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# rRNA SYNTHESIS-INHIBITING DRUGS IN ANTINEOPLASTIC THERAPY: p53 STABILIZATION LEVEL IS DIRECTLY RELATED TO THE CELL RIBOSOME BIOGENESIS RATE

Presentata da: **Dott.ssa Federica Scala**

Coordinatore Dottorato

**Chiar.mo Prof.**
**Pierluigi Lollini**

Relatore

**Chiar.mo Prof.**
**Lorenzo Montanaro**

Correlatore

**Chiar.mo Prof.**
**Massimo Derenzini**

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# INTRODUCTION

Ribosomes play an important role in the molecular life of a cell, providing the basis for protein production thus allowing cells growth and proliferation. In growing cells the synthesis of new ribosomes is fundamental and the ribosome biogenesis is one of the most energetically active processes. To understand the extent of this process, it is sufficient to consider that in an eukaryotic cell ribosomal RNA (rRNA) accounts up to 80% of total cellular nucleic acid (Lempiäinen and Shore 2009) and every second in a growing yeast cell 40 nascent ribosomes leave the nucleolus, the major site of ribosome production, for export into the cytoplasm (Harris and Levine 2005).

The close relationship between ribosome biogenesis and cell growth and proliferation is emphasized by the fact that the biological mechanisms which control cell proliferation also control ribosome biogenesis. This process in fact can be regulated by the products of the same genes that control cell cycle entry and cell cycle progression, as it will be explained later. These are the same genes that undergo mutations during the neoplastic transformation in order to promote a more rapid cell proliferation. For this reason, ribosome biogenesis process is frequently altered in cancer cells.

In active proliferating cells any stress which interferes with the ribosome biogenesis process, e.g. the inhibition of rRNA synthesis, induces a cell response causing either a block in cell cycle progression or the death of the cell (Pardee 1989).

These effects appear to be mediated by the tumor suppressor protein p53 whose levels increase in the cell following ribosome biogenesis inhibition.

## THE P53 TUMOR SUPPRESSOR

The p53 protein is considered one of the most important tumor suppressors because of its importance in preventing the growth and survival of potentially malignant cells.

In the nucleus p53 works predominantly as a transcription factor that enhances the rate of transcription of several genes connected with cell proliferation. Many types of stress activate p53, including DNA damage, oncogene activation, hypoxia and nucleolar stress. Activation of p53 can induce different responses in the cells, including differentiation, senescence, DNA repair and the inhibition of angiogenesis, but the most relevant effect is the ability of p53 to induce cell cycle arrest and/or apoptotic cell death (Ryan et al. 2001). In this way p53 inhibits the proliferation of stressed cells either by causing the cycle arrest, which might be transient to allow damages repair before cell cycle progression, or by inducing apoptosis to eliminate the damaged cell. Both responses can prevent replication of cells undergoing neoplastic transformation and in this way inhibit tumor development. Because p53 plays a role in preventing tumor cell growth at several points during the transformation process, the loss of p53 function is one of the mutation most frequently observed in cancer cells and can be found in about 50% of tumors (Levine 1997).

From a structural point of view, the human p53 protein contains 393 amino acids and has been functionally divided into four domains. The N-terminus consists of a transcriptional activator domain which interacts with the transcription machinery and positively regulates gene expression. This domain also interacts with the p53 negative regulator MDM2 (Lin et al. 1995). The sequence-specific DNA-binding domain of p53 is localized between amino acid residues 102 and 292. It is a protease-resistant and independently folded

domain containing a $Zn^{2+}$ ion that is required for its sequence specific DNA-binding activity (Cho et al. 1994). Amino acid residues 324–355 are required for the oligomerization of the protein, in fact p53 works as a tetramer. The C-terminal 26 amino acids form an open protease-sensitive domain, this domain regulates the ability of p53 to bind to specific DNA sequences at its central or core domain (Lee et al. 1995).

**Regulation of p53**

As p53 is such a potent inhibitor of proliferation, it must be tightly regulated. This control is achieved through several mechanisms, such as regulation of transcription and translation, but rapid response to cell stresses is mediated by post-transcriptional control that lead to stabilization and activation of p53 (Woods and Vousden 2001).

One of the key regulators of p53 stability is the protein mouse double minute 2 (MDM2, HDM2 in humans). It is an E3 ubiquitin ligase that works targeting proteins for proteasome degradation. MDM2 ubiquitination of p53 occurs in the C-terminus of the protein. It has been discovered that p53 ubiquitination is also essential for its nuclear export to the cytoplasm because it allows the nuclear export sequence to reveal (Boyd et al. 2000). The critical role of MDM2 in p53 regulation has been shown by the observation that *MDM2* gene deletion was lethal in mice with functional p53 during early embryogenesis, whereas simultaneous deletion of *TP53* and *MDM2* genes allowed the normal development. It means that in mice during development MDM2 is essential for negative regulation of p53 function (Jones et al. 1995).

As MDM2 is a transcriptional target of p53 it exists an auto-regulatory feedback loop in which increased p53 activity leads to increased expression of its own negative regulator (Harris and Levine 2005).

Increased stability of p53 in response to cellular stresses involves the inhibition of MDM2 function through several pathways.

Furthermore, the regulation of p53 activity is obtained through a series of post-translational modifications which involve its C-terminal domain thus affecting p53 DNA binding ability. The main modifications are phosphorylation (Meek 1999), sumoylation (Gostissa et al. 1999) and acetylation (Gu and Roeder 1997). As an example, in response to DNA damage different kinases are activated, e.g. ataxia-telangiectasia-mutated (ATM), ataxia and rad3 related (ATR), and phosphorylate p53 hindering MDM2 binding.

During neoplastic transformation the stabilization of p53 in response to abnormal proliferation associated with oncogene activation (such as RAS or MYC overexpression) does not require phosphorylation of p53, but depends to a large extent on activation of the p14ARF protein, whose function is to bind and to inhibit MDM2 directly (Sherr and Weber 2000).

**p53 and cell cycle control**

To better understand how p53 controls cell cycle progression, a short description of cell cycle phases and regulation is necessary.

The cell cycle consists of a specific sequence of events, finely regulated, that leads to cell growth and culminate with the production of two separate cells.

Briefly, the cell cycle is composed of two main stages: interphase and mitosis. During interphase, the cell grows in size and duplicate DNA through different stages: Gap1 (G1), Synthesis (S) and Gap2 (G2). During the G1 the cell grows increasing its size and produce the elements necessary for the following stage, the S phase, in which the cell duplicate its DNA. After DNA synthesis during the G2 phase the cell continues to grow in order to produce the material needed for cell division. Mitosis is the stage in which the cell effectively generates two different daughter cells. Mitosis includes prophase, metaphase, anaphase and telophase. Cells in G1 can, if not committed to DNA replication, enter a resting state called G0. Cells in G0 account for the major part of the non-growing, non-proliferating cells in the human body (reviewed in Norbury and Nurse 1992).

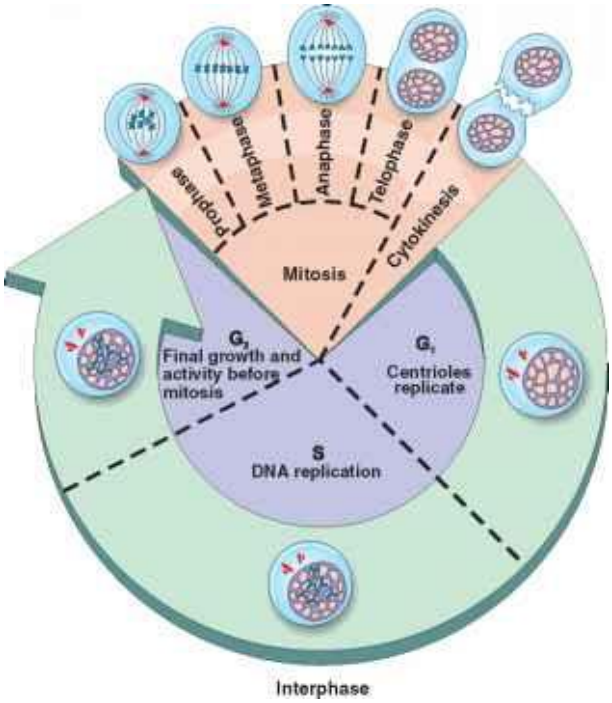



**Figure 1: Schematic representation of cell cycle phases in eukaryotic cells.**

The transition from one phase to another occurs in an orderly way and is finely regulated by different cellular proteins. The key regulators of this process are the cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. Binding to a Cyclin is necessary for the activation of CDKs (Pines 1995). Cyclin protein levels rise and fall during the cell cycle and they periodically activate CDKs (Evans et al. 1983).

Thus, the cell enters and exits the cell cycle in association with production and activation of specific Cyclins. Generally to proceed to the following phase the previous Cyclin is degraded and another one has to be synthesized (Schafer 1998).

Until now, nine CDKs have been identified and, of these, five are active during the cell cycle. Each CDK has one or more corresponding Cyclin and act in a precise step of the cell cycle (Vermeulen et al. 2003). When CDKs are active they phosphorylate target proteins resulting in changes that catalyze the process of cell division. The most studied target of CDKs is the Retinoblastoma protein (pRb). Hypo-phosphorylated pRb binds the E2F-1 transcription factor, inactivating it. During early G1, CDK4/6-Cyclin D complexes phosphorylate pRb leading to the release of E2F-1. This factor positively regulate the transcription of genes whose products are required for S phase progression, including Cyclin A and Cyclin E. pRb remains hyper-phosphorylated for the remainder of the cell cycle and CDK2-Cyclin E participates in maintaining this state (Buchkovich et al. 1989).

CDKs activity can be inhibited by negative regulator proteins, called CDK inhibitors (CKI) which bind to CDKs alone or to the CDK-Cyclin complex and regulate their activity. Two distinct families of CDK inhibitors have been discovered: the INK4 family and the Cip/Kip family (Sherr and Roberts 1995). The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), p19 (INK4d), which specifically inactivate G1

CDKs (Cdk4 and Cdk6). This CKI family forms stable complexes with the CDKs preventing the association with Cyclin D (Carnero and Hannon 1998). The second family of inhibitors, the Cip/Kip family, includes p21 (Waf1/Cip1), p27 (Cip2), p57 (Kip2). These inhibitors inactivate CDK-Cyclin complexes and are of primary importance in G1 regulation (Polyak et al. 1994). The p21 family binds to Cyclins preventing pRb phosphorylation and inhibits its dissociation from E2F-1. They inhibit to a lesser extent, CDK1-Cyclin B complexes (Hengst and Reed 1998). p21 also inhibits DNA synthesis by binding to and inhibiting the proliferating cell nuclear antigen (PCNA) a subunit of DNA Polymerase δ which has a role in DNA replication and repair (Pan et al. 1995).

To ensure a quality control on cell cycle progression and an orderly sequence of events there are some checkpoints. In response to DNA damage checkpoints arrest the cells in order to provide time to repair the problem. The two mains checkpoints are positioned before or after DNA duplication: the G1-S and the G2-M checkpoint.

The tumor suppressor p53 exerts its control on cell cycle progression mainly acting on the transcription activation of the *p21* gene (el-Deiry et al. 1993). As previously described, the induction of p21 expression results in CDKs inhibition and cell cycle arrest at the G1-S checkpoint. The p21 protein in fact inhibits the phosphorylation of the pRB protein preventing E2F-1 release.

The protein p53 may also play a role in the regulation of the G2-M checkpoint. DNA damage-dependent increase of p53 results in increased transcription of *p21* and of *14-3-3 σ*. The 14-3-3 σ protein can bind to the Cyclin B and can actively exclude it from the nucleus (Hermeking et al. 1997).

Another transcriptional target of p53 is *Gadd45* (growth arrest and DNA damage inducible gene) whose product mediates the dissociation of CDK1-Cyclin B1 complexes (Taylor and Stark 2001).

**p53 induction of apoptosis**

The first evidence that p53 induces apoptosis was given by Oren and co-workers who showed that the reintroduction of p53 into a p53-deficient myeloid leukemia cell line caused apoptosis (Yonish-Rouach et al. 1991). The main role of p53 induced apoptosis is to suppress tumorigenesis but it also contributes to chemotherapy induced cell death in human cancer. Consistent with this role the loss of p53 can be linked to chemotherapy resistance in certain type of tumors (Johnstone et al. 2002).

In human cells apoptosis can be induced following two pathways. The extrinsic pathway involves a series of cell surface receptors which activate intracellular proteins. The intrinsic pathway involves the mitochondria which contain apoptogenic factors, such as cytochrome c, second mitochondria-derived activator of caspases (SMAC), apoptosis inducing factor (AIF) and endonuclease G. Both pathways lead to the activation of a class of specific proteases, the caspases. Once the caspases are activated the process of cellular death is irreversible.

The p53 protein regulates the apoptotic pathway by modulating key control factors in the intrinsic pathway.

The protein p53 can transcriptionally activate some proapoptotic genes of the *Bcl-2* family: *BAX*, *PUMA*, *OXA* and *BID*. These proteins effects increase the quantity of pro-

apoptotic Bcl-2 proteins, thereby favoring the release of apoptogenic compounds from the mitochondria, leading to caspase activation and apoptosis (Fridman and Lowe 2003).

There is evidence that p53 can also promote apoptosis through transcription independent mechanisms. Recently a small fraction of the p53 protein was shown to allow permeabilization of the outer mitochondrial membrane directly by forming inhibitory complexes with the protective Bcl-XL and Bcl-2 proteins, leading to the release of cytochrome c (Mihara et al. 2003).

Another role for p53 in promoting apoptosis is due to changes induced in REDOX metabolism. This leads to an increase in reactive oxygen species (ROS) that, perhaps by interfering with mitochondrial function and integrity, contributes to cell death (Polyak et al. 1997).

## RIBOSOME BIOGENESIS

The ribosome biogenesis process requires the coordinated action of all three nuclear RNA polymerases (Pol I, II and III). Pol I transcribes a single 47S rRNA precursor (pre-rRNA), which is subsequently processed into the mature 18S, 5.8S and 28S rRNA. Pol II transcribes the mRNA required in the cytoplasm for the synthesis of ribosomal proteins (RPs). Pol III synthesizes the 5S rRNA and other non-coding RNAs (van Riggelen et al. 2010; Roeder and Rutter 1970; Weinmann and Roeder 1974). These components are assembled in the nucleolus to form the mature subunits that are exported to the cytoplasm where the ribosomes perform protein synthesis.

**The nucleolus**

The nucleolus is the compartment of the interphase nucleus where the ribosome biogenesis takes place. The structure of the interphase nucleolus is organized around the genes of rDNA and is maintained by transcription and ribosome assembly. In the human genome rDNA is present as head-to-tail tandem repeated genes. During mitosis the nucleolus disassembles to reassemble after cell division and the rDNA localize on the short arm of the five acrocentric chromosomes 13, 14, 15, 21 and 22, in regions called "nucleolar organizer regions" NORs (Derenzini 2000)

Observing the nucleolus by electron microscopy, it is possible to identify three major regions with different morphology:

- The fibrillar center (FC) appears as a roundish clear area characterized by low electron opacity. Generally cells with a high rate of ribosome biogenesis possess numerous small FCs, as an example cycling cells. On the contrary, cells with greatly reduced metabolic and transcription activities, present small nucleoli with one large-sized FC such as in lymphocytes (Derenzini et al. 1990).
- The dense fibrillar component (DFC) constitutes a rim intimately associated with the fibrillar centers composed of densely packed fibrils. (Derenzini 2000).
- The granular component (GC) is mainly composed of granules of 15-20 nm in diameter surrounding the DFC.

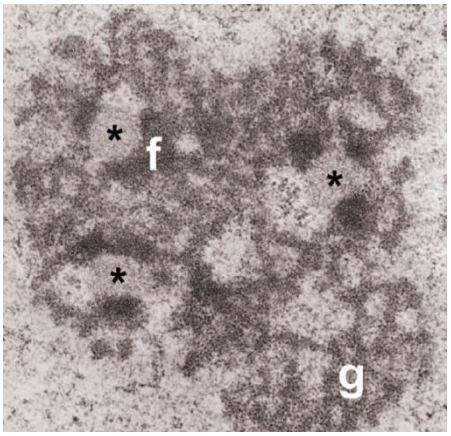



**Figure 2: Electron microscope visualization of the nucleolus**. Three fibrillar centers are present (*), with the closely associated dense fibrillar component (f). The granular component (g) is organized in cord-like structures (Montanaro et al. 2008).

The localization of the ribosome biogenesis machineries has led to assign specific functions to different compartments of the nucleolus (Derenzini et al. 1990).

Nascent transcripts appear at the junction between the FCs and DFC and accumulate in the DFC. Processing of the 47S pre-rRNA starts at the site of transcription in the DFC (Cmarko et al. 2000) and continues during the intranucleolar migration of the rRNA towards the GC. The nucleolar proteins that participate in the early stages of rRNA processing, localize in the DFC, such as Fibrillarin and Nucleolin along with the U3 snoRNAs, whereas proteins that are involved in intermediate or later stages of processing, such as Nucleophosmin (B23), have been localized to the GC (Biggiogera et al. 1989; Ginisty et al. 1998; Ochs et al. 1985; Gautier et al. 1994).

Nucleoli can be stained rather selectively within cell nuclei with reactions based on the reduction of metallic ions, the most commonly used are silver nitrate and silver ammonium salts (Schwarzacher and Mosgoeller 2000). Some of the proteins located in the fibrillar components (Nucleolin, Nucleophosmin, UBF, the largest RNA polymerase I subunit) are selectively stained by the reaction with these metallic ions, therefore these proteins are called AgNOR proteins (Sirri et al. 2000). This procedure allows visualizing at the light microscopy the interphase NORs, in fact after the staining they appear as well-defined black dots within the nucleolar body. These structures can be quantified by morphometric analysis and their size is proportional to the nucleolar transcriptional activity (Derenzini et al. 1998). AgNORs can be considered a simple method to obtain information on the ribosome biogenesis activity of the cell and can be applied to the study of nucleolar alterations in tumor cells (Montanaro et al. 2008).

**RNA polymerase I and rRNA synthesis**

The process of assembling a ribosome subunit requires the initial transcription of rDNA genes by the specific RNA polymerase I.

A human diploid cell has approximately 400 repeats of rDNA but only a subset of these (~50%) is transcribed at any given time.

In mammals, each rDNA repeat is approximately 43 kb and is composed by intergenic spacers (IGS) of approximately 30 kb and a single transcribed region of approximately 13 kb containing the 47S coding region. The gene promoters are contained in the IGS rRNA and their main regulatory elements are the core promoter and the upstream control element (reviewed by McStay and Grummt 2008).

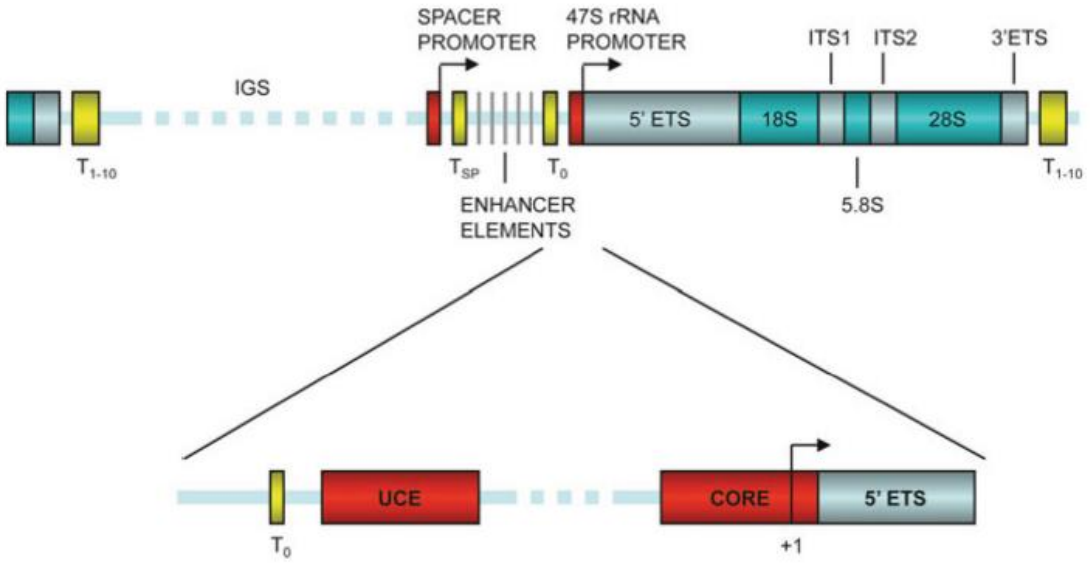



**Figure 3: General organization of a rDNA gene and promoter** (Goodfellow and Zomerdijk 2013)**.**

Specific transcription factors are necessary for accurate recruitment of Pol I and for the formation of pre-initiation complex (PIC) at the gene promoter. The main proteins involved in this process are selectivity factor 1 (SL1) and UBF. SL1 recognizes and binds

the core promoter element in the rDNA, it is essential for Pol I recruitment to the transcription start site and promotes a stable interaction between UBF and the rDNA promoter (Friedrich et al. 2005). To achieve activated transcription, UBF must also be incorporated into the PIC where it interacts with SL1 at the rDNA promoter and with the PAF53 and PAF49/CAST Pol I subunits (Hanada et al. 1996; Panov et al. 2006).

In mammalian cells, the Pol I complex exists as two distinct subpopulations: Pol Iα and Pol Iβ. Both forms are active and can catalyze the synthesis of RNA, but only Pol Iβ, which represents less than 10% of the total Pol I in a cell, can be incorporated into PICs and initiate transcription. This is due, at least in part, to the association of Pol Iβ with TIF1A which interacts with Pol I A43 subunit (Miller et al. 2001; Cavanaugh et al. 2002). Pol I must dissociate from the promoter-bound initiation factors in a process known as promoter escape. Promoter escape following transcription initiation coincides with the release of TIF1A from polymerase (Panov et al. 2006).

Transcription elongation is highly efficient with about 100 Pol I complexes transcribing each active gene, at a rate of approximately 95 nucleotides per second (Dundr et al. 2002).

Several enzymatic complexes cooperate with PolI to optimize the rRNA transcription process. For example Topoisomerase IIα is a component of Pol Iβ in human cells and is necessary in order to remove topological changes in the rDNA (Panova et al. 2006). Furthermore, various DNA repair proteins have been found in Pol I complexes thus transcription-coupled DNA repair occurs at rDNA genes (Conconi et al. 2002).

Transcription termination by Pol I is a multistep process involving specific DNA sequence elements and regulatory proteins. In mammals, transcription termination factor (TTF-I) binds to terminator elements downstream of the rRNA gene, causing polymerase

pausing. Dissociation of the paused transcription complex is then mediated by Pol I transcript release factor (PTRF) (Jansa and Grummt 1999).

**From rRNA to ribosome subunits**

The pre-rRNA 47S transcribed by the Pol I undergo chemical modifications to specific nucleotide cleavages to remove transcribed spacers, thus releasing the mature-size 18S 28S and 5.8S rRNA species.

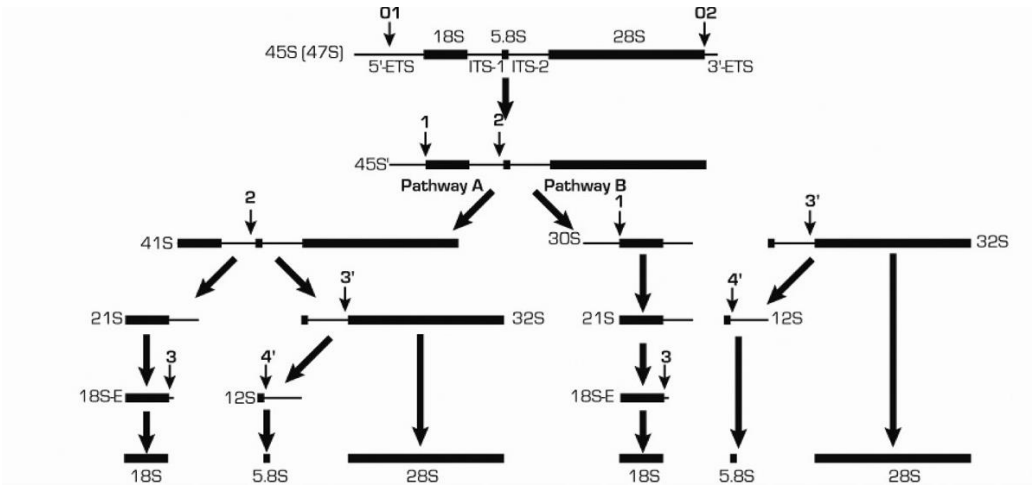



**Figure 4: Pre-rRNA processing** scheme in HeLa cells (Henras et al. 2008).

Pre-rRNA processing begins on the 47S primary transcript by snipping both ends of the molecule at sites 01 and 02 in the 5'- and 3'-ETS segments, respectively, generating the 45S pre-rRNA. The 45S precursor is matured following two major alternative pre-rRNA processing pathways that appear to differ not in the nature of the actual processing sites used but rather in the kinetics and order of cleavage. Both pathways produce three mature rRNA 18S, 5.8S and 28S as shown in fig. 4 (Mullineux and Lafontaine 2012).

There is evidence that the first processing event occurs before the pre-rRNA transcript is completed (Lazdins et al. 1997).

The pathways of pre-rRNA processing involve both endo- and exonucleases, many of this 3'→5' exonucleases are contained in particles called 'exosomes' (Allmang et al. 1999).

An important role in rRNA processing is exerted by small nuclear RNAs (snoRNAs) which are involved in different steps of the process: they can act as RNA chaperones to produce folding patterns recognized by the nucleases, they can be components of the ribonucleoprotein ribonucleases and the majority of them are guides for RNA modification (Borovjagin and Gerbi 1999). In the pre-rRNA the main modifications consist of 29-O-methylation of ribose groups and the conversion of uridine to pseudouridine (Olson et al. 2000).

There is an important group of nucleolar proteins contained in the small nuclear ribonucleoprotein (snoRNPs). Although little is known about most of them, the snoRNP U3-associated protein Fibrillarin has been studied extensively and it has been shown to be essential for pre-rRNA processing, pre-rRNA methylation and ribosome assembly (Tollervey and Kiss 1997).

Many nucleolar proteins are involved in pre-RNA processing, among them Nucleolin and Nucleophosmin (protein B23). Nucleolin primary function is to facilitate the early stages of pre-rRNA processing, possibly by first interacting with the 5'region of pre-rRNA and then recruiting processing components (Ginisty et al. 1998). Several activities of protein B23 have been identified in vitro, including nucleic acid and nuclear localization signal binding, stimulation of nuclear import, and ribonuclease and molecular chaperone activities (Szebeni and Olson 1999).

The mature rRNAs are assembled in the nucleolus with the ribosomal proteins and with the 5S rRNA. The ribosomal proteins are synthetized in the cytoplasm and then imported

in the nucleus. The 5S-rRNA is synthetize in the nucleoplasm by the action of the Pol III and then is transported in the nucleolus. The 5S genes are present in multiple copies located outside the NORs and near the telomere of the long arm of chromosome 1 (Srivastava and Schlessinger 1991).

In mammals the mature ribosome is composed of two subunits: the small 40S ribosomal subunit, which contains one 18S rRNA and approximately 32 ribosomal proteins (RPS), and the large 60S subunit, which is composed of one of each 5S, 5.8S and 28S rRNA and approximately 47 ribosomal proteins (RPL).

The subunits are assembled into the nucleolus and then exported into the cytoplasm to perform protein synthesis (van Riggelen et al. 2010).

**Ribosome biogenesis regulation**

Growing and proliferating cells need to maintain high rates of Pol I transcription to sustain the level of ribosomes required for protein synthesis. Conditions that influence cell growth and proliferation, such as growth factor, nutrients and energy availability can regulate Pol I transcription.

The main mediator between these environmental conditions and ribosome biogenesis is mTORC1 (mammalian target of rapamycin complex 1) that contains the evolutionary conserved serine/threonine kinase mTOR and can control Pol I transcription via different pathways (Kusnadi et al. 2015).

First, mTORC1 signaling activates TIF1A by increasing phosphorylation at serine 44, required for TIF1A activity, and decreasing phosphorylation at serine 199 (Mayer et al. 2004). Moreover, mTOR regulates UBF in an S6K1-dependent manner. Mitogen-

induced activation of S6K1 phosphorylates UBF in its C-terminal region, phosphorylation required for the interaction between UBF and SL1 (Hannan et al. 2003).

Another important regulator of ribosome biogenesis is the transcription factor MYC, which can be activated in response to growth factor stimuli. Elevated expression of MYC regulates Pol I transcription by two different mechanisms. First, it can act by stimulating the transcription of factors associated with Pol I, such as UBF, TIF1A and Pol I subunits (Poortinga et al. 2014). Secondly, MYC can activate PolI transcription in a double manner: by binding to the rDNA promoter region remodeling rDNA chromatin structure and by directly interacting with SL1 promoting the stabilization of the UBF/SL1 complex (Arabi et al. 2005; Grandori et al. 2005).

In addition to these two major pathways for ribosome biogenesis regulations, there are other intracellular signaling ways that can modulate this process in response to external stimuli. One of these pathways leads to ERK (extracellular signal-regulated kinase) activation. It can phosphorylate TIF1A at serine 649 and serine 633 and UBF, thus promoting Pol I transcription (Zhao et al. 2003).

Another factor involved in Pol I regulation is the serine/threonine kinase AKT, which upregulates rDNA transcription at multiple steps, including transcription initiation, elongation, and rRNA processing, through both mTORC1-dependent and independent mechanisms (Chan et al. 2011).

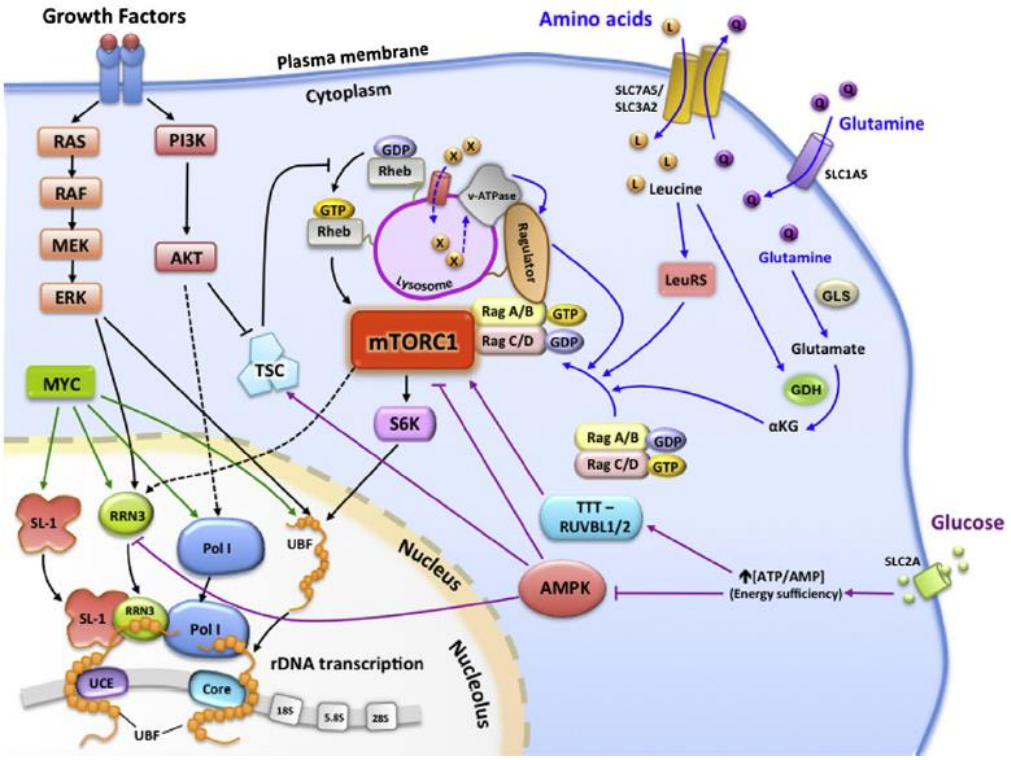



**Figure 5: the complex pathway of ribosome biogenesis regulation**. mTORC1 links the availability of growth factors, amino acids and glucose to rDNA transcription. MYC expression stimulates Pol I trough different mechanisms (Kusnadi et al. 2015).

The mechanisms which control cell proliferation also control ribosome biogenesis; this underlines the close relationship linking ribosome biogenesis to cell growth and proliferation. This process in fact can be regulated by the products of the same genes that control cell cycle entry and cell cycle progression.

Cyclin D and E can induce the phosphorylation of UBF, by Cdk4-Cyclin D1 and Cdk2-Cyclin E complexes, thus enhancing the transcription of ribosome genes (Voit et al. 1999).

There is evidence that pRB, in addition to controlling the transition from G1 to S phase, also modulates ribosome biogenesis. Active, hypo-phosphorylated pRB inhibits rRNA synthesis by binding to UBF (Cavanaugh et al. 1995). Therefore, in cycling cells hyper-

phosphorylation of pRB induces an increase of the rRNA transcription rate, along with an enlargement of the nucleolar size.

The protein p53, in addition to its role in response to cellular stresses, directly influences ribosome biogenesis. Accumulation of the wild-type p53 inhibits Pol I transcription by binding to the selectivity factor SL1 and by hindering the formation of the UBF-SL1 complex (Zhai and Comai 2000). The activity of p53 may also be activated by the tumor suppressor protein p14Arf, which also hinders the ribosome biogenesis by inhibiting UBF recruitment on the transcription complex and by lengthening rRNA processing (Bertwistle et al. 2004; Ayrault et al. 2006).

Lastly, PTEN (phosphatase and tensin homolog deleted in chromosome 10), another important tumor suppressor which inhibits cell proliferation, also represses Pol I transcription by disrupting the SL1 complex ( Zhang et al. 2005).

**Ribosome Biogenesis alterations in cancer**

Human cancer cells are frequently characterized by nucleolar hypertrophy, characteristic that is often associated with an upregulated ribosome biogenesis process. Interestingly, several human cancers have been analyzed, using AgNOR staining, and nucleolar sizes appear to be very variable within both different histotypes and the same tumor sample. This can be explained by the fact that the nucleolar changes in tumors are closely related to the number and the rapidity of proliferating cells within the cancer tissue, kinetics parameters that are highly variable in human tumors ( Derenzini et al. 1998; Derenzini et al. 2000).

It is known that neoplastic transformation is characterized by mutations which activates oncogenes or inactivate tumor suppressor genes. As explained in the previous chapter, the

products of these genes regulate not only cell proliferation but also ribosome biogenesis. Therefore these neoplastic mutation might result in the enhancement of the ribosome biogenesis process (Montanaro et al. 2008).

Among the tumor suppressor genes *RB1* and *TP53* are the most important, their inactivation is linked to the induction of genetic instability and a poor prognosis in cancer cells, but it is also responsible for the upregulation of ribosome biogenesis process (Cordon-Cardo 1995). It has been observed that in human tumors the degree of nucleolar hypertrophy was directly related to the severity of the pRB and p53 pathway alterations: cancers without change of pRB and p53 presented small nucleoli whereas cancers with *RB1* loss and mutated *TP53* showed hypertrophic nucleoli (Treré et al. 2004; Derenzini et al. 2004).

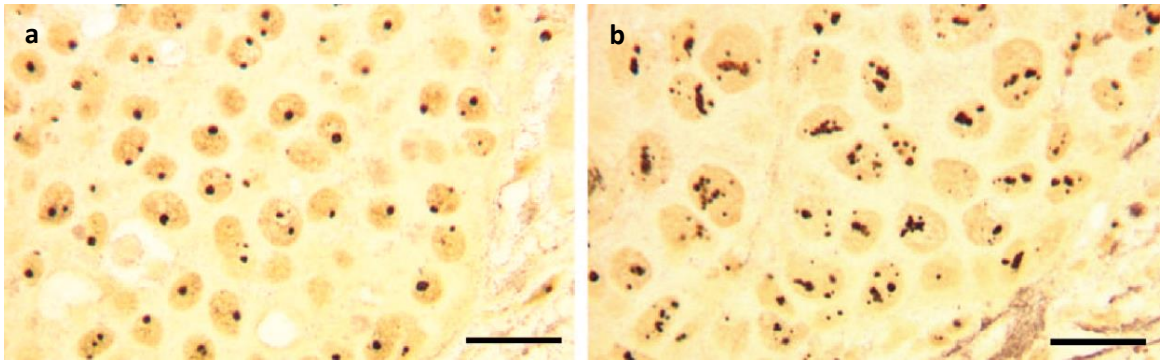



**Figure 6: Histological sections of two human breast carcinomas, stained for the AgNOR proteins.** Note the small size on the nucleoli in the case reported in **a** and the larger size of nucleoli in the case reported in **b** (Montanaro et al. 2008).

Another oncogene which is frequently altered in cancer cells is *c-MYC*, its overexpression induces cell growth and proliferation but also stimulate rRNA transcription and ribosome production.

Also Cyclin D and E, which control the cell cycle progression, may be overexpressed or altered in human tumors and can directly activate ribosome biogenesis (Montanaro et al. 2008).

The *ARF* gene may be mutated or silenced in tumor cells (Sherr 2001); the loss of p14ARF expression may be responsible for the enhancement of ribosome biogenesis both directly and through the action on p53 stabilization.

Also, the tumor suppressor *PTEN* is very frequently deleted or mutated in human cancers (Yin and Shen 2008), in which its repressive action on Pol I transcription may be lost.

According to these data, the up-regulation of ribosome biogenesis in cancer cells appears to be as an adaptation of the nucleolus to the neoplastic transformed cell. In fact, in this way the cell guarantees the production of the constituents needed for the appropriate division (Derenzini et al. 2005).

However, there is also evidence of an active role for nucleolus in cancer development. A series of human pathological conditions are linked to an increased risk of cancer onset, such, for example, tissues with chronic inflammation.

Hepatitis B (HBV) and C (HCV) viruses, which are responsible for chronic liver disease, have also been shown to upregulate the activity of Pol I and III (Kao et al. 2004; Wang et al. 1998). Interestingly, the presence of abnormally enlarged hepatocyte nucleoli represents a strong risk factor in the development of hepatocellular carcinoma, mainly in HBV-related cirrhotic livers (Trerè et al. 2003).

Moreover, a recent study shows that Interleukin-6 (IL6), which is produced in tissues with chronic inflammation, induced an rRNA transcription up-regulation with the consequent p53 degradation and activation of the epitelial-mesenchimal transition process (Brighenti et al. 2014).

Apart from the quantitative variation of the rate of ribosome synthesis, specific qualitative alterations in the process of ribosome biogenesis are responsible for a series of human diseases in which cancer incidence is very high (Narla and Ebert 2010)

The Dyskeratosis congenita is a ribosomal disorder in which the molecular connections between the qualitative alterations of ribosome biogenesis and cancer have been better characterized. It is caused by mutations in the *DKC1* gene whose product, dyskerin, is a nucleolar protein necessary for the site-specific conversion of uridine to pseudouridine presents in the rRNA molecules. The reduction in modified uridine residues in the ribosome might result in an impaired translation of specific mRNAs encoding for tumor suppressors such as p53 and/or p27, explaining the increased tumor susceptibility characteristic of this pathology (Bellodi et al. 2010; Bellodi et al. 2010).

Another well-characterized ribosome biogenesis diseases is Diamond-Blackfan anemia, a group of inherited bone marrow failure disorders (Ellis and Lipton 2008). The 25% of patients are reported to carry a mutation in RPS19, but mutations in other RPs have been reported (Gazda et al. 2008; Campagnoli et al. 2008). In conjunction with chronic anemia and congenital abnormalities, Diamond–Blackfan anemia patients also have a predisposition to development of cancer (Deisenroth and Zhang 2010).

## THE RIBOSOMAL PROTEINS-MDM2-P53 PATHWAY

The synthesis of new rRNA is an integral process of ribosome biogenesis that needs to be continually monitored to ensure appropriate balance between rRNA synthesis and RPs availability (Lempiäinen and Shore 2009).

Perturbations of many aspects of ribosome biogenesis contribute to 'nucleolar stress' which leads to the breakdown of nucleolar structure. Following nucleolar stress the RPs-Mdm2-p53 response pathway is activated and p53 levels arise in the cell. As previously described, in fact, one of the primary mechanisms to induce p53 stability is through modifications in the MDM2-p53 interaction.

HDM2 is characterized by an N-terminal domain which binds p53, a C-terminal domain which promotes p53 ubiquitination, and a central region which contains multiple sites for binding and regulation by different proteins, including pRb, ARF, p300 histone acetyltransferase and some ribosomal proteins (Zhang et al. 2003).

Marechal and colleagues were the first to report RPs interaction with Mdm2, they described the RPL5 binding to Mdm2 in a 5S rRNA ribonucleoprotein complex, but at the time, the meaning of this interaction was unclear (Marechal et al., 1994).

The interaction between ribosomal proteins and Mdm2 was deeply studied in the following years and the proteins RPL5, RPL11 and RPL23 were all reported to bind to Mdm2, inhibiting its E3 ubiquitin ligase function and promoting p53 accumulation (Y. Zhang et al. 2003; Bhat et al. 2004; Dai and Lu 2004). Subsequently, further RPs were described as Mdm2 binding partners: RPS7 (Chen et al. 2007), RPL26 (Ofir-Rosenfeld et al. 2008) and RPS3 (Yadavilli et al. 2009).

In this scenario, perturbations to the synthesis of rRNA precursors can induce ribosome biogenesis disruption with the release of ribosomal proteins that, no longer utilized for

ribosome building, are free to bind to Mdm2 inhibiting its E3 ubiquitin ligase activity thus causing p53 accumulation in the cell.

This has been repeatedly demonstrated using rRNA synthesis inhibitors. The most utilized compound which selectively impair rRNA synthesis is Actinomycin D (Act D), an anticancer antibiotic which at low doses (<10 nM) specifically inhibits the production of rRNA. Actinomycin D has a high affinity for deoxyguanine nucleotides and preferentially intercalates into the GC rich regions of rDNA to inhibit PolI-mediated transcription of nascent 47S rRNA (Perry and Kelley 1970). This interaction leads to nucleolar stress with consequent p53 stabilization. In addition to Act D, several chemical reagents have been found to trigger nucleolar stress by inhibiting rRNA processing or synthesis, such as 5-fluorouracil (5-FU) and mycophenolic acid (MPA) (Sun et al. 2008; Sun et al. 2007)

Nucleolar disruption is not always a necessary condition to induce RPs-Mdm2 interaction and p53 stabilization. A study conducted in our laboratory showed that gene silencing of POLR1A, a component of Pol I, caused rRNA synthesis inhibition without inducing nucleolar disruption and could anyway stabilize p53 through the RPs-Mdm2 interaction (Donati et al. 2011).

Recently, the research is addressing to the development of new anticancer molecules directly inhibiting RNA PolI I activity and able to activate p53 through the RP-Mdm2 interaction. Among these, CX-5461 inhibits ribosome biogenesis by disrupting the SL-1 rDNA complex. This drug was shown to induce p53-mediated death of Lymphoma cells via activation of the Rp-MDM2-p53 pathway in a mouse model of lymphoma with *MYC* overexpression (Eμ-Myc). CX-5461 was able to kill selectively transformed malignant cells sparing normal B cells. Thanks to this result the drug is used in clinical trials for the treatment of hematologic malignancies (Bywater et al. 2012).

The RPs-Mdm2 pathway seems to have relevance not only in the response to chemotherapy treatments but also in neoplastic transformation. In fact, it has been shown that stimulation of ribosome biogenesis results in a greater degradation of p53 due to the fact that the quantity of ribosomal proteins necessary for ribosome biogenesis is increased with the consequent reduction of the amount of RPs which inhibit MDM2. Stimuli which increase rRNA synthesis, such as Insulin, Insulin-like growth factor 1, Interleukin-6 downregulate the cell tumor suppressor potential by decreasing p53 levels (Donati et al. 2011; Brighenti et al. 2014).

# AIMS OF THE THESIS

Many chemotherapeutic drugs actually used in cancer treatment hinder the process of ribosome biogenesis as a direct or indirect consequence of their action (Burger et al. 2010).

In the p53 wild-type cancers the ribosome biogenesis inhibiting drugs can contribute to the therapy effectiveness promoting p53 stabilization. Indeed, as a consequence of rRNA synthesis inhibition the ribosomal proteins, no longer utilized in the ribosomes building, bind to MDM2 and inhibit its E3-ubiquitin ligase activity therefore causing p53 accumulation in the cell. Since a functional ribosome biogenesis process requires the stoichiometric production of rRNA and RPs (Donati et al. 2011), we hypothesized that in cancer cells characterized by different levels of rDNA transcription the ribosome biogenesis inhibition could lead to the release of a different amount of RPs which bind to and inactivate MDM2 causing differences in the level of p53 stabilization.

The main purpose of this work was to assess the relationship between the cell ribosome biogenesis rate and the p53 stabilization level in wild-type p53 human cancers after treatments with rRNA synthesis-inhibiting drugs. We found that the inhibition of rRNA synthesis caused greater p53 stabilization in the cells characterized by an elevated ribosome biogenesis process compared with the cells in which this process was lower.

We also found that according to the level of p53 stabilization these rRNA inhibitors may induce an arrest of cell cycle progression or the apoptotic cell death. The induction of apoptosis occurred only in cells in which the ribosome biogenesis rate, evaluated before the drug exposure, was very high, differently from the cells characterized by a low ribosome biogenesis rate in which only a cell cycle arrest was induced.

Since these data demonstrated that the induction of apoptosis was due to a high degree of p53 stabilization, we finally investigated whether the combination of rRNA synthesis inhibitors with drugs which do not inhibits ribosome biogenesis, such as Hydroxyurea, could increase the effectiveness of the treatment by increasing p53 stabilization.

# MATERIALS AND METHODS

## Cell cultures and chemical treatments

Six p53 wild-type human cancer cell lines (HepG2, HCT116, U2OS, MCF7, LoVo and ZR-75-1) were obtained from the American Type Culture Collection. HCT116 p53 − / − cell line was a generous gift of Professor Bert Vogelstein. The cell lines were cultured in monolayer at 37°C in humidified atmosphere containing 5% of CO2. HepG2, HCT116 and U2OS were grown in Dulbecco's Modified Eagle Medium. MCF7 and ZR-75-1 cell lines were cultured in RMPI-1640 and LoVo in Ham's F12 nutrient mixture. Human lymphocytes were recovered from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Histopaque-1077, according to manufacturer's instructions. The cells were grown in RPMI-1640 medium. In order to obtain monocyte depletion PBMCs were cultured for 1 h at 37°C to allow monocyte adhesion and the lymphocytes suspended in the media were collected. Lymphocyte proliferation was obtained by adding 10 μg/mL of Phytohemoagglutinin (PHA) to the medium. All culture media were addicted with 10% Foetal Bovine Serum (FBS), 1% of penicillin and streptomycin, 1% of glutamine, and 0.5% of non-essential amino acids. All media and reagents were purchased from Sigma-Aldrich (Milan, Italy). Serum starvation was obtained by culturing cells in medium containing 0.2% Foetal Bovine Serum.

Actinomycin D (Biovision, Mountain View, CA, USA) was used at the final concentration of 8 nM; Doxorubicin (Doxorubicin Hydrochloride Injection, USP, Pfizer, New York, USA) at 10 μM; 5-Fluorouracil (Fluorouracile, Teva Pharma Italia, Milan, Italy) was used at the dose of 20 μg/ml; Hydroxyurea (Sigma-Aldrich) was used at the

concentration of 3,4 mM; Nutlin-3 (Sigma-Aldrich) was used at 10 μM; CX-5461 (Selleckchem, Huston, TX, USA) was used at 1 μM.

**Analysis of rRNA synthesis by 5-FU incorporation**

Analysis of nascent rRNA was performed on cells grown on coverslips by incorporation of 5-fluorouridin accordingly to the method described by Boisvert et al. 2000. Cells were incubated for 20 min in medium containing 2mM 5-fluorouridine (5-FU) (Sigma–Aldrich) then washed in cold PBS and fixed in 2% paraformaldehyde and 1% Triton X-100. Samples were incubated for 30 min in PBS with 1% bovine serum albumin (BSA) (Sigma–Aldrich) to block nonspecific binding before incubating with the primary antibody diluted in 1% BSA-PBS overnight at 4 °C. The samples were rinsed in PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (Dako, Glostrup, Denmark) for 45 min at 37 °C in the dark in a humidified chamber. Mounting and nuclei counterstaining were performed using the 'pro long antifade reagent with DAPI' (Molecular Probes, Invitrogen) and observed under a fluorescence microscope (Carl Zeiss Italy, Milano, Italia).

**Assessment of RNA polymerase I activity**

Cell nuclei from the six cell lines were extracted in TKM buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl, 3 mM $MgCl_2$) and suspended in 10 mM Tris-HCl pH 7.4, 0.25 M sucrose and 1 mM MgCl2. RNA polymerase I activity was assayed at high ionic strength in the presence of α-amanitin ( Derenzini et al. 2005). Five million nuclei of each cell line were incubated for 10 min at 37°C in a solution containing: 50 mM Tris-HCl pH 8, 0.2 mM MnCl2, 140 mM (NH4)2SO4, 0.9 mM ATP, GTP, CTP, 18 mM unlabeled UTP, 0.05 mM 3[H]UTP, 1 μg/ml α-amanitin. Then two volumes of trichloroacetic acid 5%

were added and the radioactivity of the precipitated fraction measured. Results were expressed in dpm/μg DNA. The measurements relative to each cell line were carried out in triplicate.

**RNA extraction, reverse transcription and Real-Time RT-PCR**

Cells were harvested and total RNA extracted with TRI Reagent solution (Ambion, Austin, TX, USA) according to manufacturer's instructions. Briefly, the cells were collected in TRI Reagent solution and incubated at room temperature (RT). After centrifugation, the pellet was incubated with chloroform at RT and centrifuged in order to obtain the separation of the aqueous phase containing the RNA. This phase was transferred in a new tube and the RNA was precipitated by incubation with isopropyl alcohol and centrifugation. The RNA pellet was washed in 75% alcohol, air-dried and dissolved in DEPC water. Whole cell RNA was quantified with a Nanodrop spectrophotometer (ND1000) and 2μg of RNA for each sample were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The relative RNA amounts of 45S, RPL11, p21, BAX, PUMA, β-glucuronidase, TP53 and c-MYC were evaluated by Real-Time RT-PCR performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). β-glucuronidase mRNAs were quantified with TaQMan Gene Expression Assays primers and probe kits (Applied Biosystems); primers for SYBR Green Real-Time RT-PCR analysis of human 45S rRNA, RPL11, BAX, PUMA, p21, TP53 and c-MYC were designed using the Roche online primers design tool.

For each sample, three replicates were analyzed. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 15 s, and 60°C for 1 min.

The relative amount of each RNA sample was analyzed following the ΔΔCT method: the mean ΔCT value of the control sample was used in each experiment to calculate the ΔΔCT value of samples replicates (Livak and Schmittgen 2001).

**Protein extraction and Western blotting**

Western blotting was performed on whole cell protein extracts and on nuclear protein fractions.

Total cellular proteins were extracted in lysis buffer (KH2PO4 0.1M pH 7.5, NP-40 1%, 0.1 mM β-glycerolphosphate) supplemented with Complete protease inhibitors cocktail (Roche Diagnostics) and quantified spectrophotometrically with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hempstead, UK). Nuclear protein fractions for Western blot analysis were obtained accordingly to the method described in Abmayr et al. 2006 with slight modifications. Samples were mixed with Leammli loading buffer (10% Glycerol, 0.2% SDS, 1.5 mM Tris, 0.1% Bromophenol Blue, 1.5 M β-mercaptoethanol) and denatured at 95°C for 5 min.

Denatured protein samples were separated in SDS polyacrylamide gels and transferred to nitrocellulose membranes (GE-Healthcare). Aspecific binding sites on the membranes were blocked by incubation with 5% non-fat dry milk dissolved in TBS-T solution (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7,4). Membranes were blotted overnight at 4 °C with primary antibody suspended in 3,5% BSA. After the incubation the membranes were washed in TBS-T to remove unbound antibody and incubated for 1 h with the secondary antibody diluted in 5% milk TBS-T at RT.

The horseradish peroxidase activity was detected using the appropriate enhanced chemiluminescence substrate (Cyanagen) on Hyperfilm enhanced chemiluminescence films (Amersham). Densitometric analysis was perfomed using the GelPro analyzer 3.0

software (Media Cybernetics). Values from whole protein extraction were normalized on corresponding β-Actin expression whereas for nuclear proteins normalization was utilized Lamin B expression.

Primary antibodies utilized in this work are as follow: anti-p53 (clone BP53-12, Novocastra Laboratories, Newcastle Upon Tyne, UK), anti-β-actin (clone AC-74, Sigma-Aldrich), anti-RPL11 (clone 3A4A7, Invitrogen, Carlsbad, UK), anti-PARP-1 (Cell Signaling Technology, Beverly, MA, USA), anti-Lamin B (C-20, Santa Cruz Biotechnology, CA,USA) anti-MDM2 (clone SMP14 sc-965 and clone H-221, sc-7918, Santa Cruz Biotechnology), anti-p21 (clone SXM30, 556431, BD Pharmingen, Milan, Italy) and anti-c-MYC (9402, Cell Signaling Technology). Horseradish peroxidase-conjugated secondary antibodies were from GE-Healthcare (Milano, Italy).

**RNAi and transfection reagents**

Select stealth RNAi (Invitrogen) targeted against RPL11 and c-MYC were used, whereas a Stealth RNAi negative control was used to transfect controls. Cells were transfected with lipofectamine RNAiMAX (Invitrogen) in Opti-MEM medium (Invitrogen), accordingly to manufacturer's procedures.

**Co-immunoprecipitation**

Cells were lysed in Co-IP buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.8% NP40, 1 mM dithiothreitol, 1 mM EDTA supplemented with Complete protease inhibitor cocktail (Roche Diagnostics). Whole extracts were quantified using Bradford assay (Bio-Rad). For each sample 1.5 mg of proteins were incubated overnight at 4°C with anti-MDM2 rabbit polyclonal antibody (clone H-221, sc-7918, Santa Cruz Biotechnology). The immunocomplexes were then precipitated with Protein A/G PLUS

Agarose beads for 4 h at 4 °C. The beads were then centrifuged, washed in Co-IP buffer and suspended in Laemmli buffer for subsequent immunoblot analysis, as described in "Protein extraction and Western blotting" section.

**Flow cytometry**

Asynchronously growing HCT116 and HepG2 cells were cultured in standard and serum starved media and treated with ActD at a final dose of 8 nM. To evaluate cell cycle distribution, cells were harvested and then fixed overnight with EtOH 70% solution at -20°C. Cells were incubated with Propidium iodide/RNase staining buffer (BD Biosciences Pharmingen, San Jose, California, USA) and analyzed with FACS (Facs CantoII, BD Biosciences Pharmingen). Results were analyzed with use of ModFit LT software.

Apoptosis was determined by Annexin V–FITC and propidium iodide double staining according to the manufacturer's instructions (Roche Applied Science, Basel, Switzerland). Data were collected on a FACS flow cytometer (Facs CantoII, BD Biosciences Pharmingen).

**Clonogenic assay**

HCT116 cells were seeded in normal medium. After c-MYC siRNA interference for 48h, the cells were collected and re-seeded in triplicate at the concentration of 1000 cells/well then treated with ActD for 24 h. The drug was removed and the cells were grown for 14 days in normal media. At the end of the period the cells were fixed in methanol and stained with 0.5% crystal violet in 25% methanol for 20 min. Finally, the colonies were

counted. Results are presented as average of the number of colonies counted in each well for each condition.

**Statistical analysis**

Correlations between parameters that are considered to be continuous variables were analyzed using the Spearman rank correlation test. Differences between groups were evaluated by Student's t–test and p values $< 0.05$ were considered statistically significant.

# RESULTS

**The level of p53 stabilization after the treatment with ribosome biogenesis inhibitors is directly proportional to the amount of rRNA synthesis of the cell.**

In order to evaluate the effect of rRNA synthesis inhibition on p53 stabilization we considered six human tumor cell lines HCT116, HepG2, U2OS, MCF7, LoVo and ZR-75-1 characterized by different levels of ribosome biogenesis. We evaluated the amount of this process both by measuring the radioactivity incorporated in the cells after incubation with $^{3}$H-UTP and by the immunocytochemical analysis of 5-Fluorouridine labeling. The results, showed in fig.1 and 2, allowed us to divide the cell lines into two groups: one characterized by high levels of rDNA transcription (HCT116, HepG2 and U2OS) and one characterized by low rDNA transcription levels (MCF7, LoVo and Zr-751).

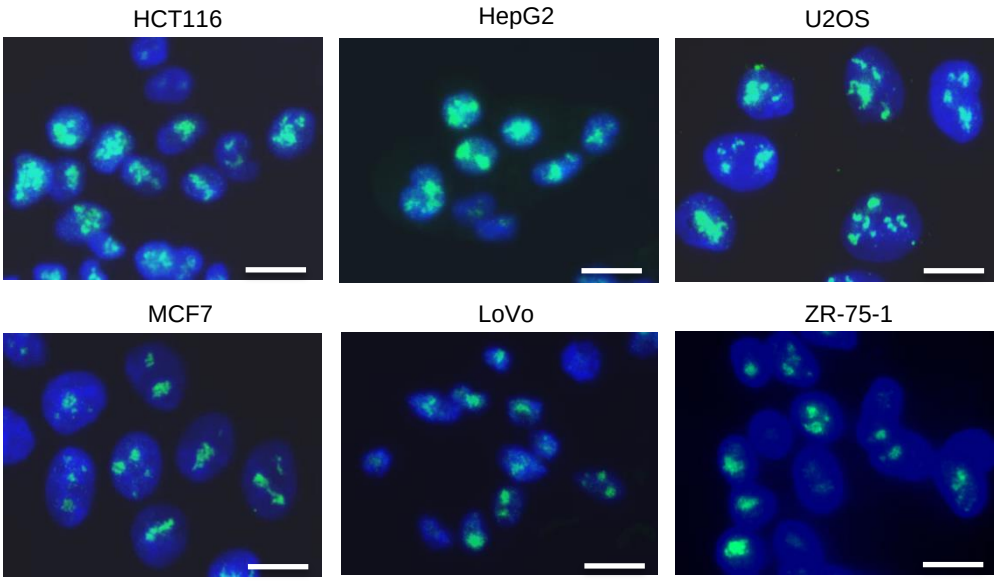



**Figure 7: Visualization of rRNA synthesis**. Cells were labeled with 5-fluorouridine for 20 minutes and the fluorescence was revealed by specific FITCH-conjugated monoclonal antibody. DAPI counter-staining. The labeling is higher in HCT116, HepG2 and U2OS cells than in MCF7, LoVo and ZR-75-1 cells. Scale bar: 10 μm.

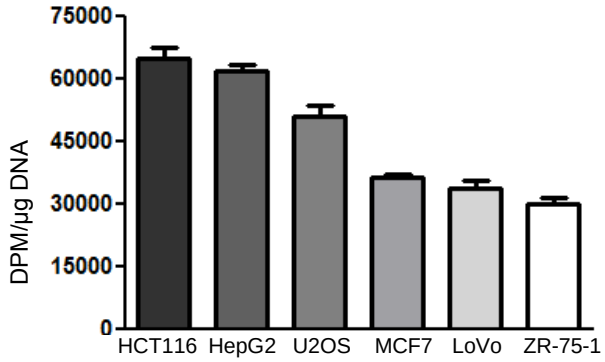
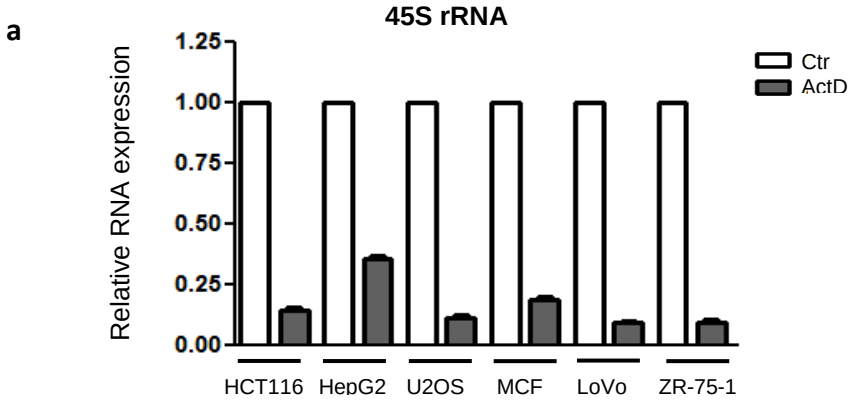



**Figure 8: Characterization of rRNA transcription level**. rRNA synthesis was measured in HCT116, HepG2, U2OS, MCF7, LoVo and ZR-75-1 cell lines by the evaluation of [3H]-uridine radioactivity incorporated in the total RNA fraction (DPM/µg DNA).

We treated all the six cell lines for 12 hours with ActD at the concentration of 8 nM, which was proved to selectively inhibit the process of rDNA transcription (Perry and Kelley 1970). The ActD effect on rRNA synthesis inhibition was evaluated by quantitative Real-Time RT-PCR of the 45S rRNA precursor: the drug induced a strong inhibition of 45S rRNA synthesis, higher than 75% in all the six lines. We performed western blot analysis to evaluate the consequences of rRNA inhibition on p53 stabilization level in all the cell lines. The level of p53 stabilization was significantly higher in HCT116, HepG2 and U2OS than in the MCF7, LoVo and Zr-75-1 and it was linearly correlated with the rRNA synthesis of the untreated cells ($r = 0.933$; $P = 0.006$). These results suggested that the level of p53 stabilization after the inhibition of rDNA transcription might depend on the rate of ribosome biogenesis of the cells.



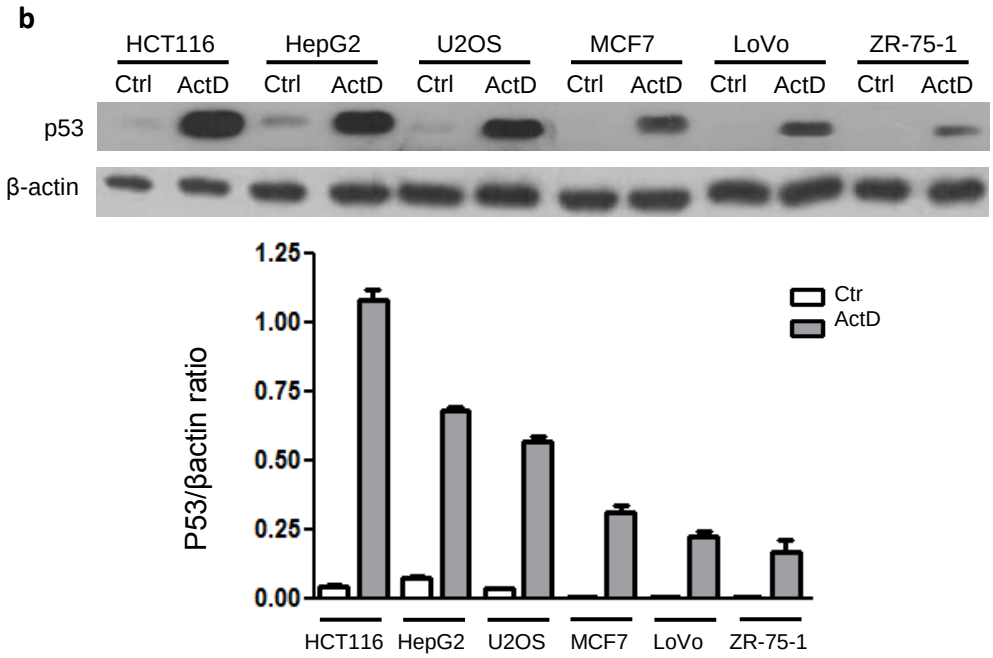



**Figure 9: The level of p53 stabilization induced by ActD is directly related to the cell rRNA transcription rate.** a) Real-time PCR analysis of 45S rRNA expression in cells treated with 8nM ActD for 12 h. The reduction of 45S rRNA after ActD exposure is similar in all cell lines. b) Representative Western blot and densitometric analysis of p53 expression in control and ActD treated cells. The level of p53 stabilization after ActD treatment is higher in HCT116, HepG2 and U2OS cells than in MCF7, LoVo and ZR-75-1 cells. Histograms show the values (mean ± s.d.) of three experiments.

To confirm these data and to exclude that genetic differences among the cell lines could have influenced the results we selected HCT116 and HepG2, the two lines with the higher levels of ribosome biogenesis, and we downregulated the synthesis of rRNA within the same cell line by growing them in serum deprived media (Donati et al. 2011).

The cells grew in normal and serum free media for 24 hours before being treated with 8nM ActD for 12 hours. The level of rDNA transcription was significantly decreased in both cell lines after serum starvation, as assessed by Real-Time PCR analysis of 45S rRNA. The treatment with ActD induced a stabilization of p53 which was higher in the cells grew in normal media than in serum starved ones; in fact the cells in normal media were characterized by higher levels of ribosome biogenesis than the cells in serum free media.

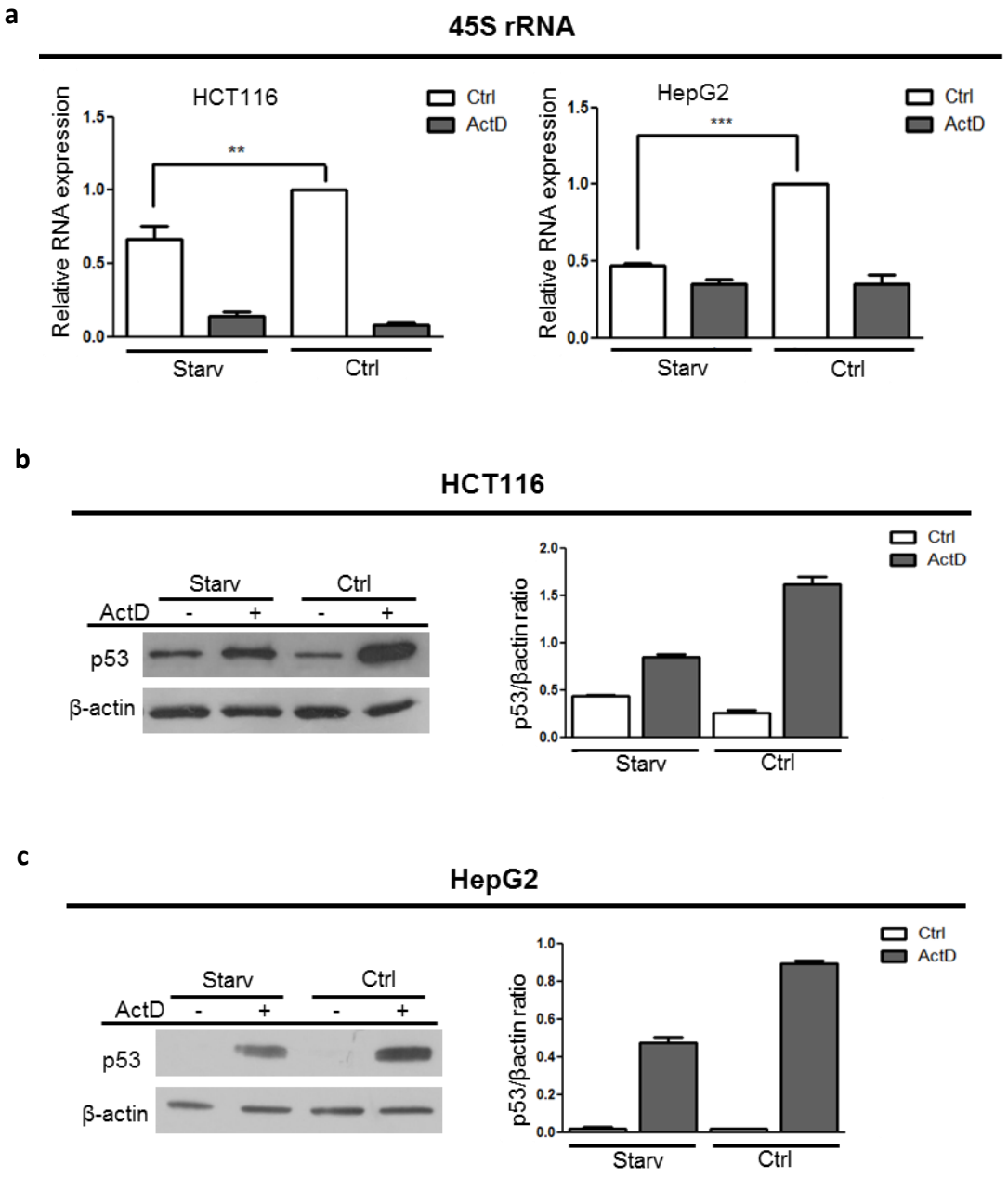



**Figure 10: Down-regulation of rRNA synthesis by serum starvation reduces p53 stabilization after ActD treatment.** a) Real time RT-PCR analysis of 45S rRNA expression in serum starved and control HCT116 and HepG2 cells. The cells were cultured at low serum concentration for 24h and then exposed to 8nM ActD for 12h. The 45S rRNA expression is lower in serum starved cells compared with control cells. ActD exposure causes a similar reduction of 45S rRNA synthesis. b) and c) Western blot and densitometric analysis of p53 expression in serum starved and control HCT116 and HepG2 cells treated with 8nM ActD for 12h. The level of p53 stabilization is significantly higher in control than in serum starved cells. Histograms show the values (mean ± s.d.) of three experiments. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

We analyzed by Real Time RT-PCR the levels of *TP53* mRNA, both in the six cell lines and in the HCT116 and HepG2 with or without serum in the media, to exclude the possibility that these results were influenced by different levels of transcription of the gene that could slow down the protein translation process.

The results showed that in the six cell lines the levels of *TP53* transcription were not correlated to the levels of protein stabilization after ActD treatment and that the serum starvation did not influence the levels of *TP53* mRNA expression.

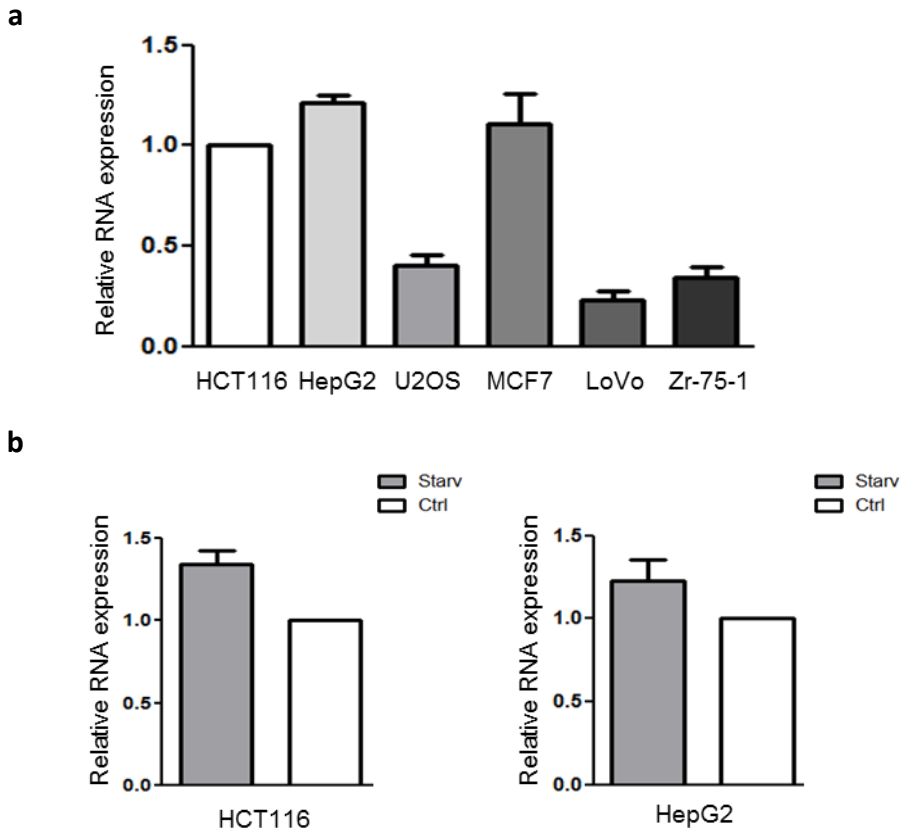



**Figure 11: TP53 rRNA expression is not correlated to the level of p53 protein stabilization after ActD exposure.** a) Real time RT-PCR analysis of *TP53* mRNA expression in a) the six cell lines and b) the serum starved and control HCT116 and HepG2 cells. The cells were cultured at low serum concentration for 24h.
Histograms show the values (mean ± s.d.) of three experiments.

We also evaluated the effects on p53 stabilization of three other drugs which hinders ribosome biogenesis: Doxorubicin (Doxo), 5-fluorouracil (5-FU) and CX-5461 (Burger et al. 2010; Bywater et al. 2012). We treated the cell lines HCT116, HepG2, MCF7 and LoVo with the tree drugs separately, at a dose able to inhibit ribosome biogenesis, and we observed in any case greater p53 stabilization in HCT116 and HepG2, which are characterized by high levels of ribosome biogenesis, then in MCF7 and LoVo.

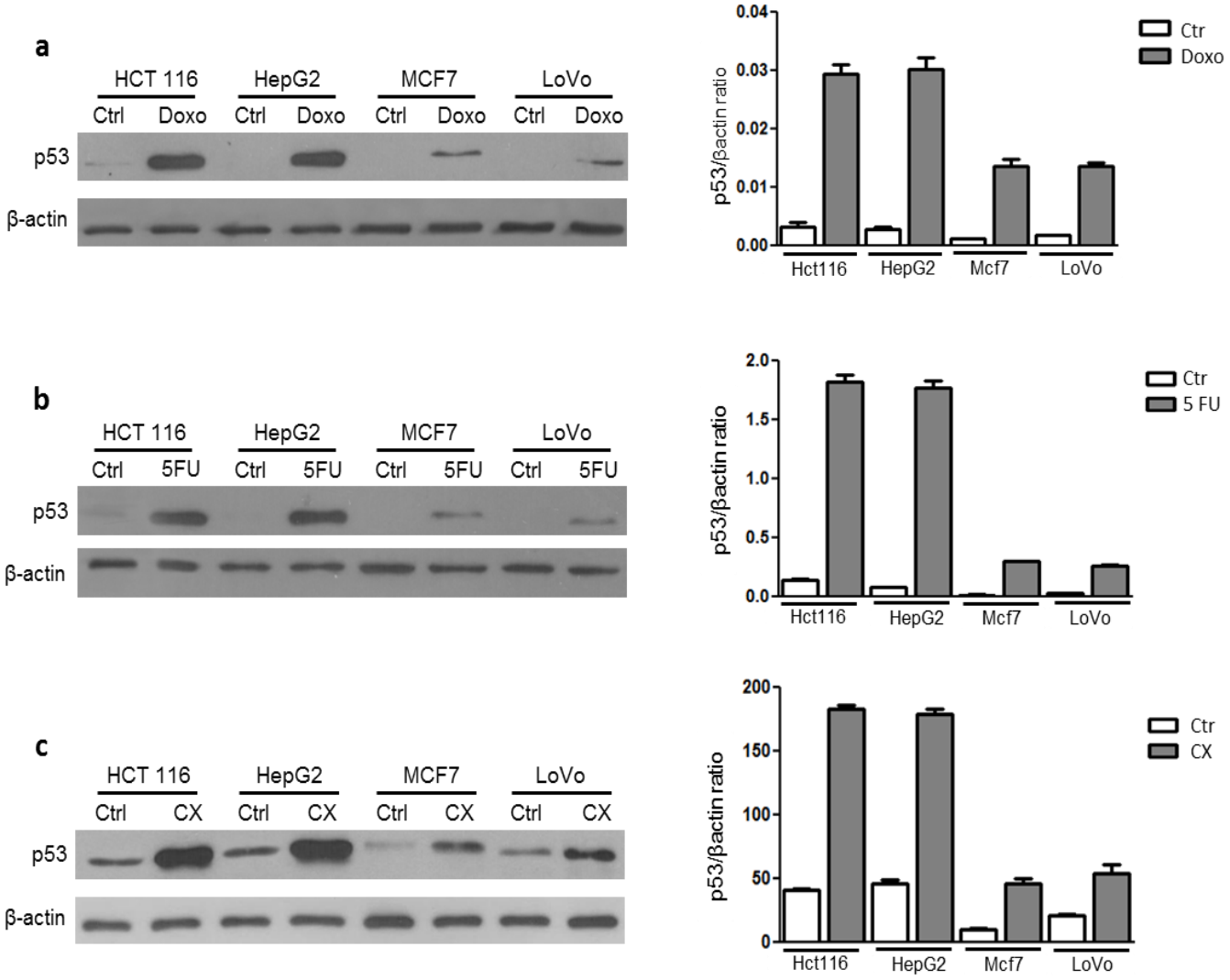



**Figure 12 : the level of p53 stabilization induced by Doxorubicin, 5-Fluorouracyl and CX-5461 is directly related to the cell rRNA transcription rate.** Western blot evaluation and densitometric analysis of p53 expression in HCT116, HepG2, MCF7 and LoVo cells treated with a) 10 µM Doxo for 12 h, b) 20µg/ml 5-FU for 12 h and c) 1µM CX-5461 for 12 h. Histograms show the values (mean ± s.d.) of three experiments.

To further investigate the relationship between ribosome biogenesis and p53 stabilization after rRNA synthesis inhibition, we isolated human peripheral blood lymphocytes form healthy donors and we incubated them with 10 μM PHA for 72 hours. The incubation with PHA increased the lymphocytes rDNA transcription rate as shown in fig. 12a. The treatment with 8 nM ActD for 12 hours caused the stabilization of p53 in a higher extent in the PHA stimulated lymphocytes compared with the control cells.

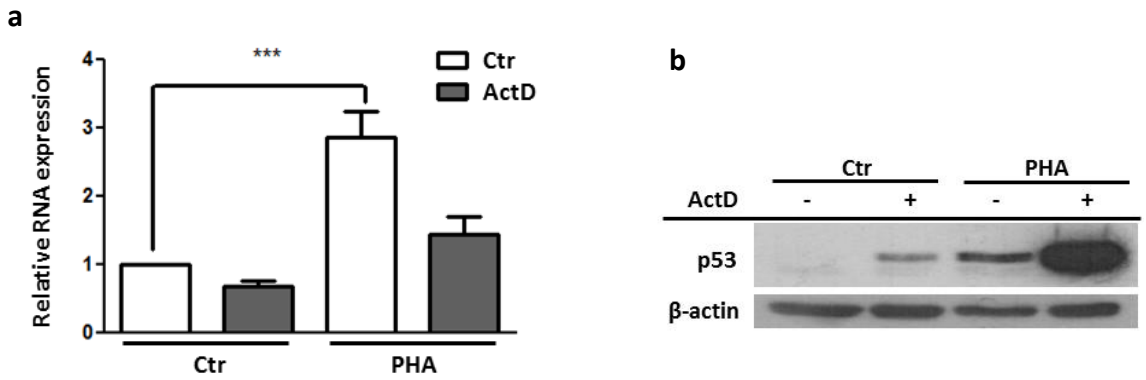



**Figure 13: p53 stabilization after ActD treatment is higher in PHA stimulated lymphocytes**. a) Real time RT-PCR analysis of 45S rRNA expression in control and PHA stimulated lymphocytes. The cells were cultured with 10 μM PHA for 72h and then exposed to 8nM ActD for 12h. The 45S rRNA expression increases after PHA stimulation and decreases after ActD treatment in both experimental conditions.
b) Western Blot evaluation of p53 expression in control and PHA stimulated lymphocites treated with 8nM ActD for 12 h. The level of p53 stabilization is higher in PHA stimulated lymphocytes than in control cells. Histograms show the values (mean ± s.d.) of three experiments. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

**The level of p53 stabilization after treatment with drugs that do not inhibit ribosome biogenesis is independent from the amount of rRNA synthesis of the cell.**

To further confirm the results obtained until now, we investigated whether the level of p53 stabilization induced by treatments with drugs that do not inhibit rRNA synthesis was independent or not from the ribosome biogenesis transcription rate of the cell.

Therefore we utilized two drugs which stabilize p53 without interfering with ribosome biogenesis: Hydroxyurea and Nutlin-3. HU inhibits the enzyme ribonucleotide reductase and decreases the production of deoxyribonucleotides, this causes the activation of ATM that phosphorylates p53 preventing its association with MDM2 (Ho et al. 2006). Nutlin-3 causes p53 accumulation by inhibiting selectively Mdm-2 (Vassilev et al. 2004).

We treated four cell lines characterized by different ribosome biogenesis rate, HCT116, HepG2, MCF7 and LoVo, with 3, 4 mM HU for 12 hours. The stabilization of p53 after the treatment was quite similar in the four cell lines and it was not proportional to the ribosome biogenesis levels. The same result was obtained after treating with HU the cells HCT116 and HepG2 grown in media with or without serum.

To exclude the possibility that the differences in p53 stabilization were caused by differences in Mdm2 amount in the cells, which after the treatment with rRNA synthesis inhibitors released different amounts of p53 protein, we treated HCT116 and HepG2 control and serum starved cells with 10 μM Nut for 12 hours. Also in this case, we found that p53 stabilization was independent from the ribosome biogenesis rate of the cell.

These results showed that p53 stabilization is proportional to the ribosome biogenesis level only when induced by treatments that inhibit rRNA synthesis or processing, but not when the drugs utilized act through a pathway different from the RP-MDM2-p53 one.

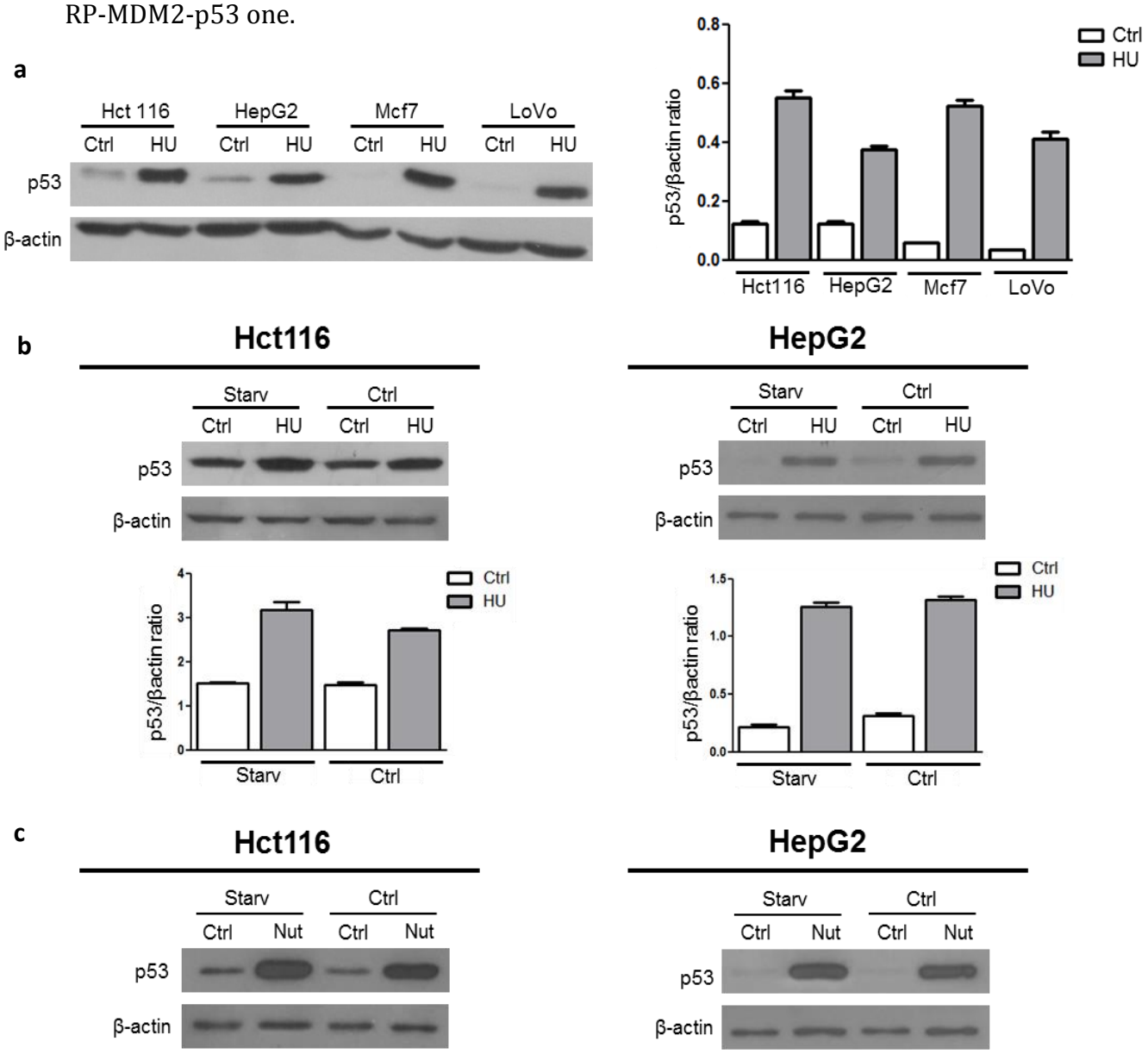



**Figure 14: The level of p53 stabilization induced by drugs that do not act on ribosome biogenesis is not related to the cell rDNA transcription rate.** a) Western blot evaluation and densitormetric analysis of p53 expression in HCT116, HepG2, MCF7 and LoVo cells after 3,4 mM HU treatment for 12 h. The amount of p53 stabilization is quite similar in all cell lines. b) Western blot and densitometric analysis of p53 expression in serum starved and control HCT116 and HepG2 cells treated with 3.4 mM HU for 12h. The amount of p53 stabilization is similar in serum starved and control cells. c) Representative Western Blot evaluation of p53 protein expression in serum starved and control HCT116 and HepG2 cells treated with 10 μM Nutlin-3 for 12 h. The level of p53 stabilization is higher in the serum starved cells than in the controls. Histograms show the values (mean ± s.d.) of three experiments. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

**The differences in p53 stabilization level after treatment with ribosome biogenesis inhibitors are caused by a different amount of RPs bound to MDM2.**

It is known that the rRNA synthesis inhibition can induce the release of ribosomal proteins that, no longer utilized for ribosome building, are free to bind to MDM2, inhibiting its ubiquitin ligase function and causing p53 accumulation in the cell.

Therefore we investigated whether the differences observed in the stabilization of p53 after ActD treatment, in cells characterized by different levels of ribosome biogenesis, were connected to this pathway.

We first silenced the ribosomal protein *RPL11* gene expression with RNA interference for 48 hours in HCT116 and LoVo cells and then we treated them with 8 nM ActD for 12 hours. We evaluated with Real Time RT-PCR the efficiency of RNA interference for *RPL11* and it resulted higher than 90% in both cell lines. The western blot analysis, performed using the nuclear proteins fraction, showed that in the interfered cells the expression of RPL11 decreased and the ActD treatment could not induce p53 stabilization, whereas in the control cells p53 stabilization was higher in HCT116 than in LoVo cells. This result confirmed that the p53 stabilization was dependent on the RPs-MDM2-p53 pathway and suggested that the differences observed in the level of p53 stabilization could be caused by a major quantity of RPL11 bound to MDM2 in the cells with high ribosome biogenesis after rRNA synthesis inhibition.

In order to demonstrate this mechanism, we performed an immunoprecipitation for MDM2 in HCT116 and LoVo cells treated with ActD and we analyzed the levels of RPL11 co-precipitated with it (fig. 15 c). We found that the amount of RPL11 bound to MDM2 was greater in the HCT116 than in the LoVo cells after ActD exposure. Indeed also the amount of p53 unbound to MDM2, thus not precipitated with it, was higher in

HCT116.

These results showed that the differences observed in p53 stabilization after the inhibition of rRNA synthesis were caused by different levels of RPL11 which bound MDM2 inactivating its ubiquitin-ligase activity.

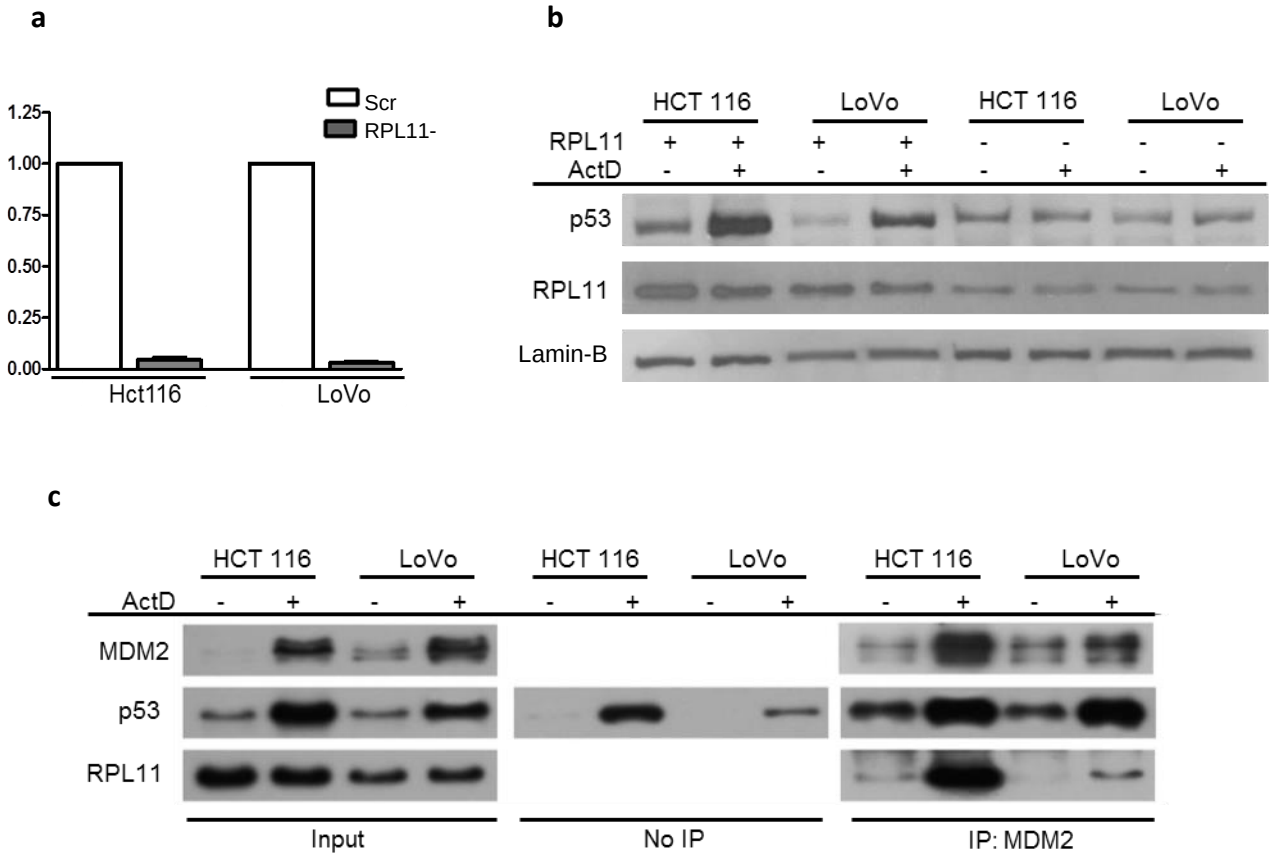



**Figure 15: The differences in p53 stabilization are caused by different amount of RPL11 bound to MDM2.** a) Real Time RT-PCR of RPL11 mRNA expression in HCT116 and LoVo cells to evaluate the efficiency of *RPL11* silencing. The cells were interfered with *RPL11* siRNA for 48h. b) Western blot evaluation of p53 expression in control and *RPL11* silenced HCT116 and LoVo cells treated with 8nM ActD for 12h after the end of the silencing procedure. RPL11 mRNA interference abolishes p53 stabilization after ActD treatment. c) Western blot evaluation of the amount of RPL11 bound to MDM2 in HCT116 and LoVo cells after treatment with 8 nM ActD for 12 h. Input shows that after ActD treatment p53 stabilization is higher in HCT116 than in LoVo cells. After immunoprecipitation with anti-MDM2 polyclonal antibody, the amount of RPL11 bound to MDM2 (IP:MDM2) is higher in HCT116 than in LoVo cells. Also the amount of p53 in the not immunoprecipitated (No IP) fraction is higher in HCT116 cells than in LoVo cells. Histograms show the values (mean ± s.d.) of three experiments.
* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

**The block of the cell cycle after rRNA synthesis inhibition is induced independently from the level of p53 stabilization.**

We wondered whether the differences observed in p53 stabilization levels after ActD exposure could cause a different effect in the progression through the cell cycle phases. To investigate this effect we measured the transcriptional activation of p21, a p53 target gene, using Real Time RT-PCR analysis in control and serum starved HCT116 and HepG2, after ActD exposure. The treatment caused an increase in p21 mRNA expression significantly higher in control than in serum-starved cells. The expression of p21 protein was also evaluated in control and serum-starved HCT116 and HepG2 cells: after ActD exposure, the amount of p21 was higher in control than in serum starved cells.

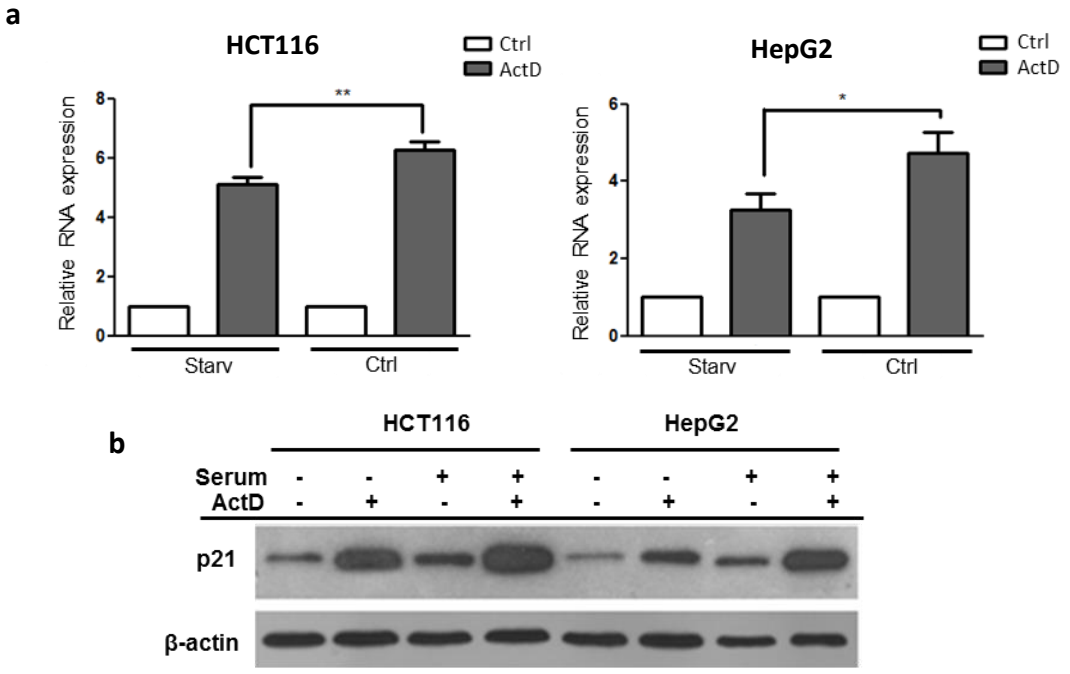



**Figure 16: p53 stabilization activates p21 mRNA transcription and protein expression**. a) Real-time RT–PCR analysis of p21 mRNA transcription in control and serum starved HCT116 and HepG2 cell lines. HCT116 and HepG2 cells were cultured at low serum concentration for 24h and treated with 8nM ActD for 12h. p21 mRNA was significantly higher in control than in starved cells. Histograms show the values (mean ± s.d.) of three experiments. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$. b) Representative Western blot of p21 expression in control and serum-starved HCT116 and HepG2 cell lines. Starved and control cells were treated with 8 nM ActD for 12h. The p21 expression after ActD exposure is higher in control than in starved cells.

The protein p21 is an inhibitor of CDK-Cyclin complexes and it is of primary importance in the transition from phase G1 to S. To investigate whether different levels of p21 could cause a different effect on cell cycle progression we performed a flow cytometry analysis. Both HCT116 and HepG2 cell lines were grown in normal or serum free media for 24 hours, then treated with ActD for 12 hours and their distribution through the cell cycle phases analyzed. Serum starvation induced in both cell lines a reduction in the number of cells in S phase. Anyway after ActD treatment the normally fed as well as the serum starved cells accumulated in the G1 phase in a similar manner. This result showed that the lower amount of p53 stabilized was sufficient to induce the block of the cell cycle progression in G1 phase at the maximum extent; indeed the higher levels of p53 did not increase this effect.

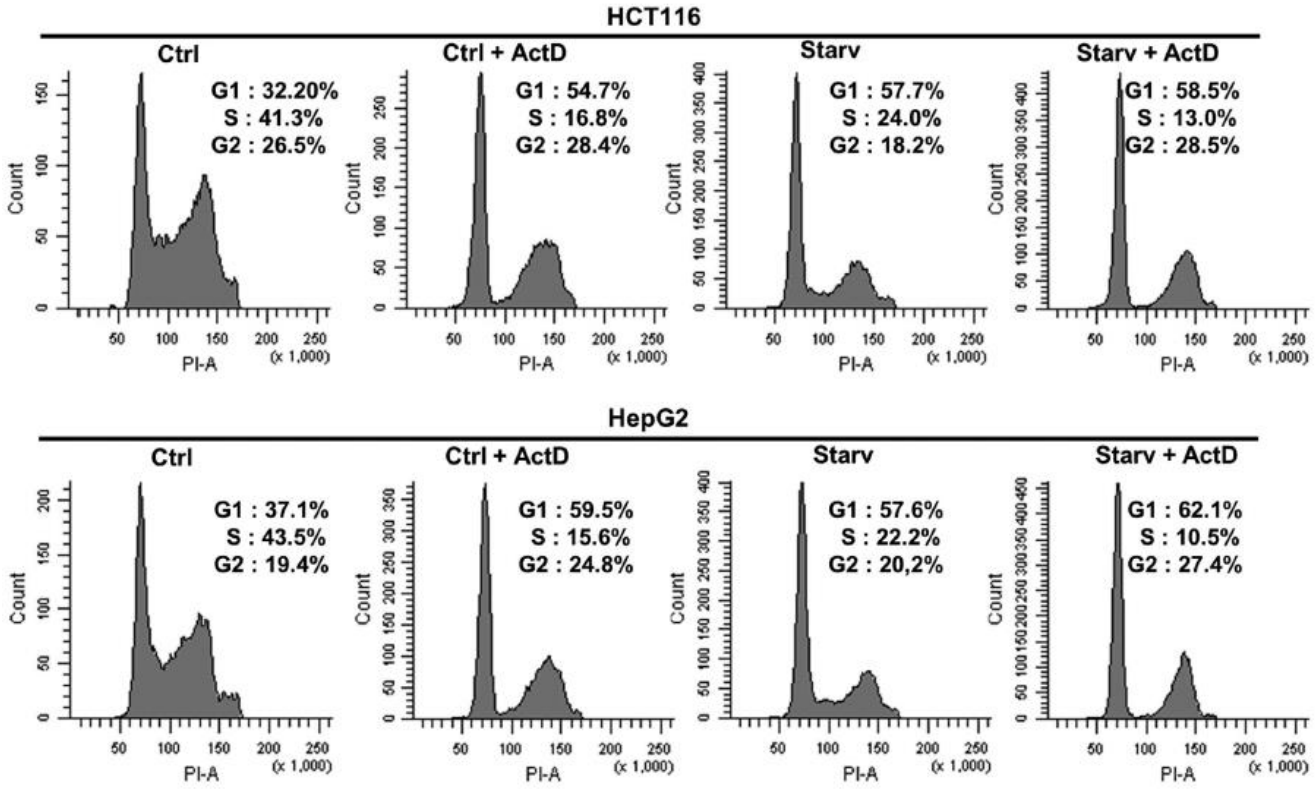



**Figure 17: rRNA synthesis inhibition causes cell cycle progression arrest.** Representative flow cytometry DNA profiles of control and serum starved HCT116 and HepG2 cells after 8nM ActD treatment for 12 hours. Cells accumulate mainly in G1/S phase. No differences were observed in the profile of serum starved and control cells after ActD exposure.

**The apoptosis after rRNA synthesis inhibition is induced only in cells with high ribosome biogenesis rate in which p53 stabilization is elevated.**

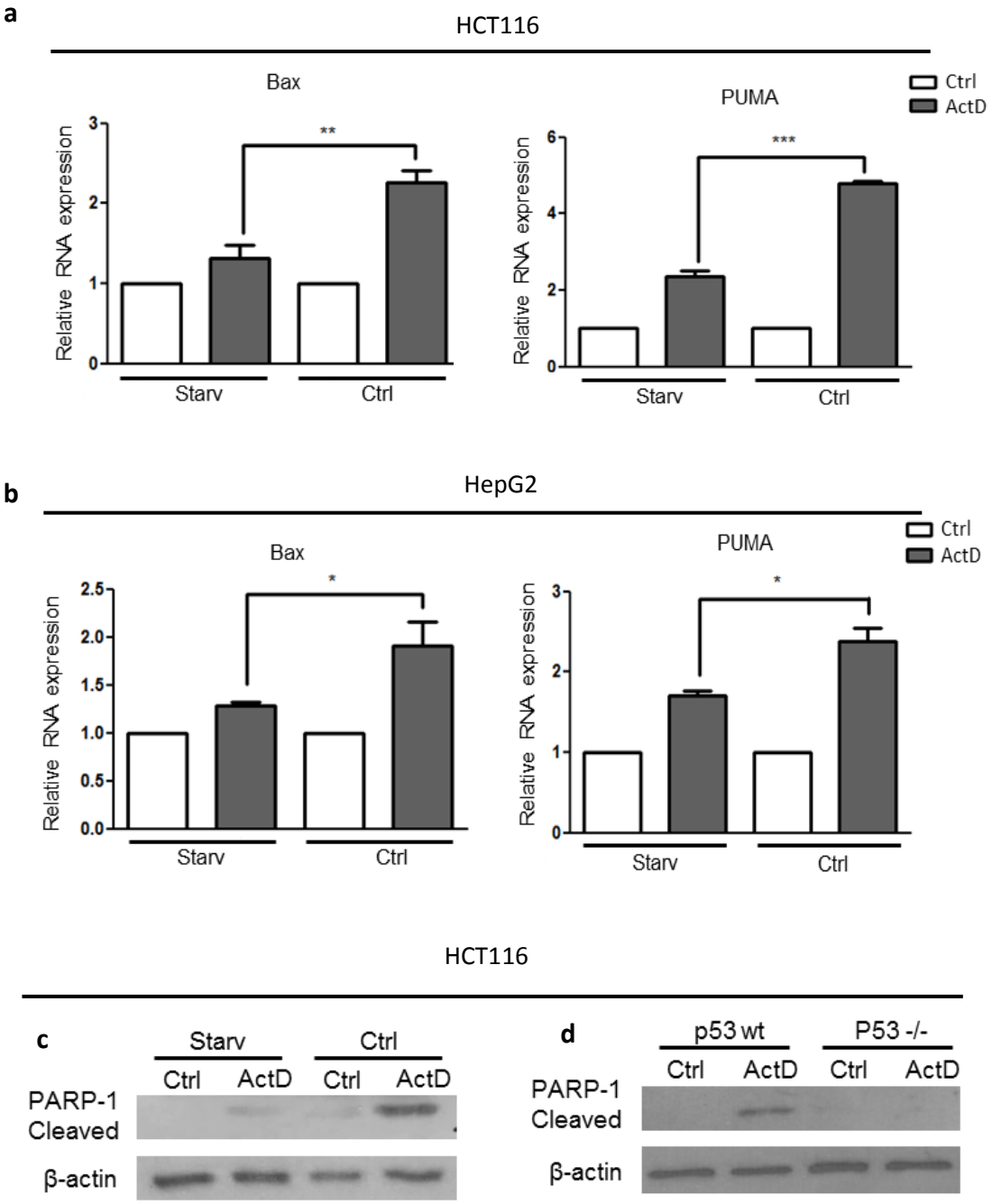
Once showed that the different levels of p53 stabilized produced the same effect on the progression through the cell cycle we wondered whether the effects on the induction of apoptosis could be different proportionally to p53 amount in the cell.

We evaluated the mRNA transcription activation of two p53 target genes connected with apoptosis induction: *BAX* and *PUMA.* In control and serum starved HCT116 and HepG2, the 12 hours treatment with 8 nM ActD caused an increase in *BAX* and *PUMA* mRNA transcription significantly higher in control than in serum-starved cells.

To investigate whether the higher transcription of pro-apoptotic genes after rRNA synthesis inhibition could influence the amount of apoptosis we analyzed the cleavage of the protein poly (ADP-ribose) polymerase 1 (PARP-1) in normal fed and serum starved HCT116 after 12 hours of 8 nM ActD exposure. PARP-1 is an early marker of chemotherapy induced apoptosis that is cleaved by activated caspases (Kaufmann et al. 1993). Only normal fed cells showed PARP-1 cleavage after ActD treatment. This result was confirmed by flow cytometry analysis of Annexin V positive cells; Annexin V binds to the phosphatidylserine (PS) expressed on the cell surface during apoptosis (Koopman et al. 1994). Only the control cells showed an increase in Annexin V positivity after ActD treatment.

To evaluate the dependence of apoptosis induction on p53 stabilization we considered HCT116 p53 null cells (p53 -/-) and we treated them with 8 nM ActD for 12 hours. The treatment did not induce any PARP-1 cleavage in the p53-/- cells whereas the cleavage was observed in wild-type (wt) p53 HCT116.

We also examined apoptosis induction after ActD treatment in the six cell lines, characterized by different ribosome biogenesis levels through the analysis of PARP-1 cleavage. The apoptosis was induced only in the cell lines characterized by high ribosome biogenesis levels HCT116, HepG2 and U2OS in which ActD treatment caused the greatest amounts of p53 stabilized.



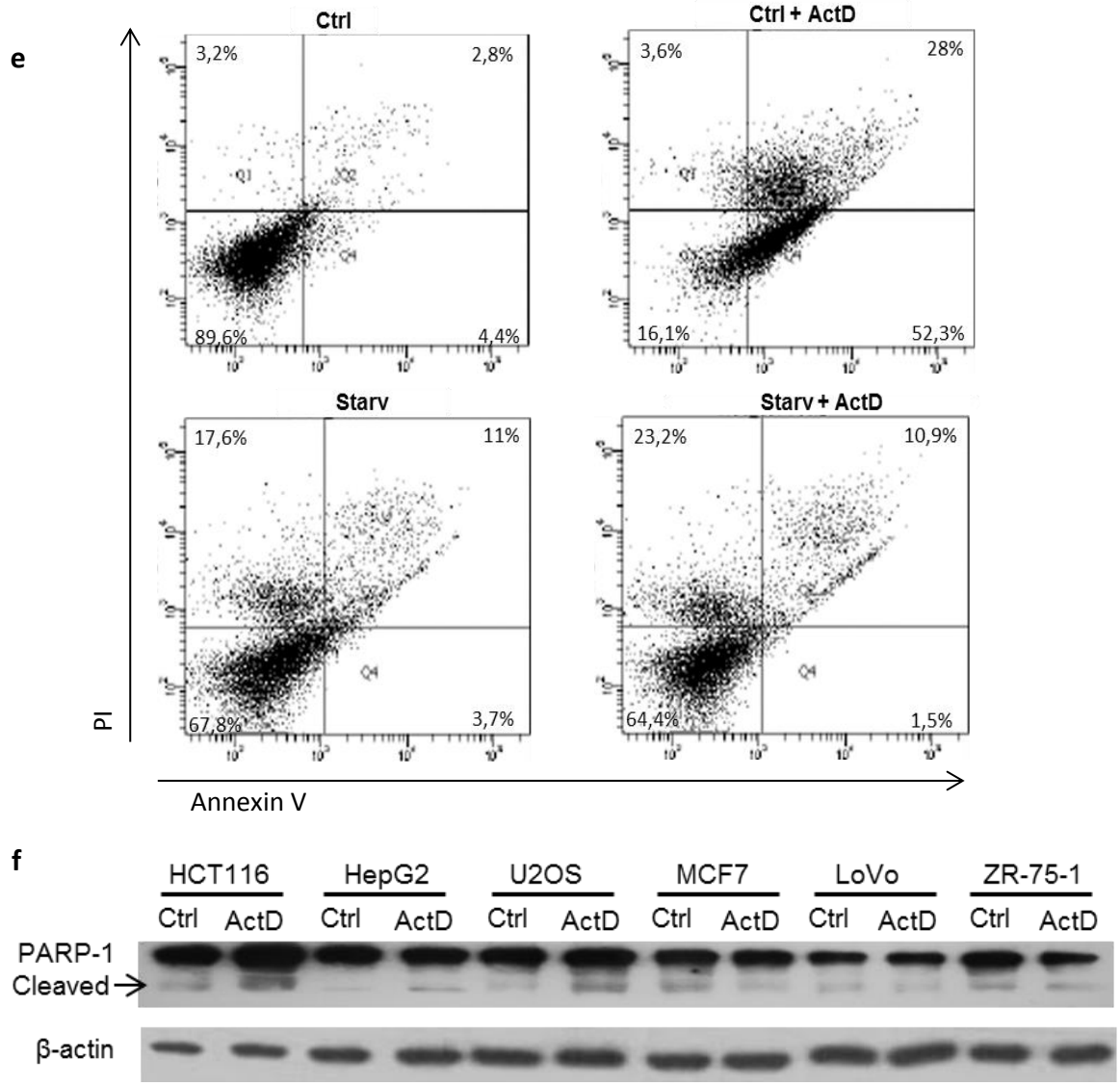
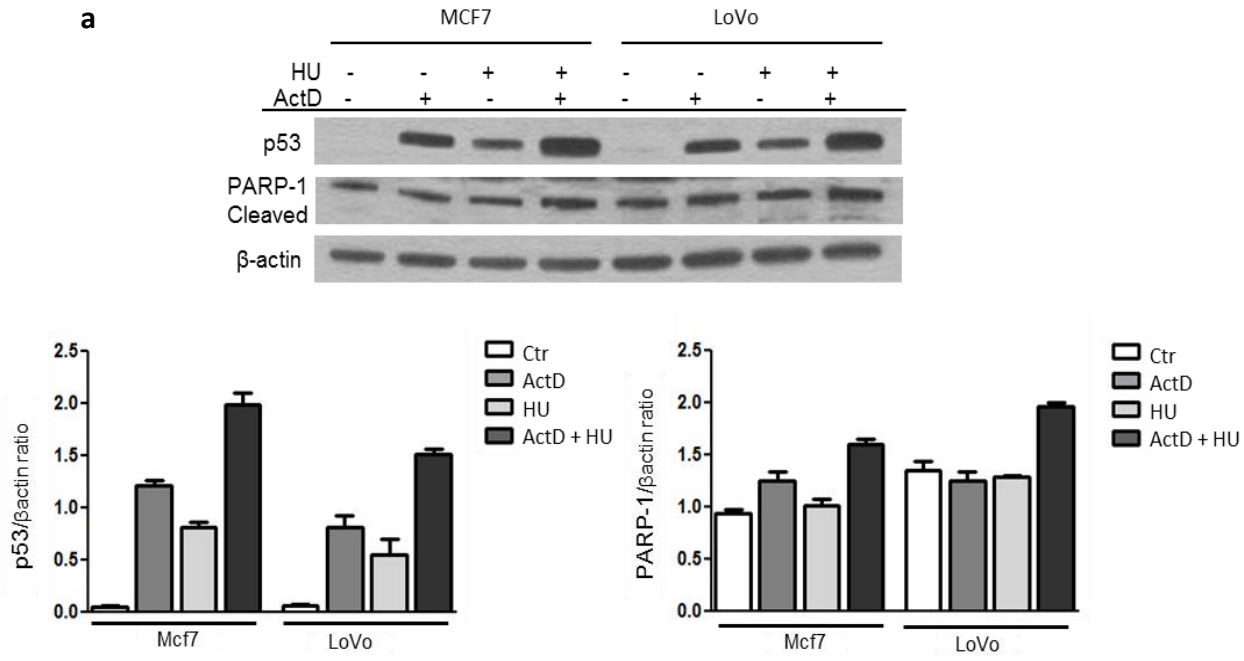



**Figure 18: rRNA synthesis inhibition induces apoptosis only in the cells with high ribosome biogenesis rate**. a) and b) Real time RT–PCR analysis of the mRNA expression of *BAX* and *PUMA* in control and serum starved HCT116 and HepG2 cell lines treated with 8 nM ActD for 12 h. *BAX* and *PUMA* mRNA was significantly higher in control than in starved cells. c) Western blot of PARP-1 cleavage in HCT116 serum starved and control cells treated with ActD. Cleaved PARP-1 is present only in control treated cells.
d) Western blot evaluation of PARP-1 cleavage in HCT116 p53 wt and p53-/- cells treated with 8 nM Act for 12 h. No cleavage of PARP-1 occurred in p53-/- cells after ActD exposure. e) Flow cytometry DNA analysis for apoptosis detection using Annexin V–FITCH/propidium iodide double staining. Serum-starved and control HCT116 were treated with 8 nM ActD for 24 h. Percentages of cells showing apoptosis (right quadrants) are shown in the boxes. f) Western blot of cleaved PARP-1expression in control and ActD treated HCT116, HepG2, U2OS, MCF7, LoVo and ZR-75-1. After 8 nM ActD treatment for 12 h, cleaved PARP -1 increases in HCT116, HepG2, U2OS cells but not in MCF7, LoVo and Zr-75-1. Histograms show the values (mean ± s.d.) of three experiments. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

**Drugs which do not inhibit ribosome biogenesis cooperate with rRNA synthesis inhibitors in the stabilization of p53.**

Taking into consideration the different mechanism of action by which HU and ActD act, we decided to evaluate whether the combination of the two drugs could induce greater p53 stabilization compared with that obtained after the single drug treatment.

We exposed the four cell lines HCT116, HepG2, Mcf7 and LoVo to 3,4 mM HU and 8 nM ActD, alone or combined, for 12 hours. The combined treatment caused an increase in p53 stabilization compared to the single drug treatments only in the Mcf7 and LoVo cell lines, the ones characterized by low levels of ribosome biogenesis. In parallel we analyzed PARP-1 cleavage, after the singles or the double treatments, and we found that only in the Mcf7 and LoVo cells the combination of the two drugs increased the protein cleavage. On the contrary, in the HCT116 and HepG2 cells no increase in p53 stabilization and PARP-1 cleavage was observed after the combined treatment compared with the single treatments.



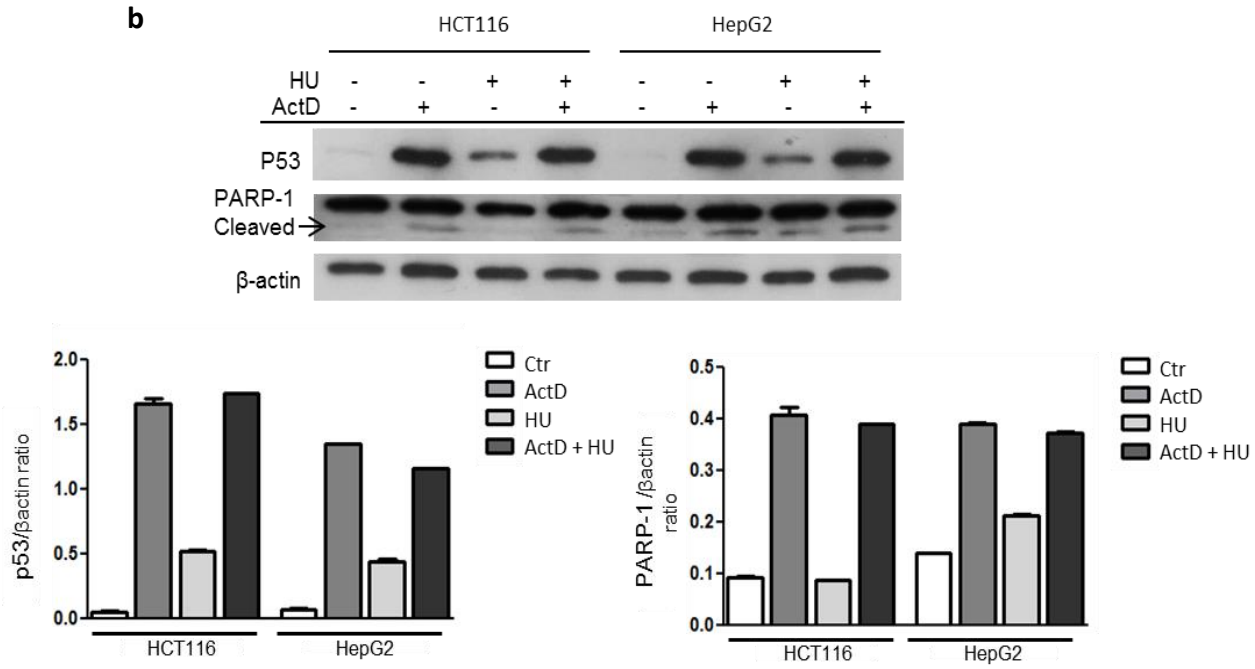



**Figure 19: Drugs not acting on ribosome biogenesis may cooperate with inhibitors of rRNA synthesis in p53 stabilization**. a) Representative western blot and densitometric analysis of p53 expression and PARP-1 cleavage in MCF7 and LoVo cells. Cells were treated with either 8nM ActD or 3.4 mM HU, separately or together for 12h. The contemporary treatment with HU and ActD causes a major p53 stabilization compared to single drugs exposure and induces an increase in PARP-1 cleavage in both cell lines.
b) Western blot and densitometric analysis of p53 expression and PARP-1 cleavage in HCT116 and HepG2 cells. Cells were treated with 8nM ActD and/or 3.4 mM HU for 12h. The contemporary treatment with HU and ActD does not increase the amount of p53 stabilization and PARP-1 cleavage in both cell lines, in comparison with that obtained after the exposure to ActD alone.
Histograms show the values (mean ± s.d.) of three experiments.

Accordingly with these results, the combination of rRNA synthesis inhibitors with drugs which do not inhibit ribosome biogenesis, such as HU, increased the effectiveness of the treatment only in the cells characterized by low ribosome biogenesis rate.

**rRNA synthesis inhibition causes apoptosis in the cells with MYC overexpression**

The research proceeded evaluating the relationship among MYC expression, the ribosome biogenesis level of the cell and the cytotoxic effects caused by p53 stabilization after rRNA synthesis inhibition. In fact, as previously explained in the introduction, the protein MYC has a stimulatory effect on ribosome biogenesis.

At first, we evaluated MYC protein expression in the six cell lines HCT116, HepG2, MCF7, U2OS, LoVo and Zr-75-1, comparing it with the rDNA transcription rate of the cells (showed in fig. 7). The result showed that the level of MYC expression was directly proportional to the ribosome biogenesis rate of the cells.

Moreover we transiently interfered *c-MYC* in the HCT116 cell line and we observed a decrease in both *c-MYC* mRNA and protein and a significant reduction of the rRNA synthesis level of the cells.

These results showed that MYC overexpression could be responsible for the differences in the levels of ribosome biogenesis among the different cell lines.

Next step was to investigate the effects of rRNA synthesis inhibition in cells characterized by different levels of MYC expression. After having reduced MYC expression by a 48 hours interference with siRNA in HCT116, which normally have high levels of MYC expression, we treated the cells with 8 nM ActD for 8 and 24 hours. The treatment induced a p53 stabilization which was greater in the not interfered cells, characterized by higher levels of ribosome biogenesis, then in the interfered cells where both MYC expression and ribosome biogenesis were reduced. We also showed that the treatment with rRNA synthesis inhibitors induced the apoptosis in the control cells but not in the *c-MYC* interfered cells. We analyzed the cleavage of PARP-1 after ActD exposure: it was higher in the control cell than in the *c-MY*C interfered cells.

Moreover we performed a clonogenic assay to confirm this result. We observed that 15 days after the treatment with 8 nM ActD for 24 hours the formation of colonies was greater in the *c-MYC* interfered cells than in the controls, probably because the drug induced the apoptotic death of a major number of cells in the control not-interfered cells than in the *c-MYC* silenced cells.

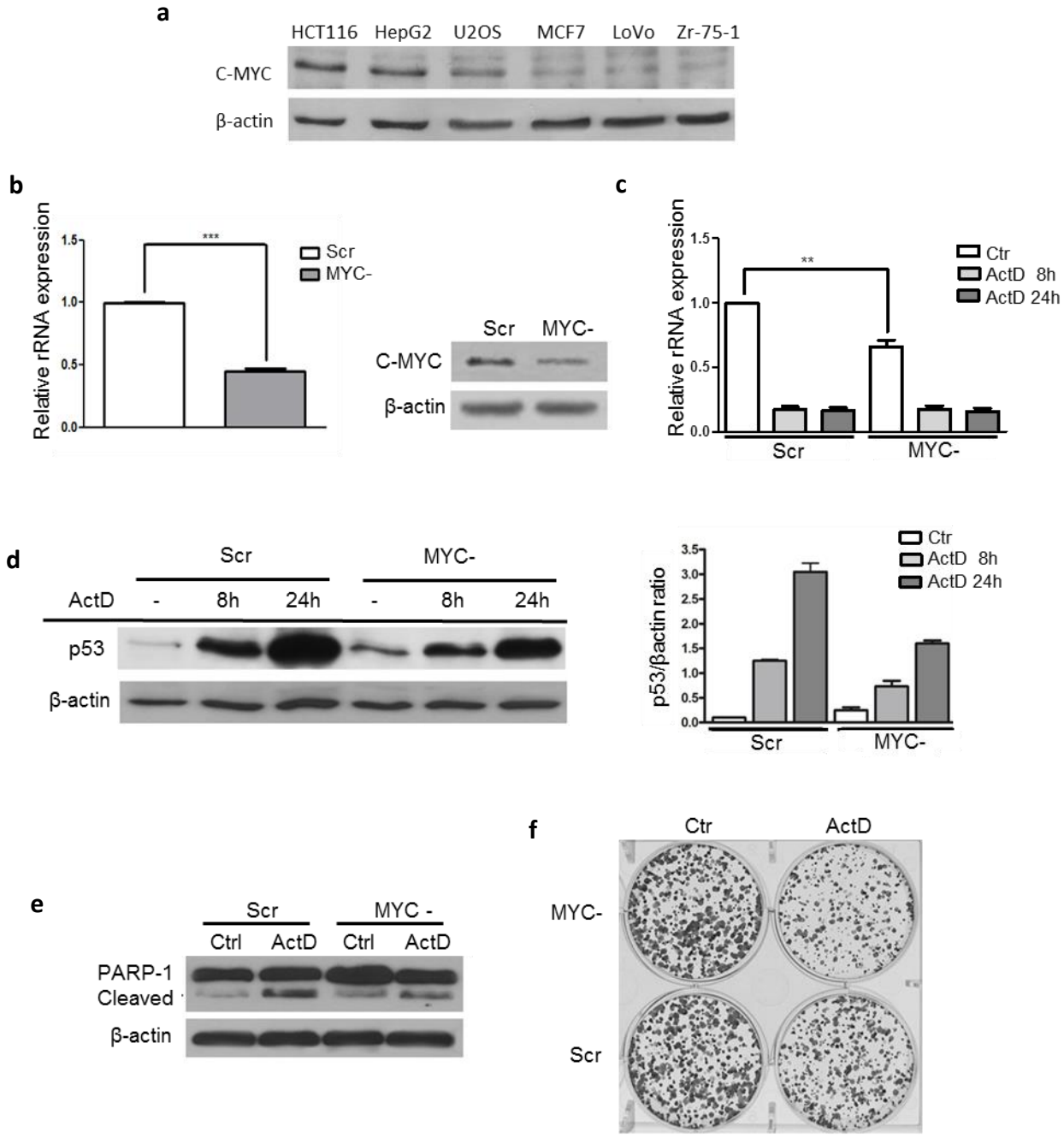


**Figure 20: rRNA synthesis inhibition induces apoptosis in the cells with MYC overexpression.** a) MYC expression is greater in HCT116, HepG2 and U2OS than in MCF7, LoVo and ZR-75-1 cells. b) Real-time RT-PCR and Western blot evaluation of *c-MYC* expression in HCT116 cells after 48h interference. c) Real time RT-PCR evaluation of 45S rRNA expression in control (Scr) and *c-MYC* silenced (MYC-) HCT116 cells treated with 8 nM ActD for 8 and 24h. The 45S rRNA is lower in interfered cells compared with control cells. d) Western blot and densitometric analysis of p53 expression in control and MYC- HCT116 cells. After ActD treatment the level of p53 stabilization is higher in control (Scr) than in MYC- cells. e) Western blot and densitometric analysis of cleaved PARP-1 in control (Scr) and MYC- HCT116 cells. After ActD treatment the expression of the cleaved PARP-1 is higher in control than in MYC- cells. f) Clonogenic assay performed with control (Scr) and MYC- HCT116 cells, treated with 8 nM ActD for 24h. After 15 days from ActD exposure the reduction of the number of colonies is greater in control than in MYC- HCT116 cells.
Histograms show the values (mean ± s.d.) of three experiments. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

These data showed that the cells in which MYC was overexpressed were characterized by high levels of ribosome biogenesis and that the treatment with rRNA synthesis inhibitors induced a great p53 stabilization that could cause the cell death by apoptosis.

# DISCUSSION

Many drugs utilized in cancer chemotherapy inhibit the ribosome biogenesis process evoking a p53 response, in p53 wild-type tumors, and the level of p53 stabilization may be important for the efficacy of the anticancer treatment. For this reason we studied the degree of p53 stabilization after treatments with rRNA synthesis inhibitors in relation with the cell ribosomal biogenesis rate, in cancer cell lines with functional p53. We showed that a direct relationship exists between the amount of p53 stabilized after rRNA synthesis inhibition and the ribosome biogenesis level of the cancer cells before the treatment.

We used several drugs which interfere with rRNA synthesis or processing: ActD, Doxo, 5-FU and CX-5461. ActD is an anticancer antibiotic which at low doses preferentially intercalates into the GC rich regions of rDNA inhibiting Pol I mediated transcription of nascent 47S rRNA (Perry and Kelley 1970). Doxorubicin is another anticancer antibiotic which intercalates in the DNA causing rRNA synthesis inhibition, but with less specificity for rDNA than ActD (Burger et al. 2010). 5-FU is a potent inhibitor of rRNA metabolism, indeed it was found to form stable adducts with dyskerin pseudouridine synthase. It confers cytotoxicity by the reduction of pseudouridylation of rRNA, which is a requirement for correct rRNA processing (Hoskins and Butler 2008). CX-5461 specifically blocks the recruitment of the SL1 complex which is essential for Pol I initiation and inhibits rRNA synthesis (Bywater et al. 2012).

When we treated with these drugs different cancer cell lines characterized by different levels of ribosome biogenesis we found that the amount of stabilized p53 was significantly greater in cells characterized by high ribosome biogenesis levels than in those with low rDNA transcription rate. The same result was obtained comparing cells

with high ribosome biogenesis rate with the same type of cells in which ribosome biogenesis rate was downregulated by serum starvation. In fact ActD treatment induced lower p53 stabilization in serum-starved cells compared with the normal feed ones.
This was not the case if drugs which do not interfere with rRNA synthesis, such as HU and Nutlin-3, were used. These drugs caused quite similar p53 stabilization levels in cell lines characterized by different ribosome biogenesis rate.

We demonstrated that the different levels of p53 stabilization induced by rRNA synthesis inhibitors in cells with high and low ribosome biogenesis rate were the consequence of the fact that different amounts of RPs, not anymore used in ribosome assembling, bind to the ubiquitin ligase MDM2 and hinder p53 proteosomal degradation. We observed that the downregulation of RPL11, one of the main RPs able to inactivate MDM2, deleted the differences observed in p53 stabilization between high and low ribosome biogenesis cells after ActD treatment. Furthermore, we showed that the amount of RPL11 that bind to MDM2, as a consequence of rRNA synthesis inhibition, was greater in cells with high ribosome biogenesis rate than in those with low ribosome biogenesis. In fact in the cells characterized by high rDNA transcription levels also a high production of RPs occurs and the inhibition of rRNA synthesis causes a large number of RPs, no longer used for ribosome building, to bind and neutralize a large number of MDM2 molecules, thus inducing high p53 stabilization. Whereas, the inhibition of ribosome biogenesis in the cells with low rDNA transcription levels induces few RPs to bind to MDM2, thus only partially neutralizing the ubiquitin ligase activity toward p53, with a consequent low-level p53 stabilization.

The following step of the present study was to assess the relationship between different amount of p53 stabilization after inhibition of rRNA synthesis and the degree of the induced cytotoxic effects. In the nucleus p53 works predominantly as a transcription factor that enhances the rate of transcription of several genes connected with cell proliferation and apoptosis (Ryan et al. 2001). We demonstrated that the mRNA transcription activation of the p53 target genes *p21, BAX* and *PUMA* was proportional to the level of p53 stabilization after rRNA synthesis inhibition.

Concerning the cytostatic effect, the protein p21 is a CDK inhibitor of primary importance in G1 regulation (Polyak et al. 1994). We observed that after rRNA synthesis inhibition the block of cell cycle progression occurred independent of the amount of p53 stabilization, with cells accumulation mainly in G1 phase.

Then, we investigated the effects on apoptotic cell death induction of different levels of p53 stabilization. We found that in cell lines with a high ribosome biogenesis rate the inhibition of rRNA synthesis resulted not only in the cell cycle arrest, but also in apoptotic cell death. In cell lines with low level of ribosome biogenesis the inhibition of rRNA synthesis did not cause apoptotic cell death. These different effects were dependent on p53 stabilization. In fact, when we treated HCT116 p53 null cells with ActD we did not observed the induction of apoptosis, at differences of p53 wild-type HCT116 cells. Altogether, these results indicate that after the treatment with rRNA synthesis inhibitors the activation of apoptosis is dependent on the level of p53 in the cell. This result is consistent with previous data described in literature (Lai et al. 2007).

These results might explain the findings by Bywater and colleagues; they studied the effects of the rRNA synthesis inhibitor CX-5461 in a mouse model of lymphoma with MYC overexpression (Eμ-Myc). This drug was able to induce a p53-mediated apoptotic cell death selectively in transformed malignant cells, sparing normal B cells. MYC in fact

is a powerful activator of ribosome biogenesis and for this reason we hypothesized that its overexpression could make the malignant cells more susceptible to treatments with rRNA synthesis inhibitors. In our study we demonstrated that the cancer cells characterized by MYC overexpression died by apoptosis after the treatment with rRNA synthesis inhibitors. Analyzing the expression of MYC protein we found a correlation with the levels of ribosome biogenesis of the cells. Moreover the *c-MYC* gene silencing decreased the rRNA synthesis rate in HCT116 cells and after ActD treatment the level of p53 stabilization and apoptosis was lower than in the normal cells.

In this study we also showed the importance of associating drugs which stabilize p53 by inhibiting rRNA synthesis to drugs which induce p53 stabilization through different pathways in cells characterized by low ribosome biogenesis rate.

We observed that the association of ActD with HU induced a p53 stabilization in the MCF7 and LoVo cells that was higher than that obtained with the single drug treatments, and induced the apoptotic cell death.

We did not observe this additive effect in the cell lines (HCT116 and HepG2) with high ribosome biogenesis rate, in which the maximum amount of p53 stabilization and apoptosis was obtained after the single ActD treatment and the combination with HU did not increase p53 stabilization. This can be explained by the fact that in cells with high ribosome biogenesis rates, the inhibition of rDNA transcription makes large amounts of RPs free to bind to MDM2 and, very likely, to neutralize its ubiquitin-ligase activity completely, with the consequent stabilization of all the synthesized p53. Therefore, the phosphorylation of p53 caused by HU cannot modify the final amount of stabilized p53. On the other hand, if the cells are characterized by a low ribosome biogenesis rate, the inhibition of the synthesis of rRNA makes small amounts of RPs free to bind to MDM2,

with a partial neutralization of its ubiquitin-ligase activity and the consequent stabilization of only a portion of p53. In this case HU exposure may increase the total amount of stabilized p53 by phosphorylating p53 thus hindering its binding to the still active portion of MDM2.

# CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented in this study allow us to conclude that, in case of wild-type p53 cancers, those with high levels of ribosomal biogenesis could strongly benefit from treatments with drugs that inhibit rRNA synthesis. In fact, in these cancers the drug treatment promotes a high activation of p53 with the consequent induction of apoptotic death. Conversely, the treatment with rRNA synthesis inhibitors of tumors characterized by low ribosomal biogenesis rate induces a low p53 stabilization level that does not induce cancer cells death, but only the arrest of the cell cycle progression. For this reason the inhibitors of the rRNA synthesis alone should be considered in the treatment of cancers characterized by high levels of ribosome biogenesis but cannot be recommended for the treatment of cancers characterized by low ribosome biogenesis levels. In this type of cancers, in order to obtain a p53 stabilization sufficient to activate the apoptotic pathway, it might be useful to combine treatments with ribosomal biogenesis inhibitors, which stabilize p53 through the RPs-MDM2 pathway, with drugs that stabilize p53 through a different pathway, e.g. by p53 phosphorylation, in order to increase p53 stabilization level and to induce apoptotic cell death.

Altogether, these results suggest that in order to choice an appropriate chemotherapy the evaluation of the ribosomal biogenesis rate should be carried out in the neoplastic tissues. This evaluation can be easily performed by measuring the size of the nucleolus on histological samples which is in fact directly proportional to the ribosomal biogenesis rate of the cell (Derenzini et al. 2009).

These findings, demonstrating the important cytotoxic effects of the inhibition of ribosome biogenesis on cancer cells, stimulate more studies aiming to identify compounds that selectively target the ribosome biogenesis.

In this context, worthy of mention are some recent works in which a small molecular compound, BMH-21, and a small-molecule peptide (22mer) have been found to stabilize p53 by inhibiting rDNA transcription. The small molecular BMH-compounds inhibit nascent rRNA synthesis and cause nucleolar stress by the proteasome dependent destabilization of the RPA194 subunit of the Pol I (Peltonen et al. 2014). The small-molecule peptide (22mer) targets the interface between RNA polymerase I and Rrn3 thus selectively inhibiting the synthesis of rRNA (Rothblum et al. 2014).

# NOTES

The work described in this thesis led to the publication of the following paper:

Scala F, Brighenti E, Govoni M, Imbrogno E, Fornari F, Treré D, Montanaro L, Derenzini M. Direct relationship between the level of p53 stabilization induced by rRNA synthesis-inhibiting drugs and the cell ribosome biogenesis rate. Oncogene. 2015 May 11.

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