In unstressed cells, Pict1 binds and tethers Rpl11 and other ribosomal proteins in the nucleolus, thereby sustaining Hdm2 activity. Pict1 levels decrease upon nucleolar stress. This results in the translocation of Rpl11 into the nucleoplasm and p53 stabilization (Sasaki et al., 2011). The nucleolar protein MYBBP1A is another important regulator of p53 levels. MYBBP1A is tethered to the nucleolus through nucleolar RNA. Upon nucleolar stress, MYBBP1A translocates to the nucleoplasm and facilitates p53 acetylation and stabilization by p300 acetyl transferase (Kuroda et al., 2011). Taken together, functional ribosome biogenesis requires an intact nucleolar structure and is a prerequisite for maintenance of low levels of p53.

The p53 response comes in different flavours

p53 is regarded as the guardian of the genome, which senses DNA damage by induction of G1-arrest or apoptosis (Carson and Lois, 1995; Levine, 1997; Vousden and Prives, 2009). The induction of p53 is a major aim in chemotherapy to trigger cell cycle arrest and/or apoptosis in tumor cells. Indeed, a strong nuclear accumulation of p53 can be detected upon treatment of cells with various DNA-damaging cancer therapy drugs (Fritsche et al., 1993). For a long time, DNA was regarded as the most important functional target of many classical, genotoxic drugs. For example, alkylating or DNA-intercalating drugs trigger a damage response involving kinases such as ataxia telangiectasia mutated kinase (ATM) or checkpoint kinase 1 (CHK1), which modify p53 at various positions. Activated p53 induces cell cycle inhibitors such as p21CIP and p27kip, which negatively regulate Cdk2 activity and arrest cells in G1-phase of the cell cycle (Lane, 1992; Harper et al., 1995; Sakaguchi et al., 1998; Ljungman, 2000; Vogelstein et al., 2000).

However, more recent data suggest that different qualities of p53 response exist depending on the type of stress. Efeyan and colleagues found that oncogenic signaling, such as induction of the tumor suppressor p14ARF, contributes to the bulk of tumor protective p53 function, whereas DNA damage by classical chemotherapy is less important for p53 function (Efeyan et al., 2006). In fact, the presence of p14ARF has been found to be critical for the survival of mice treated with DNA-damaging agents, and tumors are associated more frequently with mutations in p14ARF than mutations in kinases of the DNA-damaging pathway (Bartek and Lukas, 2003; Sharpless, 2005). p14ARF inhibits Hdm2 and can induce p53 in analogy to the nucleolar stress response (Tao and Levine, 1999; Sherr and Weber, 2000). p53 serine-15 phosphorylation, a hallmark of DNA-damaging agents, is absent after inhibition of rRNA processing (Holzel et al., 2010a), and p14ARF is not required for p53 stabilization after knockdown of RNA processing factors (Holzel et al., 2005). This suggests an Hdm2-dependent, but DNA damage and p14ARF independent pathway for p53 stabilization after inhibition of rRNA processing. It has also been shown that p53 levels are crucial for its activity, while p53 modifications rather fine-tune its activity (Blattner et al., 1999). Taken together, the quality of p53 induction by nucleolar stress differs significantly from p53 induction upon classical DNA-damaging chemotherapy. For cancer therapy, the nucleolar stress-mediated p53 response may be superior, because it induces p53 without damaging DNA. But which chemotherapeutic drugs inhibit ribosome biogenesis?

Genotoxic inhibitors of ribosome biogenesis

The increased size of nucleoli is a diagnostic marker for the proliferative state of cancer cells (Derenzini et al., 1998, 2000). Early studies utilized changes in nucleolar morphology as a marker to measure the responsiveness of tumor cells to classical chemotherapeutic drugs. For example, antibiotics such as doxorubicin, daunorubicin, mitomycin C or actinomycin D cause nucleolar segregation and translocation of the nucleolar protein nucleophosmin (NPM1) to the nucleolus (Merski et al., 1976; Daskal et al., 1978; Wassermann et al., 1986; Chan et al., 1987, 1988). Upon stress relief NPM1 relocates to the nucleolus (Chan et al., 1996). Nucleolar disruption usually is a consequence of inhibition of ribosome biogenesis. Various drugs inhibit ribosome biogenesis at the level of rRNA transcription albeit by different mechanisms.
Treatment with the anti-metabolite and dihydrofolate reductase inhibitor methotrexate disrupts nucleolar integrity and inhibits RNAPI transcription (Safa and Tseng, 1984; Greenhalgh and Parish, 1990; Thiry et al., 1997). Actinomycin D intercalates in GC-rich regions of rDNA, thereby specifically inhibiting rRNA elongation at low concentrations (Fetherston et al., 1984). The alkylating agent cyclophosphamide inhibits rRNA synthesis, reduces nucleoli in number and size, and causes nucleolar segregation in patients (Matejokova and Smetana, 1975; Kacerovska et al., 1981; Likovsky et al., 1993). Similarly, mitomycin C treatment inhibits RNAPI transcription by alkylating guanosines and inducing interstrand cross-links in rDNA (Rey et al., 1993). Irinotecan/topotecan inhibits RNAPI transcription by trapping topoisomerase I to rDNA, which causes DNA strand breaks (Pondarre et al., 1997). The impact of the platin analog cis-diamminedichloroplatinum(II) (cisplatin) on ribosome biogenesis has been studied in detail (Berry et al., 1983; Leibbrandt et al., 1995). Cisplatin induces intranucleolar DNA cross-links, inhibits RNAPII transcription, and causes a redistribution of nucleolar transcription factors. At low concentrations cisplatin specifically inhibits RNAPII, but not RNAPII transcription (Treiber et al., 1994; Jordan and Carmo-Fonseca, 1998). Cisplatin also triggers nucleolar accumulation of the small nuclear (sn)RNA biogenesis factor coilin. Cisplatin interacts with coilin and UBF, thereby inhibiting RNAPII initiation (Gilder et al., 2011). Finally, a comprehensive study of the inhibitory effect of 36 cytostatic drugs on rRNA synthesis and nucleolar integrity confirmed previous results and revealed that about one-third of the drugs inhibit ribosome biogenesis either at the level of rRNA transcription or RNA processing (Burger et al., 2010). Interestingly, inhibition of tRNA transcription and early, but not late, rRNA processing is accompanied by nucleolar disruption.

**Non-genotoxic inhibitors of ribosome biogenesis**

Classical DNA-damaging chemotherapy can cause secondary tumors, especially in survivors of childhood cancer. Therefore, alternative drugs are of particular interest. If ribosome biogenesis is a critical target of chemotherapy and p53 control, drugs are desirable for inhibition of rRNA synthesis without genotoxic side effects. In this context, the antimetabolite 5-fluorouracil (5-FU), but not its analog 5-fluorocytosine, was identified as potent inhibitor of RNA metabolism in various studies. In an analogy to nucleolar stress, 5-FU cytotoxicity is p53-dependent and relies on Hdm2 activity (Berger, 1977; Greenhalgh and Parish, 1990; Ghoshal and Jacob, 1994; Bunz et al., 1999). Interestingly, 5-FU was found to form stable adducts with dyskerin pseudouridine synthase. Depletion of dyskerin reduced 5-FU cytotoxicity (Hoskins and Butler, 2008). 5-FU probably confers cytotoxicity by the reduction of pseudouridylation of tRNA, which is a requirement for correct tRNA processing. In addition, incorporation of 5-FU into small nuclear (sn)RNA U2 prevents pseudouridylation and formation of functional snRNPs for pre-mRNA splicing (Zhao and Yu, 2007). These data suggest that 5-FU treatment strongly inhibits the metabolism of RNAs rather than interfering with the DNA metabolism.

Previous work from the Hernandez-Verdun and our laboratory showed that cyclin-dependent kinases (Cdks) regulate the formation and maintenance of the nucleolus. Specific Cdk-inhibitors like roscovitine, olomoucine, 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) or flavopiridol selectively prevent the processing, but not the synthesis of 47S rRNA (Sirri et al., 2002; Schlosser et al., 2003; Burger et al., 2010) (Figure 3). Inhibition of 47S rRNA processing by DRB is accompanied by the formation of a perinucleolar necklace structure, which contains mislocated, unprocessed pre-rRNAs and rRNA processing factors, like fibrillarin that are disconnected from the rDNA transcription machinery (David-Pfeuty et al., 2001; Louvet et al., 2002; Schlosser et al., 2003; Burger et al., 2010).

**Figure 3** Cdk9-inhibitor flavopiridol blocks 47S rRNA processing. Efficient processing of the 47S rRNA primary transcript is dependent on Cdk9 activity. An autoradiograph of a [32P]-ortho-phosphate metabolic labelling experiment in the presence of 0.1% DMSO (ctrl.) or 800 nM flavopiridol (FL) for 6 h. Treatment with the Cdk9-inhibitor FL strongly reduces pre-rRNA processing and stabilizes an unprocessed 47S rRNA precursor. Similar results were obtained after treatment with Cdk9-inhibitors roscovitine, olomoucine, or 5,6-dichlorobenzimidazole 1-beta-D-ribofuranoside (DRB) (Sirri et al., 2002; Schlosser et al., 2003; Burger et al., 2010). Ethidium bromide-stained 28S rRNA is used as loading control.
et al., 2005). Similarly, the translocation of the Cajal body marker coilin into necklace structures has been observed upon treatment with \(\alpha\)-amanitin, a specific inhibitor of RNAPII transcription (Haaf and Ward, 1996). These data suggest that ongoing RNAPII transcription is crucial for the maintenance of nucleolar structure and function. Inhibition of RNAPII transcription by Cdk-inhibitors may be a promising approach for interference with ribosome biogenesis, induction of p53, and inhibition of cellular growth and proliferation. Flavopiridol is currently tested in clinical trials for therapy of relapsed chronic lymphocytic leukemia (CLL). Patients with a genetically high-risk CLL have been reported to be highly responsive to flavopiridol (Byrd et al., 2007; Lin et al., 2009; Phelps et al., 2009).

Given that all three RNA polymerases are required for ribosome biogenesis, inhibition of transcription is a feasible approach to trigger nucleolar stress. Recent work characterized two new small molecule inhibitors for RNAPI, CX-3543 and CX-5461, which were initially developed and characterized by Cyclene Pharmaceuticals, Inc. in collaboration with the laboratory of Ross Hannan (Melbourne). CX-3543 disrupts the interaction of the nucleolar processing factor nucleolin with rDNA G-quadruplex complexes in the nucleolus and induces apoptosis in cancer cells (Drygin et al., 2009). CX-5461 specifically blocks recruitment of the SL1 complex for RNAPII initiation and induces senescence and autophagy, but not apoptosis, in solid tumor cell lines (Drygin et al., 2011). Targeting of RNAPI transcription with CX-5461 selectively kills B-lymphoma cells and prolongs survival of tumor-bearing mice. The therapeutic efficacy could be correlated with nucleolar disruption and activation of p53-dependent apoptotic signaling (Bywater et al., 2012). A group of specific RNAPI inhibitors with therapeutic potential has very recently been described with the family of ellipticines, which also described for NPM1. The compound NSC348884 binds to a hydrophobic pocket of NPM1 and inhibits its oligomerization during ribosome biogenesis. Two small molecule inhibitors have been described for NPM1. The compound NSC348884 binds to a hydrophobic pocket of NPM1 and inhibits its oligomerization. NSC348884 inhibits cell proliferation at an IC\textsubscript{50} of 1.7–4.0 \(\mu\)M, induces p53, and synergizes with the cytotoxicity of doxorubicin in distinct cancer cell lines (Qi et al., 2008).

The compound avrainvillamide is an alkaloid and has been identified in an affinity screen with NPM1. Auvainvillamide stabilizes p53 and induces apoptosis. A screen with NPM1 cysteine/alanine substitution mutants identified cysteine residue 275 in NPM1 as critical for binding of avrainvillamide (Wulff et al., 2007). However, whether the growth inhibitory activity of NPM1-inhibitors is conferred by blocking of rRNA processing has not been studied yet.

The data discussed in this review suggest that the inhibition of ribosome biogenesis is a promising approach
for cancer therapy. Although the number of non-genotoxic drugs is currently limited, an increasing amount of small molecule inhibitors might help to broaden therapeutic options. In 2005, the nucleolar proteome with 692 proteins became available (Andersen et al., 2005). Interestingly, a large panel of the nucleolar factors is associated with cell cycle control, including kinases and phosphatases with potential regulatory function in ribosome biogenesis. Further research is required to elucidate the function of nucleolar enzymes and to open doors for the development of novel therapeutic approaches.

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References


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