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THE BALANCE BETWEEN rRNA AND RIBOSOMAL PROTEIN SYNTHESIS UP- AND DOWN-REGULATES THE TUMOUR SUPPRESSOR p53 IN MAMMALIAN CELLS

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INTRODUCTION

Nucleolus and ribosome biogenesis

The nucleolus is the most distinctive nuclear sub-compartment and is the site of the ribosome biogenesis. The nucleolus contains hundreds of ribosomal genes that are tandemly repeated in several chromosomes and which account for one-half of the total cellular RNA production. These chromosomal gene clusters identified in mitotic chromosomes are known as NORs (nucleolar organizer regions). NORs were first described by Heitz and by McClintock in plant cells, as chromatinic regions around which nucleoli reform during telophase. These regions correspond to secondary constrictions of metaphase chromosome of eukaryotic cells (Howell 1982). In man secondary constrictions are localized on short arms of chromosomes 13, 14, 15, 21 and 22. NORs are characterized by the presence of proteins that are selectively stained by silver methods (Howell 1982). During interphase, the nucleolus is the only site where both ribosomal genes and silver-stained proteins are located. The fibrillar components of the nucleolus are the interphase counterpart of the metaphase NORs. Interphase NORs have become an object of attention for pathologists because their distribution in the nucleolus has been shown to constitute a useful tool for differentiating, at the optical level, malignant from benign lesions in histological and cytological routine preparations. Abnormalities of the nucleolar morphology characterize neoplastic cells: hypertrophied and irregularly shaped nucleoli are frequently observed in cancer cells

(Koller 1963). A higher quantity of interphase NORs is generally observed in neoplastic cells than in the corresponding non-neoplastic cells (Derenzini 1991).

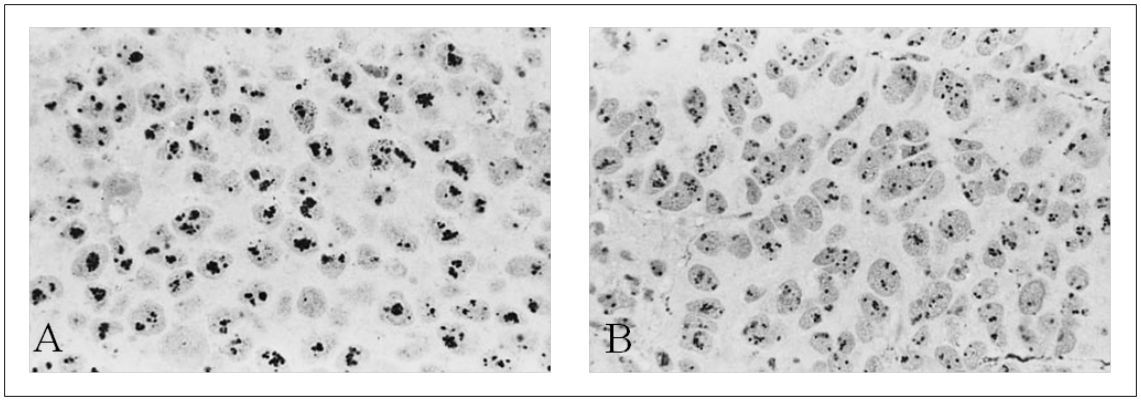

Figure 1 Silver staining for the AgNOR proteins. A, A large amount of AgNOR proteins is present within nucleoli of the rapidly proliferating tumor mass, while **B,** only a few silver stained dots are present within cancer cells of the slowly proliferating mass.

Only a subpopulation of rRNA genes is active at any given time. Indeed ribosomal RNA genes exists in two different types of chromatin, an open conformation that allow transcription and a closed conformation that is transcriptionally inactive. DNA methylation and histone modifications are the epigenetic mechanism responsible for the alterations in the chromatin structure resulting in the activation or silencing of rRNA genes (Grummt 2003).

The biogenesis of ribosomes requires the initial transcription of rDNA genes by a specialized RNA Polymerase, RNA Polymerase I. It requires auxiliary factors that mediate promoter recognition, promote transcription elongation, and facilitate transcription termination. Together with the Polimerase I, upstream binding factor (UBF) and the promoter selectivity factor (SL1) are recruited in the promoter region enabling the formation of an active transcription complex. This pre-initiation complex

synthesizes the 47S precursor rRNA that contains in addition to the 18S, 5.8S and 28S rRNA sequences, internal and external transcribed spacer sequences (Moss et al. 2007).

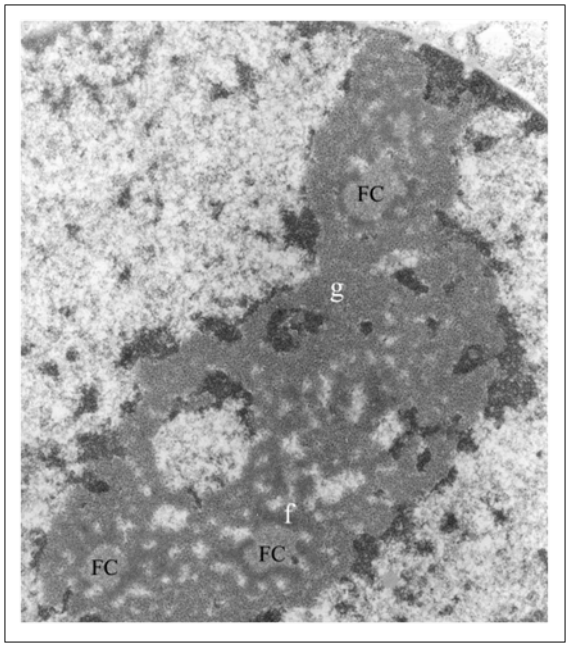

Figure 2 Electron microscopy of a nucleolus from human cells. The granular (g) and dense fibrillar (f) components as well as the fibrillar centers (FC) are shown.

Production of ribosome is largely controlled at the level of rRNA transcription: in addition to the epigenethic mentioned above, alterations in the extent of rDNA transcription can also occur via changes to the rate of transcription from each active rRNA gene. In mammalian cells rDNA transcription is a cell-cycle regulated process. Cell cycle control elements, like cdk-cyclin complexes and various oncogenes and oncosuppressors, regulate the production of rRNA. Moreover transcription rate varies during different phases of the cell cycle: it's absent during mitosis, when the concomitant disappearance of the nucleolus is observed, gradually increases during G1 and peaks in the S and G2 phases (Russell et al. 2005; Weisenberger et al. 1995).

The biogenesis of mature ribosomal RNA is a complex process (Fatica and Tollervey 2002; Tschochner and Hurt 2003) that necessitates non-ribosomal proteins and ribonucleoproteins (RNPs) containing large varieties of small nucleolar RNAs (snoRNAs): endonucleases and exonucleases remove the non-coding transcribed spacer sequences before the assembly of the small and large ribosomal subunits in nucleolus; pseudouridine synthases and methyltransferases are involved in the rRNA modifications of about 200 nucleotides. 5S rRNA, synthesized in the nucleoplasm by a different enzyme, RNA Polymerase III, and ribosomal proteins, synthesized in the cytoplasm, are then recruited into the nucleolus to become integrated into preribosomal particles.

The steps of the ribosome biogenesis occur in distinct compartments of the nucleolus, which can be distinguished by their morphology using electron microscopy: fibrillar centres (FCs), dense fibrillar components (DFCs) and granular components (GCs). Transcription of ribosomal rDNA repeats by RNA polymerase I occurs either at the boundary between the FC or in the DFC alone (Derenzini et al. 2005; Boisvert et al. 2007). The pre-ribosomal RNA transcripts are spliced and modified by small nucleolar ribonucleoproteins (snoRNPs) in the DFC. Final maturation of the pre-ribosomal ribonucleoprotein and assembly with ribosomal proteins occurs mostly in the GC region. In the GC, the 5.8S and 28S ribosomal RNAs (rRNAs) assemble with the 5S rRNA transcript to form the 60S subunit, whereas the 18S rRNA alone assembles into the 40S ribosome subunit. The 40S and 60S ribosome subunits are both exported to the cytoplasm, where they bind to mRNA to form functional ribosomes. (Tschochner and Hurt 2003; Derenzini et al. 2006).

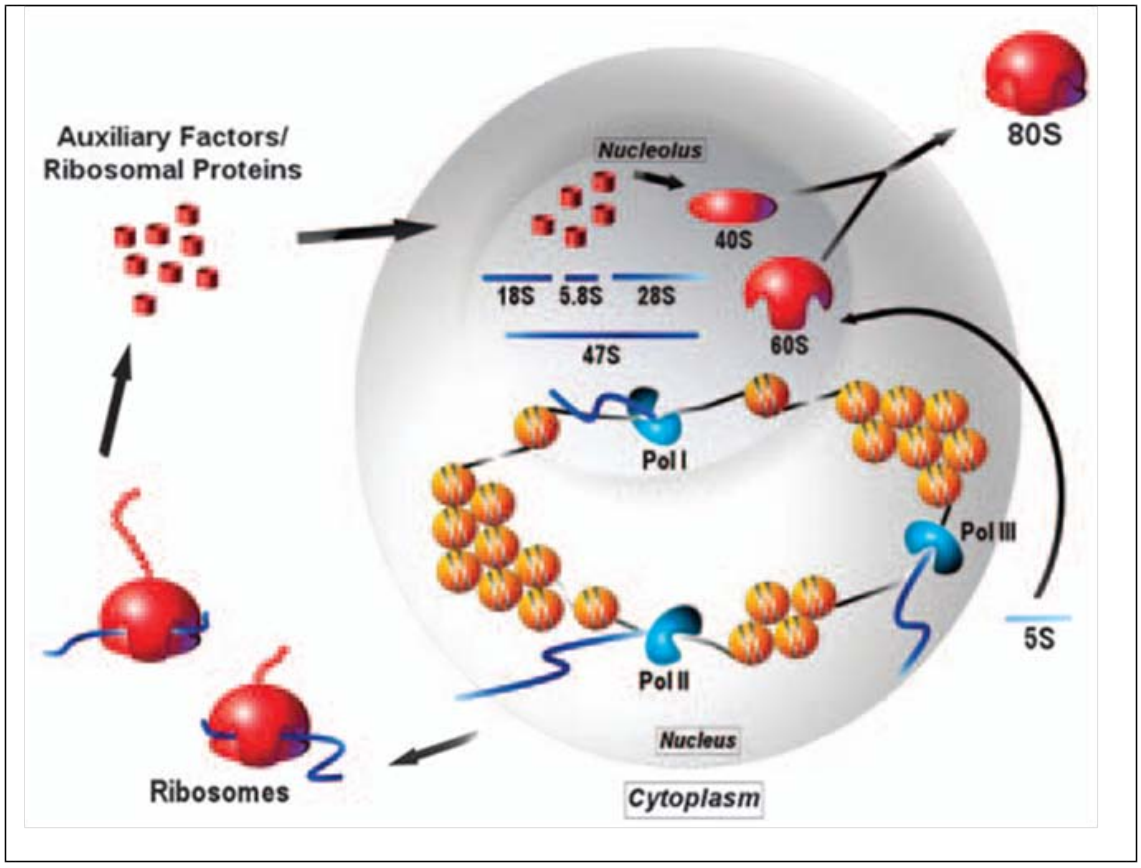

Figure 3 A model for human ribosome biogenesis. Ribosome biogenesis requires coordinated expression of precursor 47S rRNA by RNA Pol I, RPs and auxiliary factors by RNA PolII, and 5S RNA by RNA PolIII. Processing and assembly of the ribosomal subunits occur in the nucleolus, followed by export of the subunits and assembly into mature ribosomes in the cytoplasm.

Although the best characterized function of the nucleolus is the ribosome biogenesis, several lines of evidence show that this dynamic organelle is involved in additional processes. Among these there are the regulation of mitosis, the cell-cycle progression and proliferation, tumorigenesis, aging, some forms of stress response in particular those involving p53 activation (Boisvert et al. 2007).

Ribosome biogenesis and cell proliferation

The terms ‘growth’ and ‘proliferation’ have often been used interchangeably, without close attention to their actual meanings. Growth is an increase in cell mass and proliferation is an increase in cell number: they represent two distinct but interdependent events. In order to proliferate a cell must increase in size otherwise it will divide in ever smaller cells (Thomas 2000). This increase in cellular components requires an increase of protein synthesis which in turn requires new ribosomes. Given that the ribosome biogenesis has to precede the proliferative response, the first products of the protein-synthesis apparatus are the translational components themselves, most notably ribosomal proteins (Amaldi et al. 1997). As a consequence, ribosomal-protein messenger RNAs in proliferating cells have the added characteristic that they monopolize the cell’s translational capacity. In this way only when the cell has established a substantial capacity for protein synthesis the key components of the cell-cycle control system can be synthesized (Thomas 2000).

Given this strict relationship between ribosome biogenesis and proliferation and considering that making ribosomes consumes approximately 80% of the energy of a proliferating cell, it’s not surprising that ribosome biogenesis directly control cell cycle progression. Ribosome biogenesis rate influences the length of the G1 phase as observed in study of rat hepatoma cells treated with Actinomicyn D, a polypetdide antibiotics that selectively inhibit Polymerase I activity when used at low doses (Derenzini et al. 2005). Only when an adequate ribosome complement is achieved the cell can pass to the S phase. Indeed at the end of G1 phase, the restriction point defines a limit beyond which the cell is committed to divide independently of growth (Riddle et

al. 1979; Pardee 1989). Several studies demonstrated that the transcription activity of RNA Polymerase I was closely related to the cell doubling time (DT) in human cancer cell lines growing in vitro and in vivo: the greater the rRNA transcription rate, the shorter the cell DT (Derenzini et al. 1998, 2000). It's now well established that the inhibition of the ribosome biogenesis cause the cell cycle arrest in the G1 phase in a p53/pRb dependent manner (Pestov 2001; Montanaro et. al. 2007).

Cell cycle control elements can in turn affect ribosome biogenesis. Mitotic silencing of RNA Polymerase I transcription and reactivation during the transition from mitosis to the G1 phase of the cell cycle are controlled at multiple levels (Sirri 2002). Phosphorylation of SL1 by cdc2-cyclin B during metaphase correlates with the inactivation of SL1, an inability of SL1 to interact with UBF and mitotic repression of rDNA transcription. Therefore, productive pre-initiation complex formation, initiation and/or promoter escape and clearance might be down-regulated in mitosis. UBF inactivation during mitosis also correlates with its phosphorylation status (Grummt 2003). Activation of rDNA transcription, upon serum stimulation of serum starved NIH3T3 cells, correlates with phosphorylation of UBF by the G1-specific complexes cyclin-dependent kinase cdk4-cyclin D1 and cdk2-cyclin E and cdk2-cyclin A required for the interaction of UBF with Pol I (Klein and Grummt 1999; Voit 1999). The tumor suppressors pRb and p53, master key in cell cycle regulation, play an important role in the regulation of Pol I activity. Indeed they can directly interfering with the assembly of a productive transcriptional machinery on the rRNA promoter (Zhai 2000; Voit 1997).

The p53 tumor suppressor protein

The discovery of p53 as a cellular SV40 large T antigen-binding protein in 1979 marks the beginning of a dynamic era in cancer research that was expected to have a major impact in the clinic. p53 has established itself as a key tumor suppressor, potent apoptosis-inducer, and prognostic marker in cancer. The p53 gene is mutated in around 50% of human tumors (Soussi and Wiman 2007). This emphasizes the central role of the p53 pathway in regulation of cell growth and survival. Moreover, the p53 status of a tumor may have a strong influence on sensitivity to commonly used anticancer drugs and radiotherapy. The inactivation of p53 results in a failure to respond properly to a variety of stress signals, leading to increased genomic instability and eventually cancer (El-Deiry 1998). Although mice that are homozygous null for p53 are developmentally competent, they are highly predisposed to tumors, indicating that p53 has an important function in protecting cells against aberrant cell growth and neoplastic transformation (Donehower et al. 1992).

p53 is a nuclear transcription factor that accumulates in response to cellular stress, including DNA damage and oncogene activation. Several forms of DNA damage have been shown to activate p53, including those generated by ionizing radiation (IR), radio-mimetic drugs, ultraviolet light (UV) and chemicals. Recently, a new type of stress signal generated by disrupting ribosomal biogenesis and mediated by several ribosomal proteins has been shown to inhibit MDM2 and activate p53. Ribosomal biogenesis can be disrupted by serum starvation, depletion of nucleotides, agents such as Actinomycin D or 5-Fluorouracil, malfunction of nucleolar proteins involved in ribosome biogenesis (such as the dominant-negative mutant Bop1) (Pestov et al. 2001), inhibition of B23

(also known as nucleophosmin) activity by ARF (Itahana et al. 2003), and reduction of RPS6 (Fumagalli et al. 2009; Volarevic et al. 2000); all of these have been shown to generate nucleolar stress that signals to p53. This allows cells to halt proliferation under unhealthy and poor ribosomal biogenesis conditions. All these stimuli triggers transcriptional trans-activation of p53 target genes such as p21, GADD45, Bax, Puma, and Noxa, leading to cell cycle arrest, senescence and/or apoptosis (Vousden and Prives 2009). *MDM2* gene, whose protein product ubiquitinates p53 and targets it for proteasome-mediated degradation, is another p53 transcriptional target. Therefore, p53 and MDM2 form a negative regulatory loop that down-regulates p53 expression. p53 can also perform various functions in the cytoplasm (Green and Kroemer 2009). Translocation of p53 to mitochondria promotes apoptosis through transcription-independent mechanisms (Mihara et al. 2003). Oncogene activation leads to aberrant DNA replication with stalled replication forks, which triggers a DNA damage response (DDR) involving activation of ATM and Chk2 kinases and accumulation of p53 (Bartkova et al 2005; Gorgoulis et al. 2005). Oncogene activation may also induce expression of the p14Arf protein (p19Arf in the mouse) that inhibits MDM2, leading to p53 accumulation (Sherr and Weber 2000). Accumulation and activation of p53 triggers cellular senescence or cell death by apoptosis. Thus, activation of p53 upon oncogenic stress serves to eliminate nascent tumor cells by apoptosis and/or senescence, forming a critical barrier against tumor development.

A vast majority of p53 mutations in human tumors are single missense mutations that cluster in the p53 core domain (residues 100–300) that recognizes p53 binding motifs in DNA (Hainaut and Wiman 2005). In general, mutant p53 proteins are deficient for specific DNA binding, arguing that DNA binding and transcriptional regulation of

target genes are crucial for p53-mediated tumor suppression (Soussi and Wiman 2007). Moreover, it is becoming increasingly clear that the mutations may endow p53 with various gain-of-function activities (Brosh and Rotter, 2009). These include for instance enhanced NF-kB activation (Weisz et al. 2007) and promotion of tumor invasion by affecting integrin and epidermal growth factor receptor (EGFR) trafficking (Muller et al. 2009). In addition in many different cancers p53 it's rendered inactive by a range of indirect mechanisms like MDM2 amplification or loss of ARF (Olivier et al. 2004).

An important role of p53 in processes such as cell cycle arrest, senescence and apoptosis is firmly established. However, recent studies have painted a more complex picture of p53 as regulator of diverse biological processes, such as autophagy, metabolism, and aging (Vousden and Prives 2009). It has been known for decades that cancer cells have changes in multiple metabolic pathways, including the usage of glycolysis also under conditions of normoxia. Such changes seem to support dysregulated cell growth, allow synthesis of macromolecules, and protect cancer cells from oxidative stress. Interestingly, p53 has been shown to have a direct impact on metabolism. Lack of nutrients or ‘‘metabolic stress" can induce a p53 response via activation of AMP-activated protein kinase (AMPK) and inhibition of the kinase AKT that promotes MDM2- mediated p53 degradation in the proteasome. This leads to upregulation of p53 target genes, among which TSC2 inhibits the mTOR protein that stimulates protein synthesis and inhibits autophagy, a process that leads to digestion of cellular components in lysosomes. As a consequence, cell growth is suppressed (Vousden and Ryan 2009).

p53 can also regulate cellular redox status. High levels of p53 provide a pro-oxidant function through up-regulation of genes such as Bax, Puma, PIG3, and other genes,

resulting in the production of reactive oxygen species (ROS) (Bykov et al. 2009). In addition, p53 is itself regulated in a redox-dependent manner. Efficient p53 DNA binding requires a reducing environment. Another exciting aspect of p53 is its emerging role in aging and longevity. In vivo studies have indicated that overactive p53 can suppress tumor formation but also induce an early aging phenotype (Tyner et al. 2002).

Considering the multiple roles of p53 in a wide range of biological processes and its ability to control life and death of cells, it is not surprising that p53 is tightly regulated. Having a short half-life, p53 is normally maintained at low levels in unstressed mammalian cells by continuous ubiquitylation and subsequent degradation by the 26S proteasome (Chene 2003). This is primarily due to the interaction of p53 with the ring-finger ubiquitin E3 ligase MDM2. When the cell is confronted with stress, however, p53 ubiquitylation is suppressed and p53 is stabilized and accumulates in the nucleus, where it forms a homotetrameric complex (Yang et al. 2004). Only tetrameric p53 seems to be fully active as a transcriptional activator or repressor of distinct target genes that contain p53 sequence-specific DNA binding sites.

Active p53 is subject to a complex and diverse array of covalent post-translational modifications, which markedly influence the expression of p53 target genes. The most commonly reported post-translational modifications of p53 include phosphorylation and acetylation that generally result in p53 stabilization, accumulation and activation in the nucleus. (Vousden and Prives 2009). Post-translational control, rather than transcriptional regulation of the p53 gene, provides a particularly rapid, sensitive and readily-reversible mechanism for regulating p53 activity (Bode and Dong 2004).

Nonetheless, recent studies have revealed critical regulatory circuits that target p53 at the RNA level via both RNA-binding proteins and regulatory RNAs. Proteins such as HuR, L26, RPL26, nucleolin, and Wig-1 (Zhang and Chen 2008,) can bind to the 50 or 30 untranslated regions (UTR) of p53 mRNA and control its stability or translation through various mechanisms.

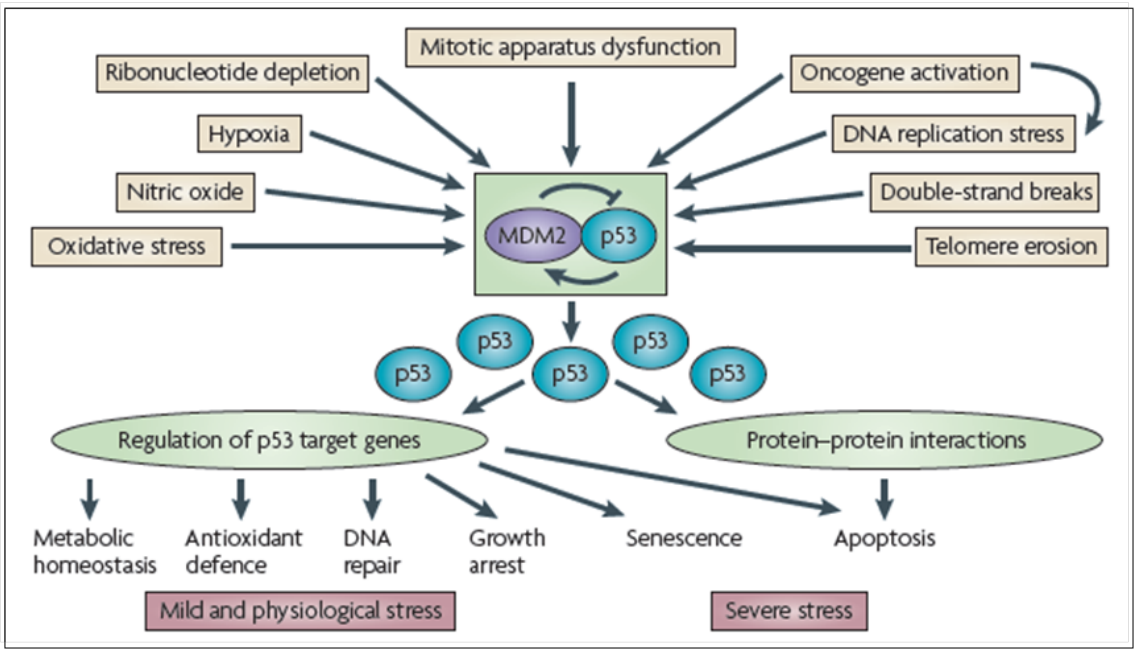

Figure 4 Simplified scheme of the p53 pathway. The p53-MDM2 feedback is the "heart" of the p53 pathway. Under normal conditions, it maintains constantly low steady-state p53 levels and activity. Various stress signals impinge on this central loop to release p53 from MDM2-mediated inhibition. It is generally believed that the nature of the phenotypic response to p53 activation is at least partially proportionate to the amplitude, duration and nature of the activating signal. Severe stress induces more extreme, usually irreversible, responses namely apoptosis and senescence while milder stress leads to a transient growth arrest coupled to an attempt to deal with the cause of the stress and repair the damage caused by it. Recent evidences indicate that p53 has also an important role in enabling the cell to adjust its metabolism in response to mild normal physiological fluctuations, including those in glucose and other nutrients levels, oxygen availability and reactive oxygen species levels.

p53 and ribosome biogenesis control

p53 protects against cancer through its capacity to induce cell cycle arrest or apoptosis under a large variety of cellular stresses. However, more than 20 years after the discovery of the protein, it is not known how such diversity of signals can be integrated by a single molecule. Numerous studies indicate that the common denominator of all p53-inducing stresses, is that they all cause nucleolar disruption and compromise nucleolar function. The nucleolus is thus a sensor responsive to a wide range of cellular stresses (Rubbi and Milner 2003). The proposed model predicts that p53 should be stabilized whenever nucleolar disruption occurs, even in the absence of DNA damage, of phosphorylation inhibition or of metabolic stresses. Strong evidence of the involvement of the ribosome biogenesis in p53 activation came from the observations on the nucleolar protein Bop1, implicated in pre-rRNA processing. When a dominant-negative Bop1 mutant was introduced into cells, it interfered with nucleolar function, inducing p53 stabilization and a p53-dependent cell cycle arrest in G1 (Pestov et al. 2001). Moreover microinjection of antibodies against the nucleolar protein upstream binding factor (UBF) was sufficient to induce nucleolar disruption and induction of p53 (Rubbi and Milner 2003). Available data indicate that the mechanism of p53 activation is mainly the consequence of changes in functional and physical interactions of the tumor suppressor with MDM2. Indeed disrupting the nucleolar structure would cause the release of several ribosomal proteins that bind to MDM2 and relieve its inhibitory activity toward p53 (Zhang and Lu 2009; Deisenroth and Zhang 2010). In response to nucleolar stress induced by a low dose of Actinomycin D (5 nM) (Dai and Lu 2004; Dai et al. 2004; Jin et al. 2004) and 5-Fluorouracil (Gilkes et al. 2006; Sun et al. 2007), by

serum depletion and contact inhibition (Bhat et al. 2004), by mycophenolic acid-mediated depletion of GTP (Sun et al., 2008), or by interfering with nucleolar function via ectopic overexpression of nucleostemin (Dai et al. 2008), an increased binding of RPL5, RPL11, and RPL23 to MDM2 was observed. In addition to the three RPs, RPS7 (Zhu et al., 2009), RPL26 (Ofir-Rosenfeld et al. 2008) and the nucleolar protein Nucleophosmin have also been shown to interact with MDM2. The correlation between impairment of nucleolar function and p53 stabilization can be extended further: mammalian cells lose their nucleoli during mitosis, and full nucleolar functionality, in the form of the maximum level of rRNA synthesis, is not achieved until late in G1 phase (Klein and Grummt 1999). This period of recovery of nucleolar functionality is precisely the window in which p53 levels are increased during the cell cycle (David-Pfeuty 1999). Moreover, agents that arrest cells in mitosis (e.g. nocodazole) induce a p53 response (Pluquet and Hainaut 2001) and at the same time prevent nucleolar reformation.

The nucleolar disruption model of p53 stabilization offers a unifying explanation for the induction of p53 under a wide range of cellular stresses. However, according to this view, a stress must always acutely damage a fundamental cellular function in order to induce p53 activation, while it's becoming clear that p53 activity may be far broader than simply to promote a tumor suppressive response to acute stress (Vousden and Prives 2009). Indeed, the ability to prevent cancer has been suggested to be an "evolutionarily late" cooption of p53 activities that had initially evolved to protect the germline and monitor development (Aranda-Anzaldo and Dent 2007; Vousden and Lane 2007). It is now becoming apparent that p53 functions are involved in diverse aspects of health and disease (Fig. 5) that do not require acute stress. Therefore, we

wondered whether quantitative alteration in the production of rRNA must necessarily disrupt the nucleolus and induce ribosomal protein leakage to stabilize and activate p53. For this purpose we induced a specific down-regulation of rRNA synthesis by silencing the RNA polymerase I catalytic subunit and then we up-regulated rRNA synthesis using three different experimental conditions. We found that quantitative variations in rRNA synthesis were able to influence p53 activity by altering the binding of MDM2 with the ribosomal proteins without inducing nucleolar disruption.

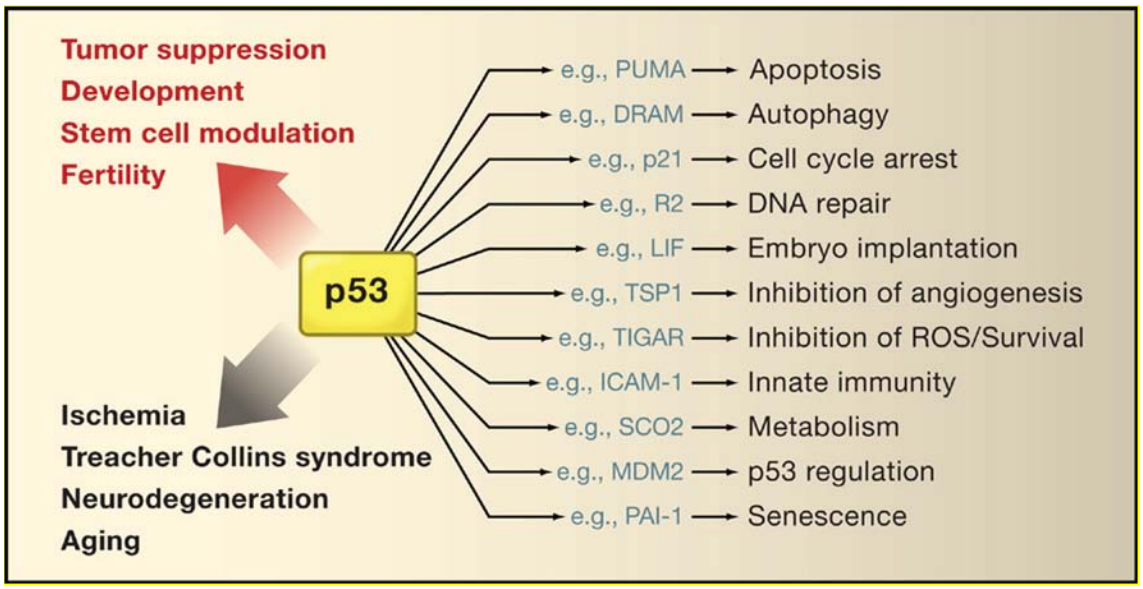

Figure 5 p53 contributes to multiple normal processes and disease pathologies. In addition to its well-known role as a tumor suppressor, p53 also regulates other cellular (right) and developmental processes (left). These include processes that result in positive outcomes (red arrow) and those that result in diseases or other unfavorable outcomes (black arrow). Examples of p53 target genes (blue) that are regulated by p53 to produce the indicated cellular outcomes of p53 induction are shown. Note that, in many cases, numerous targets have been identified to mediate a specific outcome, even though only one example target gene is shown here.

AIM OF THE THESIS

Common denominator in the majority of p53-inducing stresses is nucleolar disruption. Alterations in the nucleolar structure lead to a release of ribosomal proteins to the nucleoplasm triggering their specific binding to MDM2, thus inhibiting MDM2's E3 ubiquitin ligase function toward p53 with consequent p53 stabilization and activation (Lindstrom et al., 2007).

Purpose of our work was to assess whether this mechanism could be extended further and could be able to regulate p53 balance even in milder stressful conditions. We wonder whether quantitative alterations in the production of rRNA were sufficient to modulate p53 even in the absence of severe nucleolar alterations. We investigated the consequences of a selective down-regulation and up-regulation of rRNA synthesis on p53 level and activity. To down-regulate rRNA synthesis we decided to silencing the POLR1A, the RNA polymerase I catalytic subunit, obtaining a selective reduction of the 45S rRNA. In this condition we evaluated the level of p53 and the mechanism responsible of p53 regulation upon rRNA synthesis inhibition. In particular we focused our attention on the relationship between ribosomal proteins-MDM2-mediated p53 stabilization. To up-regulate rRNA production we used three different experimental conditions: cancer cells treated with insulin or exposed to the insulin-like growth factor 1, rat liver stimulated by cortisol and regenerating rat liver after partial hepatectomy. In all the conditions we performed a series of studies in order to elucidate whether the up-regulation of rRNA synthesis was responsible of p53 change through the mechanism involving MDM2 binding with ribosomal proteins.

MATERIALS AND METHODS

Cell lines, culture conditions and drug treatments

U2OS, HCT-116 and MCF7 human cancer-derived cell lines and H4-II-E-C3 rat hepatoma cell line were cultured in monolayer at 37°C in humidified atmosphere containing 5% CO2 in DMEM (Euroclone, Milan, Italy) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone), except MCF7 cells, which were grown in RPMI 1640 (Euroclone) plus 5% FBS (Euroclone). Rat hepatocytes were isolated as previously described (Derenzini and Moyne, 1978). Serum starvation was obtained by culturing cells in medium containing 0.2% FBS. Actinomycin D (BioVision) was used at a final concentration of 8 nM; 5-fluorouracil (Teva Pharma Italia, Milan, Italy) was used 75 μM; Hydroxyurea (Sigma-Aldrich, Milan, Italy) was used 3.4 μM; Gemcitabine (Gemzar 200mg; Eli Lilly Italia spa) was used at the concentration of 10 μg/ml; Rapamycin (Cell Signaling Technology, Beverly, MA, USA) was used 20 nM. α-amanitin (Boehringer Mannheim, Mannheim, Germany) was dissolved in the culture medium at the concentration of 5μg/ml. Human recombinant Insulin-like Growth Factor (Sigma- Aldrich) was added to growth medium at a final concentration of 100 ng/mL; human recombinant insulin (NovoNordisk Farmaceutici, Rome, Italy) was used 40 nM in growth medium; the proteasome inhibitor MG-132 (Calbiochem, Merck, Nottingham, UK) was used at a final concentration of 10 μM; the ribosome inhibitor Cycloheximide (Sigma-Aldrich) was used at a concentration of 20 μg/mL.

Animals

Four male Wistar rats (weighing 180–200 g) rats underwent partial (2/3) hepatectomy under anaesthesia, according to Higgins and Anderson (1931) and 3 rats were intraperitoneally injected with cortisol (Sigma-Aldrich) at the dose of 10 mg/100 g body weight. Hepatectomised rats were killed 12 hours after operation and 3 sham-operated rats were used as controls. Cortisol treated rats were killed 8 hours after drug injection and 3 rats injected with saline were used as controls. Experimental procedures were approved by the Ethical Committee of the University of Bologna.

RNAi, plasmids and transfection reagents

Select Stealth RNAi (Invitrogen, Carlsbad, UK) targeted against catalytic subunit of RNA Polymerase I (POLR1A) and siRNA against *rpL11* (described in Barkic et al. 2009, target sequence 1) were used, while Stealth RNAi Negative Control (Invitrogen) or a scrambled sequence siRNA were used to transfect controls, respectively. Cells were transfected with Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM medium (Invitrogen) accordingly to manufacturer's procedures.

RNA extraction, reverse transcription and real time PCR

Cells were harvested and total RNA extracted with TRI reagent (Ambion, Austin, TX, USA) according to manufacturer instructions. Whole cell RNA was quantified spectrophotometrically 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 amounts of POLR1A , 45S rRNA, rpL11, p21, Bax, PUMA, β-Glucuronidas (GUSb), rat 45S rRNA, p21, Bax, PUMA, β-actin and 18S RNAs were evaluated by Real Time PCR performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with the 2-ΔΔCT method (Livak and Schmittgen 2001). The mean ΔCT value of the control sample was used in each experiment to calculate the ΔΔCT value of samples replicates. POLR1A, p21 and the internal control GUSb mRNAs were quantified with TaQMan Gene Expression Assays primers and probe kits (Applied Biosystems); primers and UPL probe (Roche Diagnostics, Milan, Italy) for rpL11 and rpL5 were chosen with the Roche online primer design tool, primers for SYBR Green Real Time PCR analysis of human Bax, PUMA and rat p21, Bax, and PUMA were designed using the same online tool; primers for human and rat 45S rRNA have been already described in (Murayama et al. 2008) and (Grandori et al. 2005) respectively. All sequences are available upon request.

Isolation of polyribosomal mRNA

Subconfluent cells were washed in PBS at 4°C. The cellular pellet was lysed in 2 volumes of 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L MgCl2 0.5% NP40 for 10 minutes at 4°C. The lysates were then centrifuged at 14,000 x *g* for 10 minutes at 4°C and the supernatant was used for the isolation of polyribosomes. Lysates were stratified onto a 15% to 50% sucrose gradient in 30 mmol/L HEPES/KOH (pH 7.5), 80 mmol/L KCl, 1.8 mmol/L Mg-cetate, and centrifuged at 4°C for 15 hours at 40,000 x *g*. From gradients, 1-mL fractions were collected and their absorbance was read at 260 nm. Polyribosomal fractions were pooled and centrifuged at 100,000 x *g* for 15 hours at 4°C. RNA was extracted from pellets using Trizol reagent.

Electron microscopy

Cells were immediately fixed in a solution of 4% formaldehyde in 0.1 M Sörensen buffer, pH 7.2 and postfixed in 1% osmium tetroxide in the same buffer. All the samples were dehydrated in alcohol and embedded in Epon. Ultrathin sections were double stained with uranyl acetate and lead citrate.

Immunofluorescence

Cells grown on glass coverslips were fixed and permeabilised in PBS containing 2% paraformaldehyde and 1% Triton X-100 for 5 minutes at room temperature. Samples were incubated for 30 minutes in PBS plus 1% Bovine Serum Albumin (BSA) Sigma-Aldrich) to block unspecific binding before incubating with the primary antibody diluted in PBS 1% BSA overnight at 4°C. The samples were rinsed in PBS and then incubated with FITCconjugated 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).

Analysis of rRNA synthesis by 5-fluorouridine incorporation

Analysis of nascent rRNA was performed on cells grown on coverslips by incorporation of 5- fluorouridine accordingly to the method described in (Boisvert et el. 2000). Briefly, cells were incubated for 15 minutes in medium containing 2mM 5-fluorouridine (Sigma-Aldrich), then washed in cold PBS and fixed in 2% paraformaldehyde and 1% Triton X-100 for subsequent immunofluorescence, performed as described above, using a monoclonal antibody specific for 5- fluorouridine.

Western blotting

Whole cell protein extracts and subsequent SDS-PAGE and immunoblot analyses were made as already described (Montanaro et al. 2007). Briefly, total cellular proteins were extracted in lysis buffer (KH2PO4 0.1M pH 7.5, NP-40 1%, added with Complete protease inhibitors cocktail (Roche Diagnostics) and 0.1 mM β-glycerolphosphate) and quantified spectrophotometrically with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hempstead, UK). Nuclear proteins fraction for Western blot analysis was obtained accordingly to the method described in (Abmayr et al. 2006) with slight modifications. Proteins immobilized on nitrocellulose membranes (GE-Healthcare) were blotted overnight at 4 °C with primary antibody. Horseradish peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA) and GE-Healthcare (anti-mouse).

Immunoprecipitation

Cells were lysed in buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.8% NP40, 1mM DTT, 1mM EDTA and Complete protease inhibitors cocktail (Roche Diagnostics). For each sample, 2.0 mg of proteins were pre-cleared with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) at 4 °C for 30 minutes and subsequently incubated for 6 hours at 4 °C with anti-MDM2 polyclonal antibodies. The immunocomplexes were then precipitated with Protein A/G PLUS-Agarose beads for 2

hours at 4 °C, The beads were then centrifuged, washed four times in lysis buffer and resuspended in Laemmli buffer for subsequent immunoblot analysis.

Antibodies

Antibodies used in this work: mouse monoclonal anti-p53 (clone BP53-12, Novocastra Laboratories, Newcastle upon Tyne, UK), anti-p21 (clone SX118, Dako), anti-β-actin (clone AC-74, Sigma-Aldrich), anti-Nucleophosmin (clone Fc-61991, Zymed Laboratories, San Francisco, CA), anti-MDM2 (clone SMP14, Santa Cruz Biotechnology), anti-ribosomal protein S14 (a gift from U. Scheer, described in (Kruger et al. 2007), anti-halogenated uridine (BU-33, Sigma-Aldrich), rabbit polyclonal anti-ribosomal proteins L11 and L5 (Panic et al. 2007), anti-MDM2 (H- 221, Santa Cruz Biotechnology), goat polyclonal anti-Lamin B (C-20, Santa Cruz Biotechnology).

RESULTS

POLR1A silencing causes rRNA synthesis reduction and stabilizes p53

In order to selectively inhibit the rRNA production we silenced the POLR1A gene by the siRNA procedure. A preliminary study demonstrated that in human cancer cell lines the silencing of the *POLR1A* gene by the siRNA procedure inhibited the expression of POLR1A mRNA and gave rise to a specific inhibition of rRNA synthesis (Donati et al. 2010). The Polymerase I reduction, analyzed by Real Time PCR, was observed 24 hours after *POLR1A* siRNA and was still evident after 96 hours (Fig. 6A).

POLR1A silencing caused a progressive reduction in the synthesis of rRNA in U2OS human cancer cells as measured by Real Time PCR evaluation of the 45S rRNA (Fig. 6B). rRNA synthesis was also evaluated by 5-fluorouridine nucleolar incorporation. Immunofluorescence analysis revealed that, 48 hours after *POLR1A* siRNA, fluorouridine incorporation in the nucleoli was reduced in comparison with control cells, after 72 hours only a few foci in the nucleolar peripheral area showed fluorouridine incorporation (Fig. 6C). We also used the silver staining to evaluate nucleolar activity, this technique revealed a reduction in the number of the nucleoli in cells where Polimerase I was silenced (Fig. 6D).

We observed that in consequence of rRNA synthesis reduction p53 level was increased. The p53 accumulation level paralleled the reduction of rRNA synthesis. We found that p53 was accumulated as early as 24 hours after the end of the *POLR1A* silencing

procedure and its amount was increased in 48 hour-silenced cells, as revealed by Western blot analysis (Fig. 7).

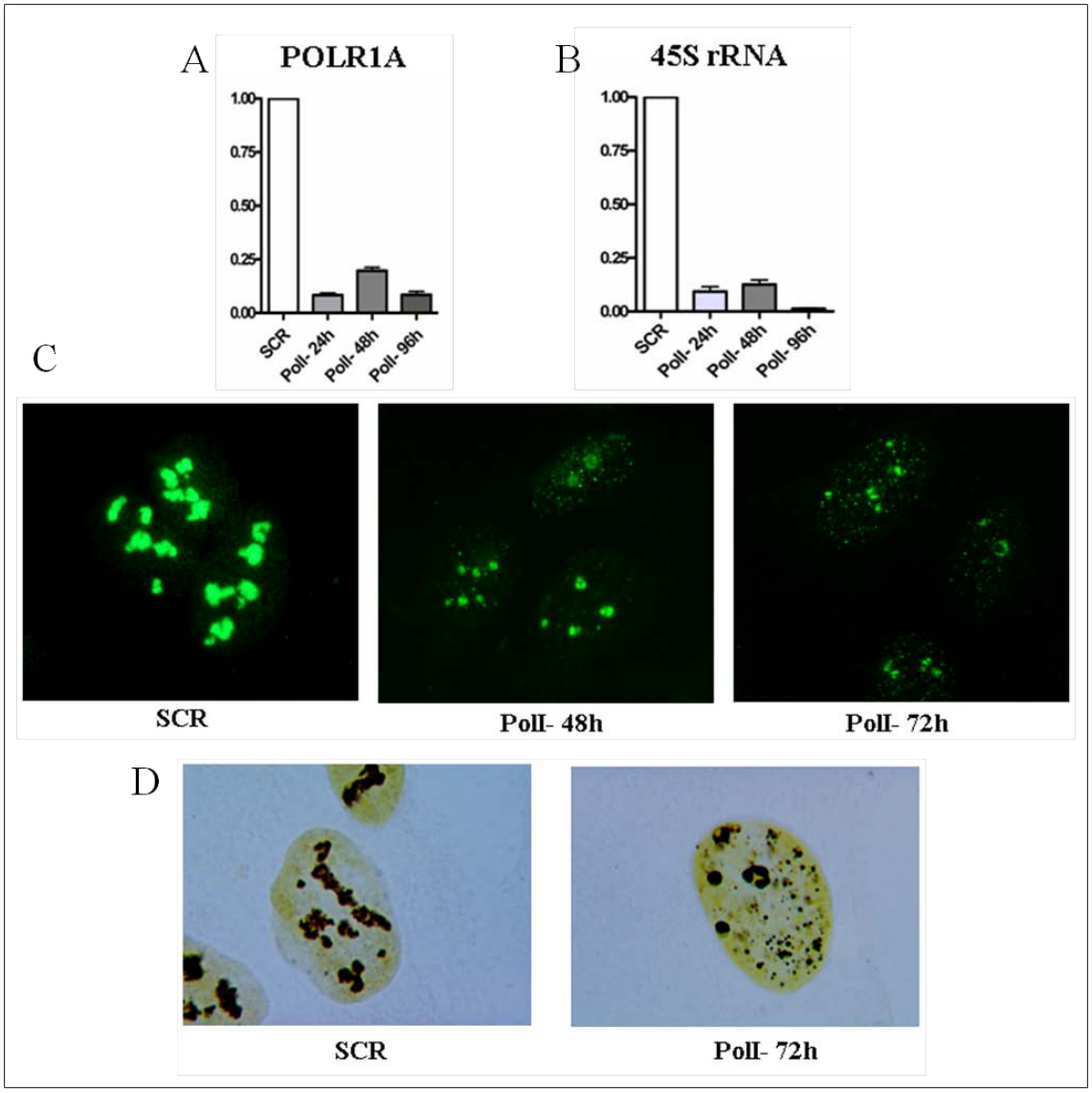

Figure 6 *POLR1A* silencing down-regulates rRNA synthesis. A, Real Time PCR analysis of *POLR1A* levels in U2OS cells after 24, 48 and 96 hours the siRNA procedure. **B**, Real Time PCR analysis of 45S rRNA in *POLR1A* silenced cells. **C**, immunofluorescence analysis of fluorouridine incorporation in nucleoli of *POLR1A*-silenced cells. **D**, silver staining of NORs in control and U2OS cells, 72 hours after *POLR1A* silencing.

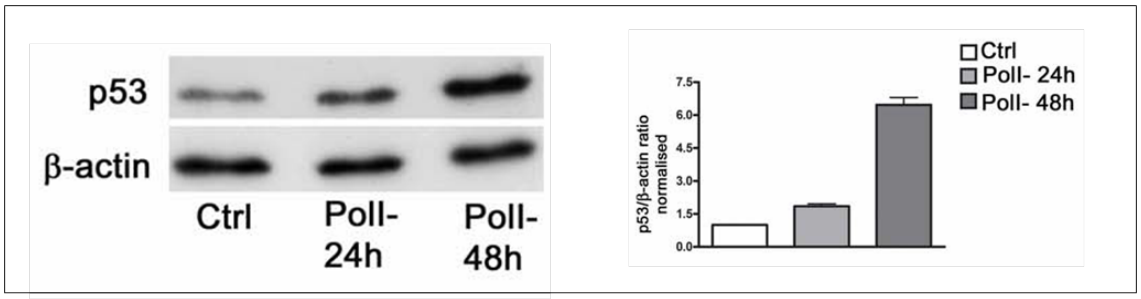

Figure 7 *POLR1A* silencing stabilizes p53. Western blot and densitometric analysis of p53 level in U2OS cells after 24 and 48 hours *POLR1A* silencing.

p53 stabilization after *POLR1A* silencing doesn't require the disruption of the nucleolar structure

48 hours after *POLR1A* silencing, electron microscopy (Fig. 9A) showed that, in comparison with U2OS cells transfected with control sequences, the nucleoli of *POLR1A*-silenced cells appeared to be smaller in size with a reduced amount of fibrillar components (fibrillar centres plus the associated dense fibrillar component) which represent the structural-functional units of the nucleolus (Montanaro et al. 2008). However, the ribonucleoprotein components did not exhibit any change, such as segregation or fragmentation (examples shown in Fig. 8), of their distribution within the nucleolar body, which are indicative of disrupted nucleolar structure. Since nucleophosmin translocation from the nucleolus to the nucleoplasm is considered to represent a marker of nucleolar changes (Chan et al. 1996), we analyzed the distribution of nucleophosmin in 48 hour-*POLR1A*-silenced cells. We found that after *POLR1A* silencing only a reduction of the immuno-cytochemical labeling of nucleophosmin was observed in nucleoli without any diffusion of the protein to the nucleoplasmic compartment (Fig. 9B). Lastly, we wondered whether *POLR1A* silencing induced

changes in the location of ribonucleoproteins to the nucleolus. We analyzed the distribution of the rpS14, a ribosomal protein incorporated early in ribosome formation (Kruger et al. 2007). 48 hours after the end of *POLR1A* silencing procedure no translocation of rpS14 from the nucleolus to the nucleoplasm was detected in U2OS cells (Fig. 9C). All together these data led us to conclude that the inhibition of rRNA synthesis by *POLR1A* silencing induced p53 stabilization without any evidence of nucleolar disruption and nucleolar protein leakage.

In order to extend our analysis to the relationship among rRNA synthesis inhibition, nucleolar disruption and p53 stabilization, we also evaluated the effect of 5-Fluorouracil exposure on p53 expression and nucleolar structural organization in U2OS cells. The reduction of rRNA synthesis induced by the drug was associated with p53 stabilization without alteration, once again, in the nucleolar ultrastructural morphology and nucleophosmin translocation to the nucleoplasm (Fig. 10).

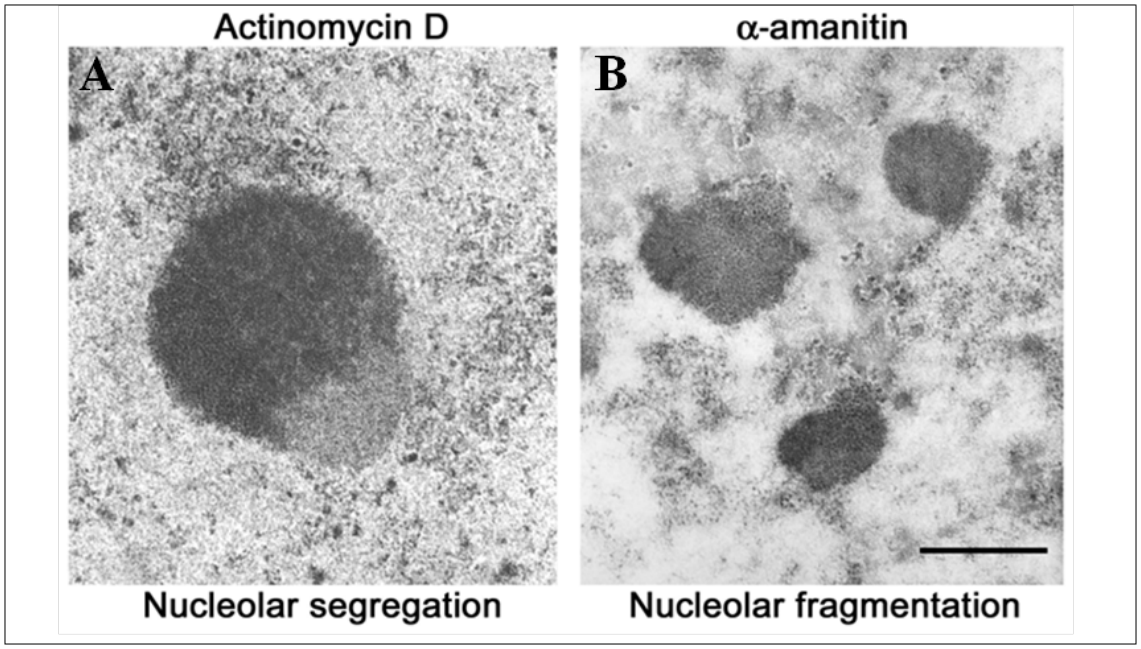

Figure 8 Ultrastructural patterns of the "nucleolar disruption". A U2OS cell after 4 h exposure to 8 nM Actinomycin D. The ribonucleoprotein structure of the nucleolus appear to be segregated into three main components: a light fibrillar, a dense fibrillar and a granular component. Uranium and lead staining. **B**, Isolated rat hepatocyte treated with α- amanitin at the dose of 5μg/ml for 3 h. The nucleolar body appears to be disrupted into three fragments in which the ribonucleoprotein components are segregated. Uranium and lead staining. Scale bar, 0.5 μm.

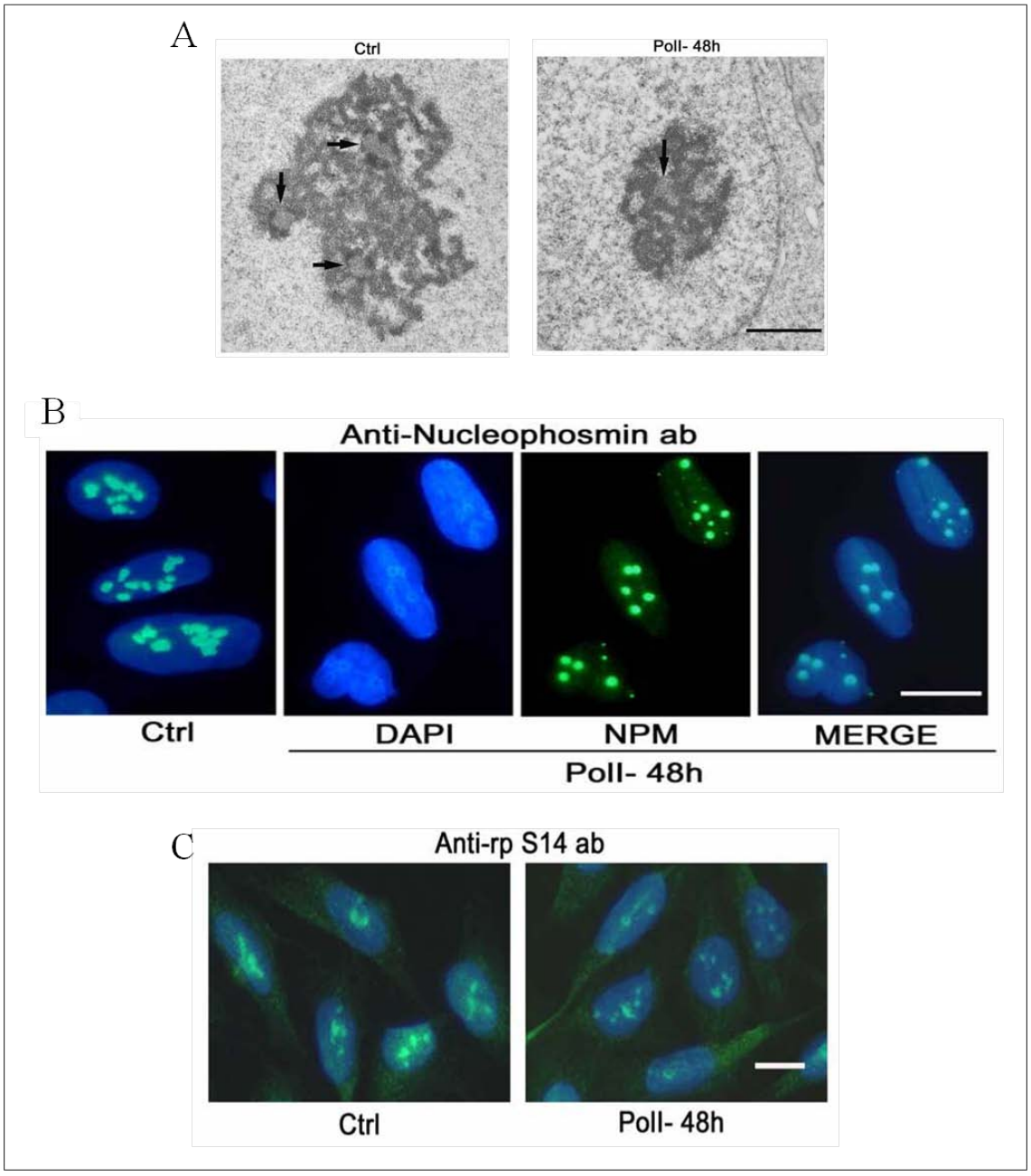

Figure 9 Nucleolar structure is preserved after *POLR1A* silencing. A, Electron microscopy visualization of nucleolar structural organization in control and *POLR1A*-silenced U2OS cells, 48 h after the silencing procedure. Arrows indicate the fibrillar centres, surrounded by the ribonucleoprotein fibrillar and granular component. **B**, Visualisation of nucleophosmin (NPM) distribution in control and 48 h *POLR1A*-silenced U2OS cells. Cells were labeled with monoclonal antibodies versus nucleophosmin; the antibodies were revealed by FITCH-conjugated secondary antibodies. Nuclei were stained with DAPI (DAPI). **C**, Visualization of rp S14 distribution in control and 48 h *POLR1A*silenced U2OS cells. Cells were labeled with

antiribosomal protein S14 antibodies; the antibodies were revealed by FITCH-conjugated secondary antibodies. DAPI nuclear staining. Scale bar, 10 μm.

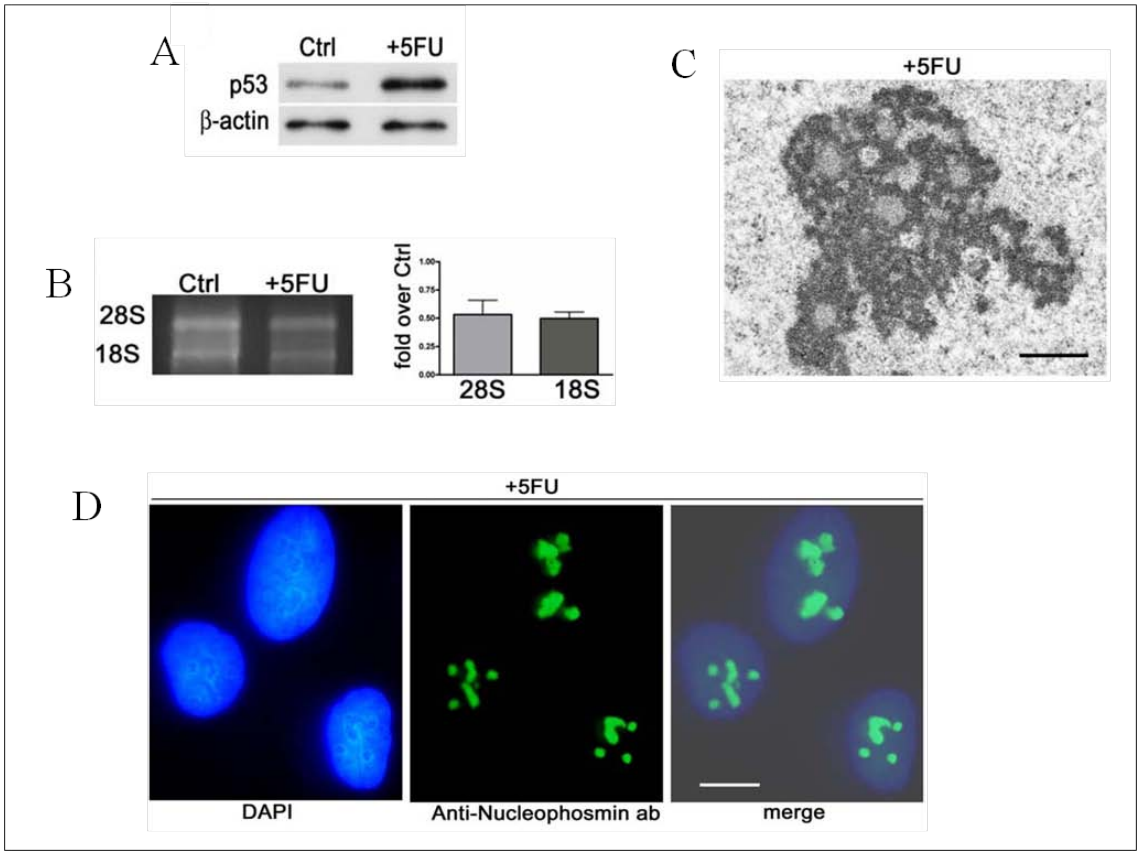

Figure 10 Fluorouracil inhibits the synthesis of rRNA and stabilizes p53 without inducing the "nucleolar disruption". A, Representative Western blot of p53 expression in U2OS cell exposed to 75 μM 5-fluorouracil for 4 h. Ctrl, control cells; +5FU, cells treated with the drug. **B**, Quantitative analysis of 28S and 18S rRNA of control and 5FU- treated U2OS cells. RNA was size-separated on 1% agarose gel and stained with ethidium bromide. Two bands corresponding to 28S and 18S rRNA were visible in each lane. Histograms show the values (mean ± s.d.) of three experiments. **C**, Nucleolar fine structural organization of a U2OS cell, treated with 5FU. Arrows indicate the fibrillar centres, surrounded by the ribonucleoprotein fibrillar and granular component. Uranium and lead staining. Scale bar, 0.4 μm. **D,** Visualization of nucleophosmin distribution in U2OS cells treated with 5-FU: 1) DAPI staining, 2) nucleophosmin labeling with monoclonal antibodies versus nucleophosmin, revealed by FITCH-conjugated secondary antibodies 3) merging of image 1 and 2. Scale bar, 10 μm.

p53 stabilization after *POLR1A* silencing is due to MDM2 inactivation by ribosomal proteins

p53 levels are mainly regulated by the ubiquitine ligase MDM2. Alterations in the ribosome biogenesis are sensed by p53 by interfering with Mdm2 mediated degradation (Momand et al. 1992; Haupt et al. 1997; Kubbutat et al. 1997). Ribosomal proteins can in fact inhibit Mdm2 function after a perturbation of ribosome biogenesis, thus leading to p53 stabilization and activation (Zhang and Lu 2009); therefore we wondered whether the reduction of rRNA synthesis induced by *POLR1A* silencing caused p53 stabilization following the same mechanism.

First we wanted to evaluate if a reduced proteasome mediated degradation was responsible for the increased p53 levels in *POLR1A* silenced cells. In order to do this we first evaluated the half-life of p53 by time course Western blot analysis in control and *POLR1A*-silenced U2OS cells after treatment with Cycloheximide at a dose capable of completely inhibiting protein synthesis. We found that the half-life of p53 in *POLR1A*-silenced cells was longer than that of control cells (Fig. 11A). The involvement of proteasome was demonstrated by blocking the proteasome activity with the small inhibitor molecule MG-132. After inhibiting p53 degradation with MG-132 we observed that the level of p53 in *POLR1A*-silenced cells for the time-intervals considered did not change while it progressively increased in control cells, as evaluated by Western blot analysis (Fig. 11B).

To understand the mechanism involves in regulating p53 levels after *POLR1A* silencing, we wonder whether *POLR1A* silencing induced quantitative alterations also in the ribosomal proteins. Real Time PCR analysis showed that the level of the rpL5 and

rpL11 mRNAs associated to polyribosomal fractions was similar both in control and in 48 h-*POLR1A*-silenced cells (Fig. 12A), demonstrating that *POLR1A* interference did not induce variations in rpL5 and rpL11 synthesis. At this point we looked for quantitative changes in the binding of rpL5 and rpL11 to MDM2 by co-immunoprecipitation analysis. We observed that the amount of both proteins co-immunoprecipitated with MDM2 increased in *POLR1A*-silenced cells in comparison with control cells (Fig. 12B).

We demonstrated the importance of the ribosomal proteins in determining p53 stabilization after *POLR1A* silencing by observation in *RPL11*-silenced cells. We silenced *RPL11* alone, or both *RPL11* and *POLR1A*; the silencing strongly reduced the expression of the relative mRNAs (Fig. 12C). Western blot analysis of p53 expression showed that *POLR1A* interference did not stabilize the protein in *RPL11*-silenced cells (Fig. 12D), thus demonstrating that rpL11 availability was necessary for p53 stabilization in *POLR1A*-silenced cells.

Other experimental models showed that p53 stabilization in *POLR1A*-silenced cells was due to the inactivation of MDM2 consequent to its nucleolar sequestration (Lohrum et al 2003; Bernardi et al. 2004). In order to exclude this, we evaluated the distribution of MDM2 in control and *POLR1A*-silenced U2OS cells by immunocytochemistry. We found that *POLR1A* silencing did not induce any accumulation of MDM2 in nucleoli (Fig. 13). Therefore, all together these data were consistent with a ribosomal-protein mediated mechanism of p53 stabilization after rRNA synthesis down-regulation.

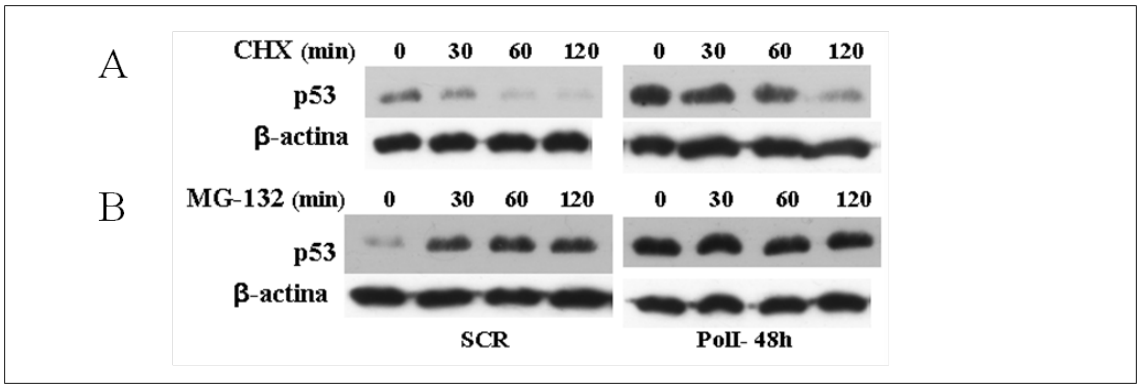

Figure 11 Reduced proteasome mediated degradation is responsible for the increased p53 levels in *POLR1A*-silenced cells. A, Time course analysis of p53 protein expression in control and *POLR1A*-silenced U2OS cells, 48 h after the silencing procedure, exposed to Cycloheximide (CHX) at a concentration of 20 μg/mL. **B**, Time course analysis of p53 protein expression in control and *POLR1A*-silenced U2OS cells, 48 h after the silencing procedure, exposed to the proteasomal inhibitor MG- 132 at the concentration of 10 μM, for 2 hours.

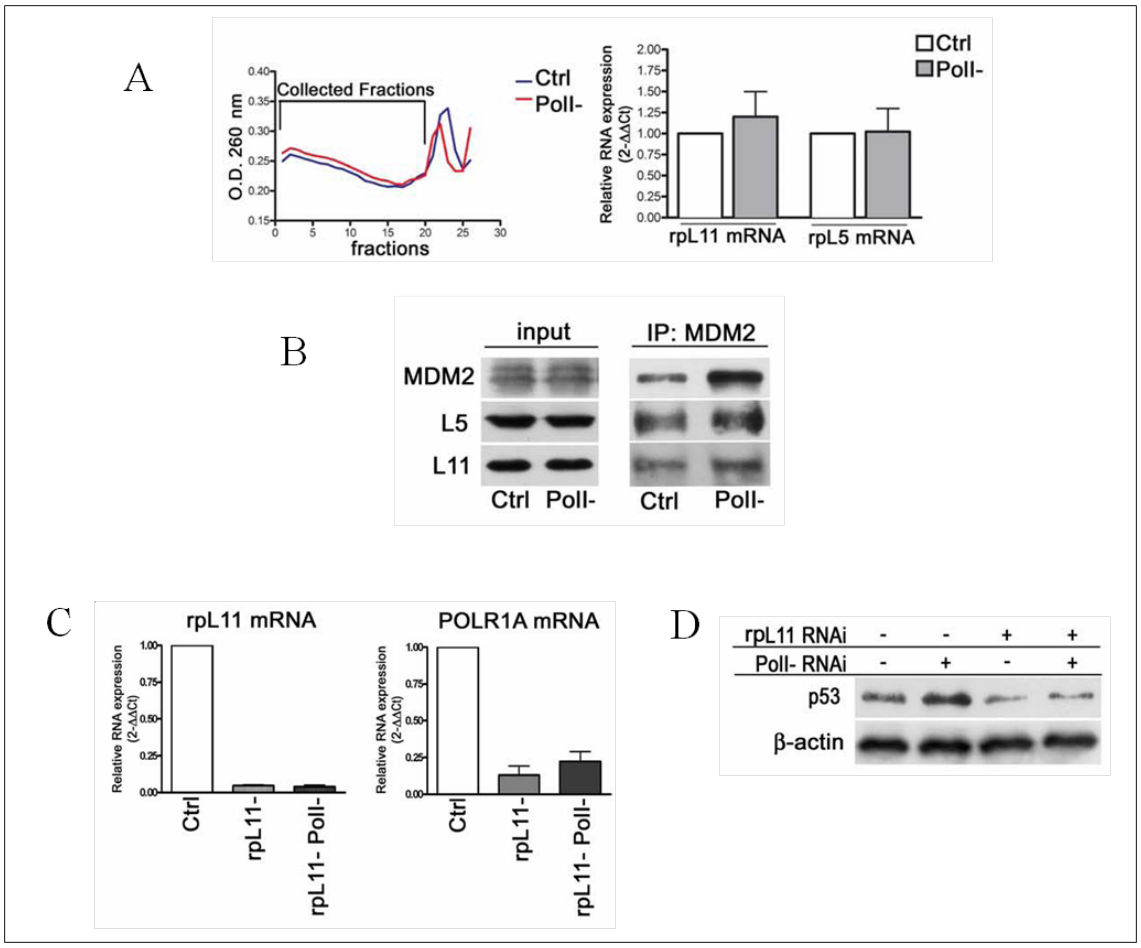

Figure 12 MDM2 inactivation by ribosomal proteins is responsible for p53 stabilization after rRNA synthesis up-regulation. A, Polysomal profiles and relative changes of the total amount of rpL11 and rpL5 mRNAs associated with polysomal fractions from control and *POLR1A*-silenced cells. **B**, Western blot of the amount of rpL5 and rpL11 bound to MDM2 in control and *POLR1A*-silenced U2OS cells, 48 h after the silencing procedure. *Input*, amount of rpL5, rpL11 and MDM2 before the immunoprecipitation; *IP:MDM2*, amount of rpL5, rpL11 and MDM2 after the immunoprecipitation with MDM2 antibody. **C,** Real Time PCR analysis of rpL11 and POLR1A mRNA in control and in U2OS cells silenced for either RPL11 alone or both RPL11 and POLR1A expression (POLI-). **D**, Western blot of p53 expression in control and *POLR1A*-silenced cells, either or not silenced for rpL11 expression.

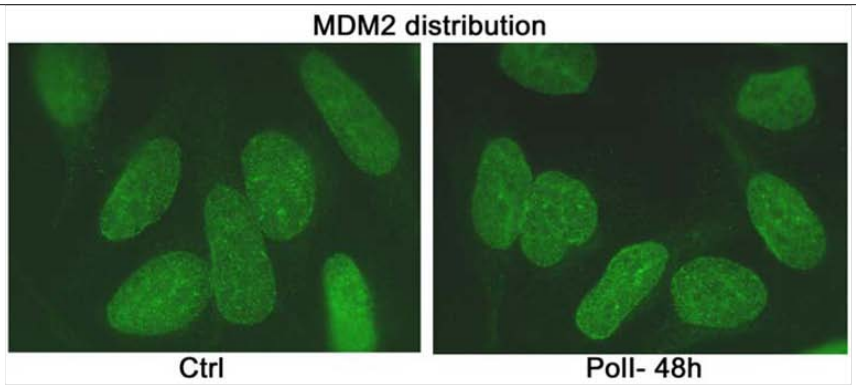

Figure 13 *POLR1A* interference does not modify the distribution of MDM2 in the U2OS cell nuclei. Distribution of MDM2 in control and in *POLR1A*-silenced U2OS cells, 48 hours after the end of the silencing procedure, as visualized by anti-MDM2 rabbit polyclonal antibody immunostaining revealed by FITCH-conjugated secondary antibodies. Scale bar, 10 μm.

The contemporary reduction of rRNA and protein synthesis does not stabilize p53

Our results strongly suggest that the increased p53 levels consequent the rRNA synthesis down-regulation is due to a reduced protein degradation mediated by MDM2 that, in turn, is due to an increased availability of ribosome free-protein that bind and inhibit MDM2. To further demonstrate this mechanism we wondered what happened to p53 when both rRNA and protein synthesis are down-regulated. For this purpose we used either the serum starvation procedure or the Rapamycin treatment. The amount of 45S rRNA, detected by RT-PCR analysis after 24 hours of serum starvation, was reduced in U2OS cells (Fig. 14A) as well as 5-fluorouridine incorporation into nucleolar rRNA detected by immunofluorescence (Fig. 14B). Evaluation of p53 by Western blot

analysis showed a reduction (Fig. 14C). At that time, the translation level of the rpL5 and rpL11 mRNAs was also markedly reduced (Fig. 14D), as well as the nuclear amount of these proteins (Fig. 14E). Regarding the experiments with Rapamycin, MCF-7 cells were used because of the very low sensitivity of the U2OS cell to the drug exposure. Treatment with 20 nM Rapamycin for 24 hours strongly reduced both the amount of 45S rRNA as detected by RT-PCR analysis (Fig. 15A) and the nucleolar 5-fluorouridine immunofluorescent staining (Fig. 15B). Rapamycin treated cells showed a reduction in p53 expression (Fig. 15C) and in the translation of the rpL5 and rpL11 mRNAs (Fig. 15D). Therefore both experimental conditions, serum starvation and Rapamycin treatment, conducted for a period of 24 hours were able to induce a reduction of rRNA and ribosomal protein synthesis. In this conditions we couldn't observe an increase in p53 expression levels as previously observed in consequence of rRNA down-regulation alone; in this case p53 levels were reduced likely as a consequence of the reduced p53 mRNA translation.

Given the above results we wondered what happened to p53 levels in *POLR1A*-silenced cells after serum starvation. We found that serum starvation prevented p53 accumulation also in *POLR1A*-silenced cells, thus indicating that p53 accumulation after the inhibition of rRNA synthesis by *POLR1A* interference was strictly dependent upon availability of newly synthesized ribosomal proteins (Fig. 16). To exclude the possibility that the reduced p53 levels observed in this experimental condition was not due to a reduced p53 protein synthesis induced by the serum starvation, we decided to performed a further experiments using Actinomycin D (ActD). ActD selectively inhibits rRNA transcription (Montanaro et al. 2007) and disrupts the nucleolar structure causing ribosomal protein leakage from the nucleolus to the nucleoplasm (Rubbi and Milner

2003) when used at low doses. Serum-starved and Rapamycin-treated cells, analyzed by 5-fluorouridine immunofluorescence staining, showed a complete inhibition of rRNA transcription when treated with ActD (Fig. 17). In ActD exposed cells p53 accumulation occurred again as observed by Western blot analysis (Fig. 18). The increased p53 levels were very likely a consequence of MDM2 inactivation caused by the released nucleolar ribosomal proteins.

Therefore, all together these results led us to conclude that p53 stabilization after the reduction of rRNA synthesis by *POLR1A* interference was actually due to a relatively increased availability of ribosomal proteins for MDM2 binding as a consequence of their reduced use in ribosome building.

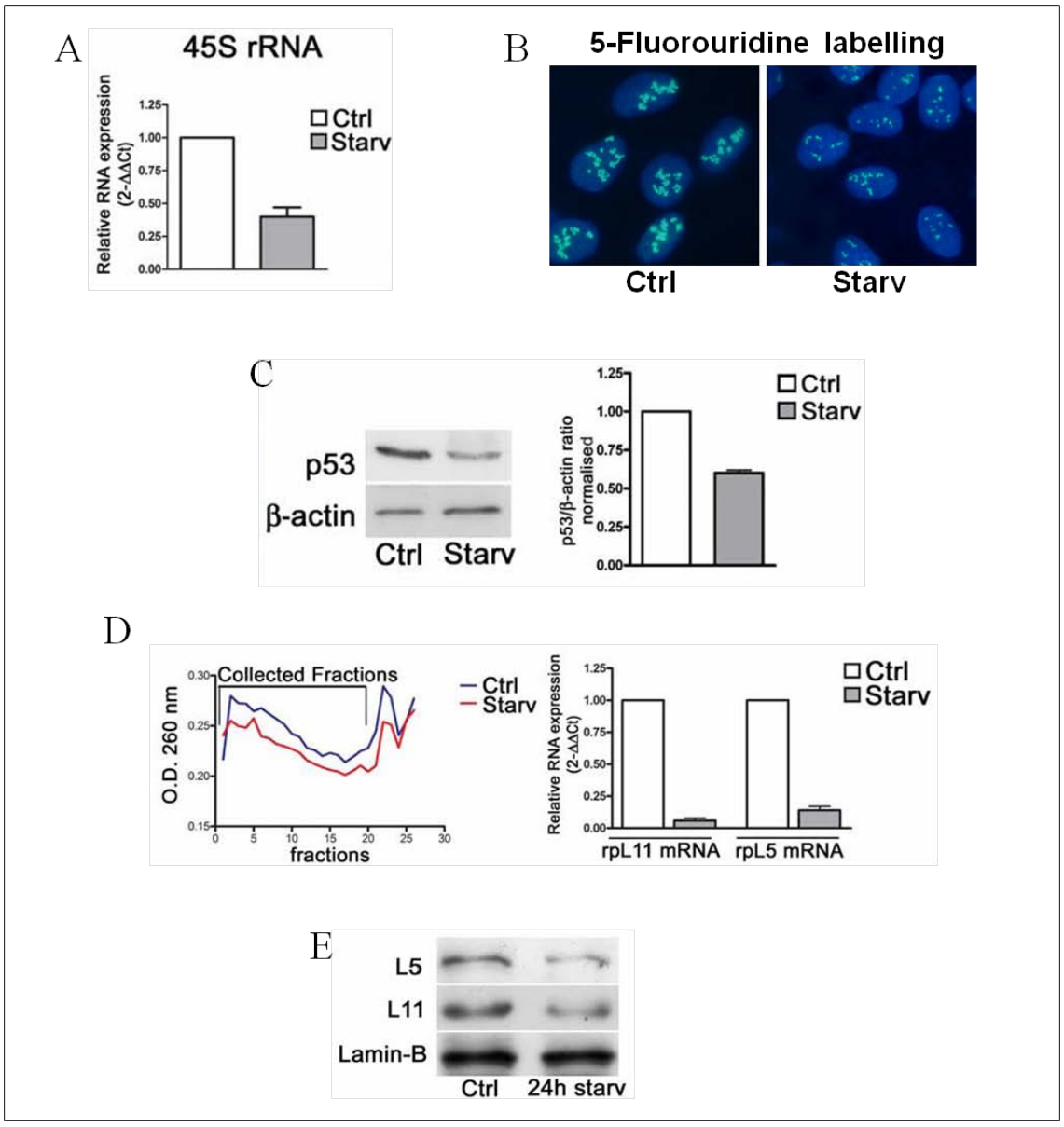

Figure 14 Serum starvation reduces both rRNA synthesis and ribosomal protein production and does not stabilize p53. A, Real-time RT-PCR analysis of the 45S rRNA expression and visualization of rRNA synthesis in control and serum-starved (starv) U2OS cells. Cell were starved for 24 h. **B**, to visualize rRNA synthesis cells were labeled with 5-fluorouridine for 15 min, and 5-fluorouridine revealed by specific FITCH-conjugated monoclonal antibodies. DAPI counter-staining. Scale bar, 10 μm. **C,** Representative Western blot and densitometric analysis of p53 expression in control and 24 h serum-starved U2OS cells. **D**, representative polysomal profiles (left) and relative changes of the total amount of rpL11 and rpL5 mRNAs (right) associated to polysomal fractions from control and 24 h serum starved U2OS cells. **E**, representative Western

blot of the nuclear amount of rpL5 and rpL11 in control and 24 h serum-starved U2OS cells.

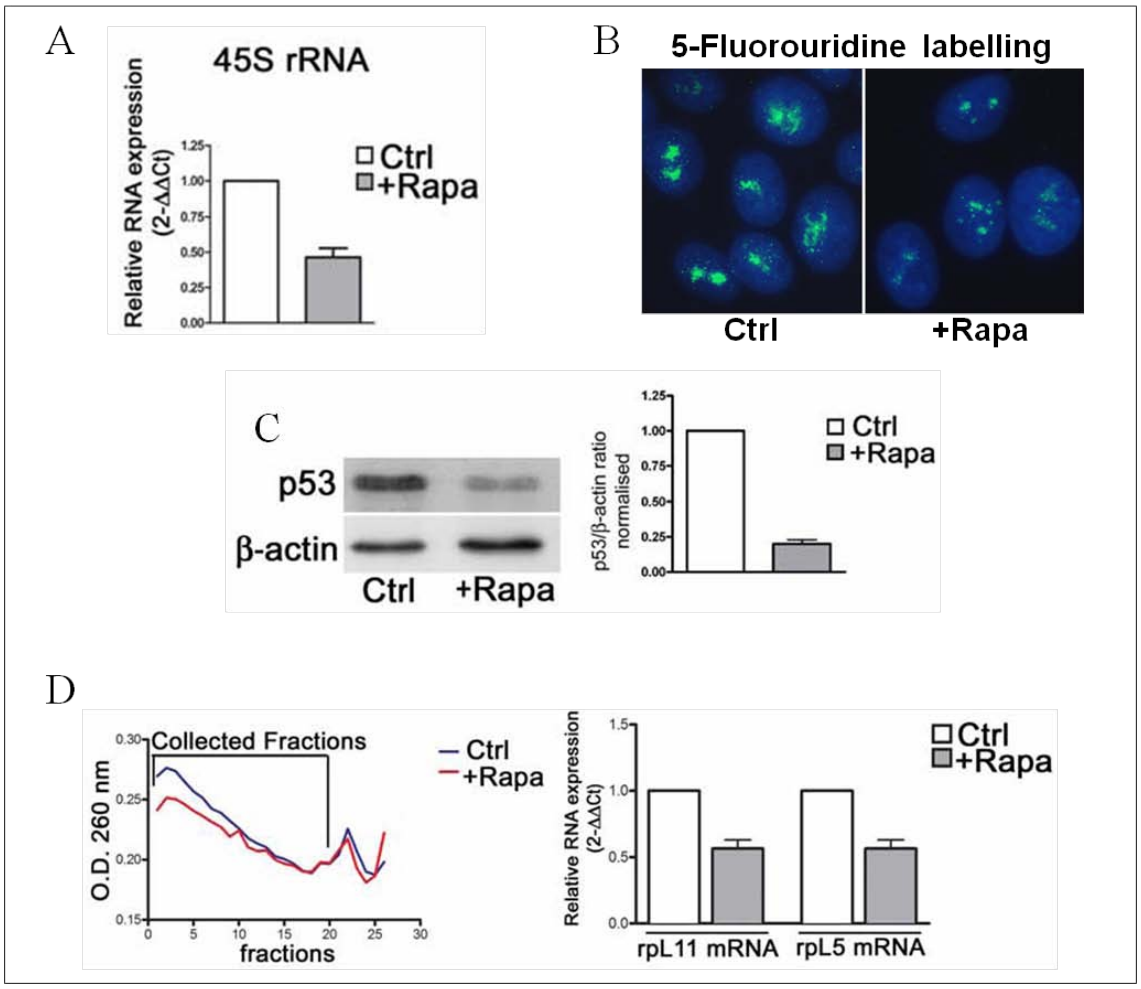

Figure 15 Rapamycin treatment reduces both rRNA synthesis and ribosomal protein production and does not stabilize p53. A, Real-time RT-PCR analysis of the 45S rRNA expression and visualisation of rRNA synthesis in control and Rapamycin-treated (+Rapa) MCF-7 cells. Rapamycin was used at a concentration of 20 nM for 24 h. **B**, Cells were labeled with 5-fluorouridine for 30 min, and 5-fluorouridine revealed by specific FITCH-conjugated monoclonal antibodies. DAPI counter-staining. Scale bar, 10 μm. **C**, Representative Western blot and densitometric analysis of p53 expression in control and Rapamycin-treated MCF-7 cells. **D,** Representative polysomal profiles (left) and relative changes of the total amount of rpL11 and rpL5 mRNAs (right) associated with polysomal fractions from control and Rapamycin-treated (+Rapa) MCF-7 cells.

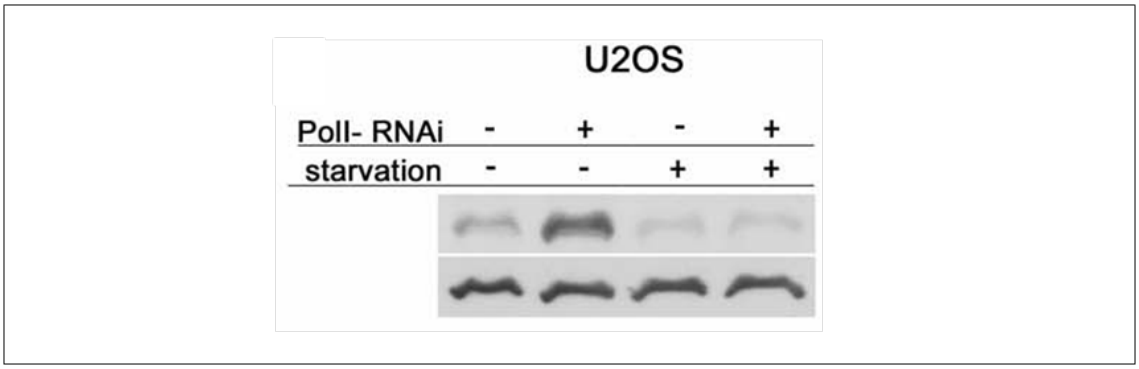

Figure 16 Serum starvation prevents p53 accumulation also in *POLR1A*-silenced U2OS cells. Western blot analysis of p53 level in *POLR1A*-silenced and control U2OS cells, in normal growing medium or 24 hours after serum starvation.

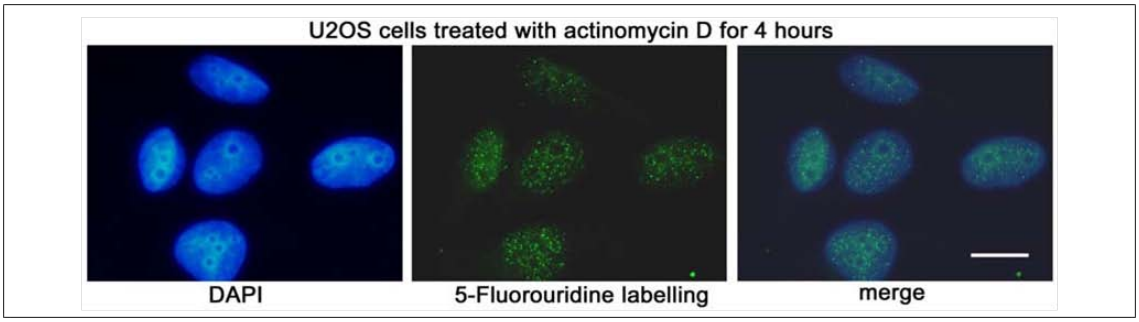

Figure 17 Actinomycin D treatment abolishes rRNA transcription in U2OS serum-starved cells. Serum-starved U2OS cells were exposed to 8 nM Actinomycin D for 4 h. 1) DAPI staining reveals unstained round structures corresponding to nucleolar bodies. 2) 5-fluorouridine immunofluorescent staining is uniformly distributed through the nucleoplasm, but not in the nucleolar bodies which are unlabelled. 3) Merging of image 1 and 2. Scale bar, 10 μm.

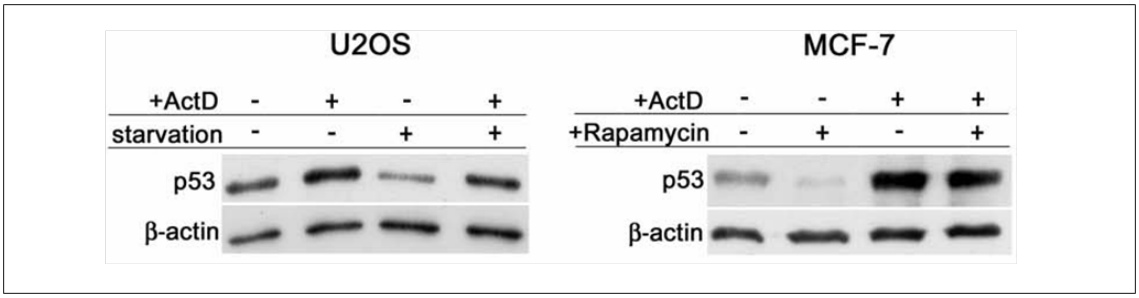

Figure 18 Effect of Actinomycin D on serum starved and Rapamycin treated cells. Western blot analysis of p53 level in serum starved U2OS and Rapamycin treated MCF-7 cells exposed to 8 nM Actinomycin D.

rRNA synthesis up-regulation reduces p53 expression levels

Considering the results observed in consequence of a selective reduction of the rRNA synthesis on p53 expression level, we wondered whether an up-regulation of rRNA synthesis was able to influence p53 level. To study the effect of a stimulation of rRNA synthesis on p53 stabilization we used three different experimental conditions: 1) cancer cell lines exposed either to insulin or to the insulin-like growth factor-1 (IGF-1), 2) regenerating rat liver after partial hepatectomy, and 3) rat liver after intra-peritoneal cortisol injection. Insulin and IGF-1 stimulate the activity of polymerase I transcription by elevating both UBF protein level and phosphorylation (Hannan et al. 1998). Partial hepatectomy induces a progressive increase in rRNA synthesis (up to 24 hours after operation) in regenerating rat hepatocytes (Sirri et al. 1995) very likely due to the induction of c-myc (Thompson et al. 1986) which controls the RNA polymerase I activity (Arabi et al. 2005; Grandori et al. 2005). Cortisol at the dose used in the present experiments stimulated rRNA transcription in rat hepatocytes (Sirri et al. 1995) by activating the glycocorticoid receptor signaling (Glibetic et al. 1993), while inhibiting their proliferation (Nadal 1995).

We first evaluated the effect of an increase in rRNA synthesis in cancer cell lines: MCF-7 and H4-II-E-C3 cells. We used insulin to stimulate rRNA synthesis in H4-II-E-C3 hepatoma cells, while MCF-7 mammary carcinoma cells that showed no response to the hormone were stimulated with Insulin Like Growth Factor 1(IGF-1). The evaluation of rRNA transcription by real time RT-PCR analysis of 45S rRNA indicated that rRNA synthesis was significantly increased in MCF-7 cells exposed to IGF-1 and in H4-II-E-C3 cells treated with insulin, after 12 hours following the administration (Fig. 19A). In

all these experimental conditions we found that the expression of p53, as measured by Western blot analysis, was reduced in cells in which rRNA synthesis was stimulated in comparison to the relevant controls (Fig. 19B).

Since in the above reported experimental conditions a series of signaling pathways are activated that might led to p53 down-regulation independent of rRNA synthesis stimulation, we evaluated the effect of IGF-1 stimulation on p53 expression in MCF-7 cells after 48 h *POLR1A* silencing. We found that the synthesis of rRNA was down-regulated both in control and IGF-1 treated cells silenced for POLR1A expression as evaluated by Real Time PCR analysis of 45S rRNA. IGF-1 exposure did not modify the level of p53 expression in *POLR1A*-silenced cells, thus indicating the key role of rRNA synthesis up-regulation in determining the reduction of p53 expression after IGF-1 stimulation (Fig 20).

Then we analyzed the consequences of rRNA synthesis stimulation in two different *in vivo* experimental conditions: regenerating rat liver after partial hepatectomy and rat liver after intra-peritoneal cortisol injection. Both the experimental conditions were efficient in inducing the increase in the amount of rRNA in comparison with the normal liver. The evaluation by Real Time PCR analysis of 45S rRNA showed an increase in the rRNA 12 hours after partial hepatectomy and 8 hours after intra-peritoneal hormone administration (Fig. 21A). Western blot analysis of p53 expression levels confirmed the reduction of the oncosuppressor previously observed in the cell lines, in the experimental conditions in which rRNA synthesis was stimulated (Fig. 21B).

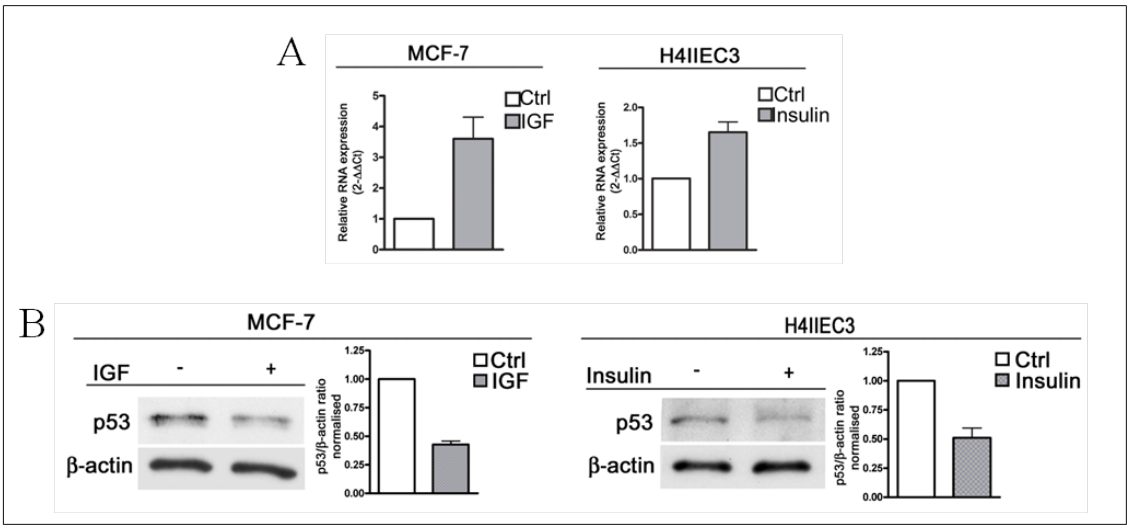

Figure 19 rRNA synthesis up-regulation causes p53 reduction in cancer cell lines. A, Real Time PCR analysis of 45S rRNA 12 hours after IGF or Insulin administration in MCF-7 and H4-II-E-C3 cells respectively. **B**, p53 levels analyzed by Western blot in control and stimulated cells.

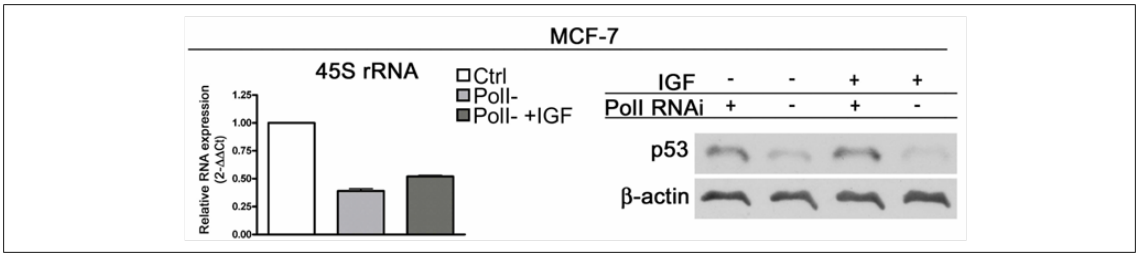

Figure 20 IGF-1 exposure does not modify the level of p53 in *POLR1A*-silenced cells. Left panel shows a real-time RT-PCR analysis of the 45S rRNA expression in control, in 48 h-*POLR1A*silenced and in *POLR1A*-silenced MCF-7 cells exposed to IGF-1 at a dose of 100 ng/ml for 12 h. The right panel shows a representative Western blot of p53 expression of MCF-7 cells in the same experimental conditions as in the left panel. Histograms show the values (mean ± s.d.) of three experiments.

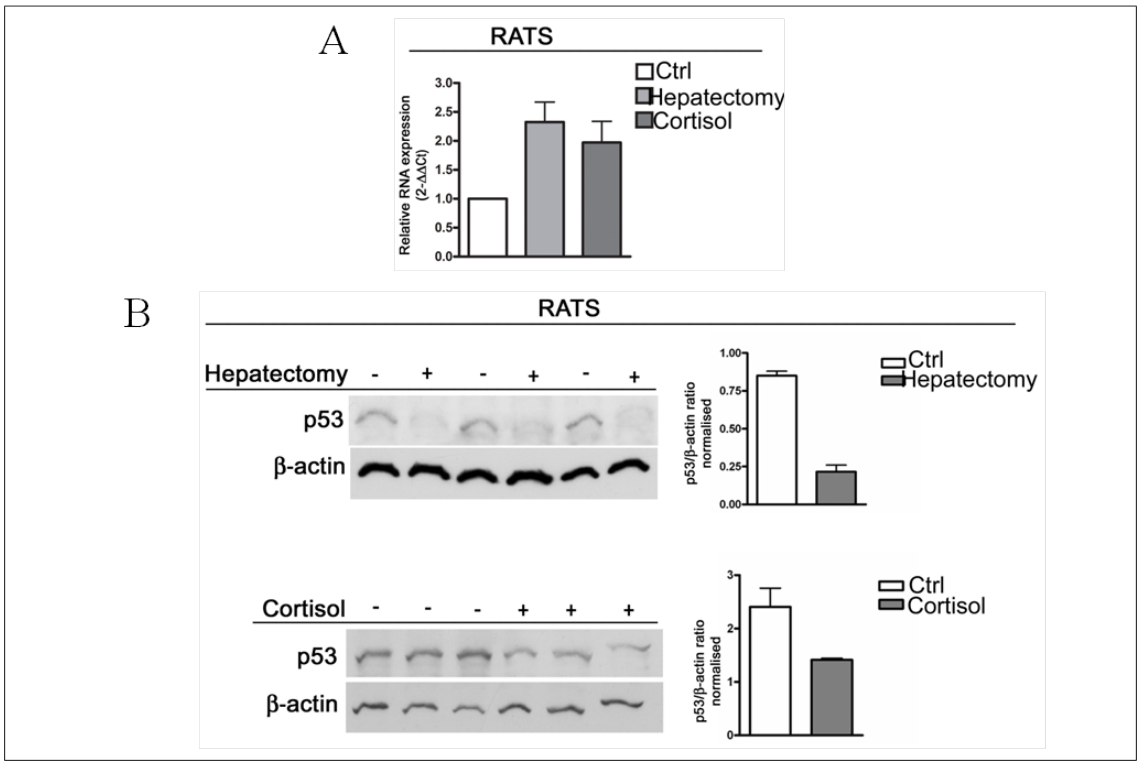

Figure 21 rRNA synthesis up-regulation causes p53 reduction in rats. A, Real Time PCR analysis of 45S rRNA in control rat liver, in regenerating rat liver 12 h after partial hepatectomy and in rat liver 8 h after cortisol intra-peritoneal injection at the dose of 10 mg/100 g body weight.. **B**, p53 levels analyzed by Western blot in control and treated animals.

Increased proteasome degradation is responsible for p53 reduction after rRNA synthesis stimulation

In order to investigate the mechanism involved in the reduction of the p53 level after stimulation of rRNA synthesis we first analyzed the expression of the p53 mRNA in the MCF-7 cells exposed to IGF-1, the H4-II-E-C3 cells treated with insulin, the regenerating rat liver and the rat liver after cortisol treatment. The evaluation of the p53 mRNA expression by Real Time PCR analysis indicated that, in all the experimental conditions of rRNA synthesis stimulation, no quantitative changes in *TP53* transcription

occurred (Fig. 22). Therefore, we considered the possibility that the reduced level of p53 might be due to its increased proteasomal degradation. For this reason we treated both IGF-1-stimulated MCF-7 cells and insulin-exposed H4-II-E-C3 cells with the proteasome inhibitor MG-132. We found that, in both experimental conditions, the inhibition of proteasomal degradation cancelled the difference between the p53 expression of control and stimulated cells, as evaluated by Western blot analysis (Fig. 23). This data clearly indicated that the changes in p53 level were actually due to an increased protein degradation.

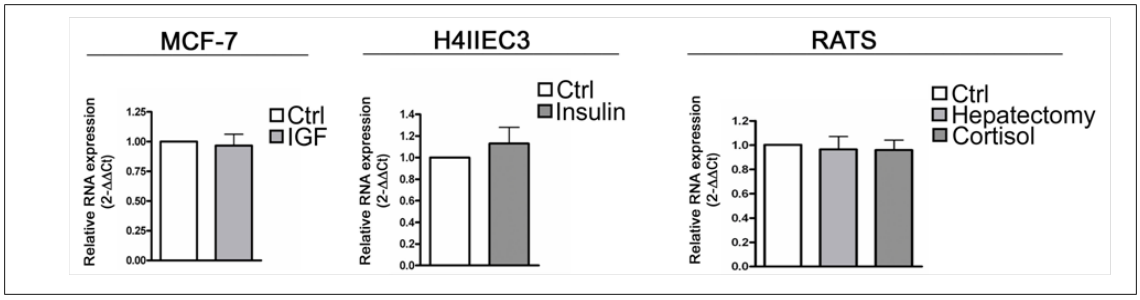

Figure 22 p53 transcription is not altered after rRNA synthesis stimulation. Real Time PCR analysis of p53 transcription level in control MCF-7 and H4-II-E-C3 cells and treated with 12 hours IGF or insulin, and in control, hepatectomized and cortisol treated rat liver.

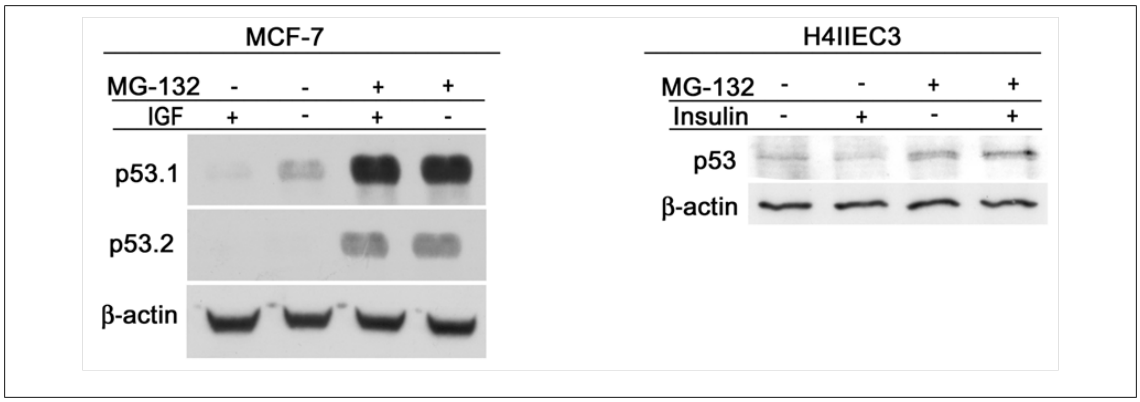

Figure 23 Increased protein degradation is involved in regulating p53 level after rRNA up-regulation. Representative Western blot of the effect of 2 h treatment with the proteasomal inhibitor MG-132 at the concentration of 10 μM, on p53 expression in MCF-7 either or not stimulated by IGF-1 (p53.1: long exposure; p53.2: short exposure) and in H4IIEC3 cells either or not stimulated by insulin.

p53 reduction after rRNA synthesis up-regulation involves MDM2 inactivation with ribosomal proteins

p53 expression levels are controlled by a mechanism that involves MDM2. We previously demonstrated the involvement of MDM2 increased binding to the ribosomal proteins in increasing p53 levels after selective reduction of rRNA synthesis. Therefore we wonder whether the same mechanism might regulate p53 level after a stimulation of rRNA synthesis. We performed co-immunoprecipitation analysis to evaluate the quantitative changes of two ribosomal proteins bound to MDM2 after rRNA synthesis stimulation. We found that the amount of the ribosomal proteins rpL5 and rpL11 co-immunoprecipitated with MDM2 was reduced both in IGF-1-stimulated MCF-7 cells and in insulin-exposed H4-II-E-C3 cells in comparison to control cells (Fig. 24A), thus demonstrating that the same mechanism observed in consequence of a reduction of the synthesis of rRNA was valid even when the rRNA synthesis is stimulated. To demonstrate that the reduced binding of the analyzed ribosomal protein to MDM2 wasn't the consequence of a reduced protein synthesis, we analyzed the rpL5 and rpL11 mRNAs bound to polyribosomes. The analysis performed by Real Time PCR showed an increase of the level of rpL11 mRNA in stimulated versus control cells (Fig. 24B), whereas we noticed no significant changes in the rpL5 mRNA level.

To better define the mechanism of p53 stabilization after rRNA up-regulation, we performed experiments with the MDM2 inhibitor Nutlin-3 in control and IGF-stimulated MCF7 cells. Nutlin-3 binds MDM2 in the p53-binding pocket, thus inhibiting MDM2-mediated p53 degradation and resulting in p53 stabilization (Vassilev et al. 2004). Western blot analysis of p53 expression after Nutlin-3 exposure indicated

that p53 stabilization was similar both in control and IGF-1-stimulated MCF-7 cells (Fig. 25). This strongly suggested that the reduction of p53 expression after stimulation of rRNA synthesis was actually the consequence of an increased binding of MDM2 to p53.

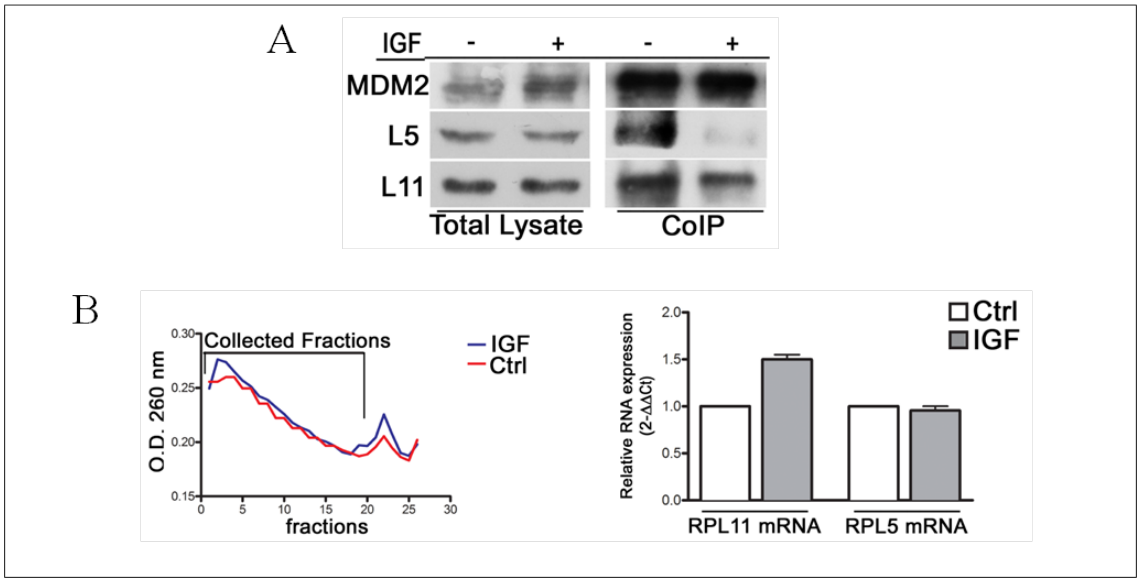

Figure 24 After rRNA synthesis up-regulation the binding between the ribosomal proteins and MDM2 is reduced. A, Western blot analysis of the amount of rpL5 and rpL11 bound to MDM2 in control and IGF treated MCF7 cells, 12 h after IGF administration. *Input*, amount of rpL5, rpL11 and MDM2 before the immunoprecipitation; *IP:MDM2*, amount of rpL5, rpL11 and MDM2 after the immunoprecipitation with MDM2 antibody. **B**, Polysomal profiles and Real Time PCR analysis of the total amount of rpL11 and rpL5 mRNAs associated with polysomal fractions from control and IGF treated MCF7 cells.

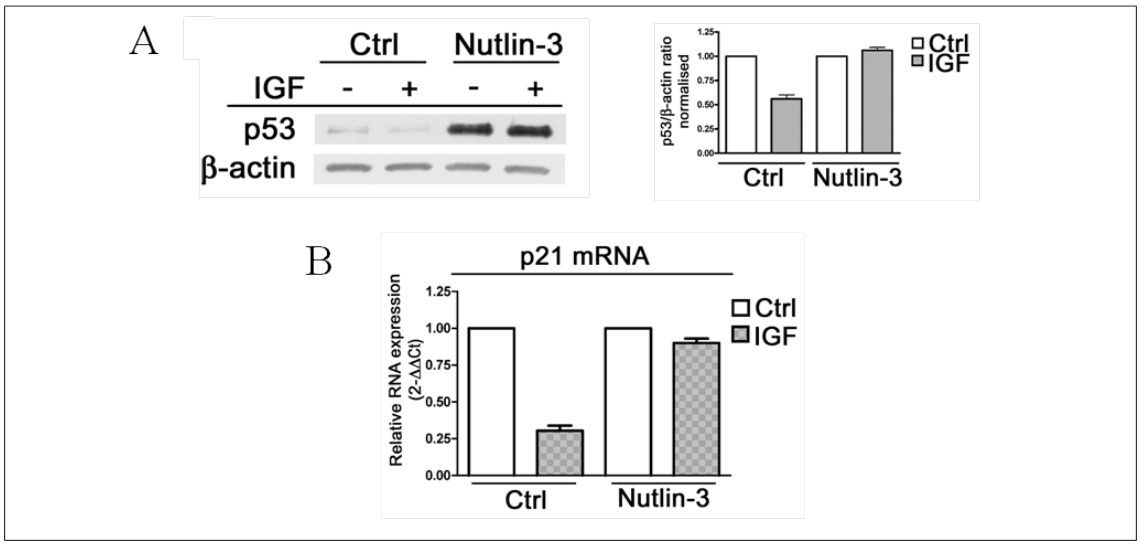

Figure 25 After Nutlin-3 treatment p53 level is the same in control and IGF-stimulated cells. MCF-7 cells stimulated by IGF-1 and treated with Nutlin-3 at the concentration of 5 μM for 16 hours**: A**, Western blot analysis of p53 expression. **B,** Real-time RT-PCR analysis of p21 mRNA expression.

p53 target genes expression is reduced after rRNA synthesis up-regulation

The observation that the level of p53 was reduced in cells in which the rRNA synthesis was stimulated prompted us to investigate whether the activity of p53 was also reduced in these cells. In order to evaluate p53 function we analyzed the expression of three p53-target genes. We chose genes whose products have a tumor suppressor function: p21, BAX, PUMA (Burns and El-Deiry 1999; Sax and El-Deiry 2003). We analyzed the mRNA expression level of these target genes by Real Time PCR. In MCF-7 and H4-II-E-C3 cancer cell lines the expression level of all the analyzed genes resulted significantly reduced when the rRNA was up-regulated by IGF and insulin stimulation respectively (Fig. 26).

Regarding the regenerating rat liver and the rat liver after cortisol treatment we observed a reduction only in BAX and PUMA expression level. p21 mRNA expression appeared to be reduced only in cortisol treated tissue, in fact in the regenerating rat liver p21 expression level was highly increased in comparison with control and resting liver (Fig. 26B). This observation is consistent with previous data showing that in regenerating hepatocytes after partial hepatectomy the p21 mRNA was up-regulated with enhanced expression during G1 phase (Albrecht et al. 1997). For this reason we used regenerating rat livers which are in G1 phase, 12 hours after partial hepatectomy, while the synthesis of DNA began only 15 hours after surgery (Sirri et al. 1995).

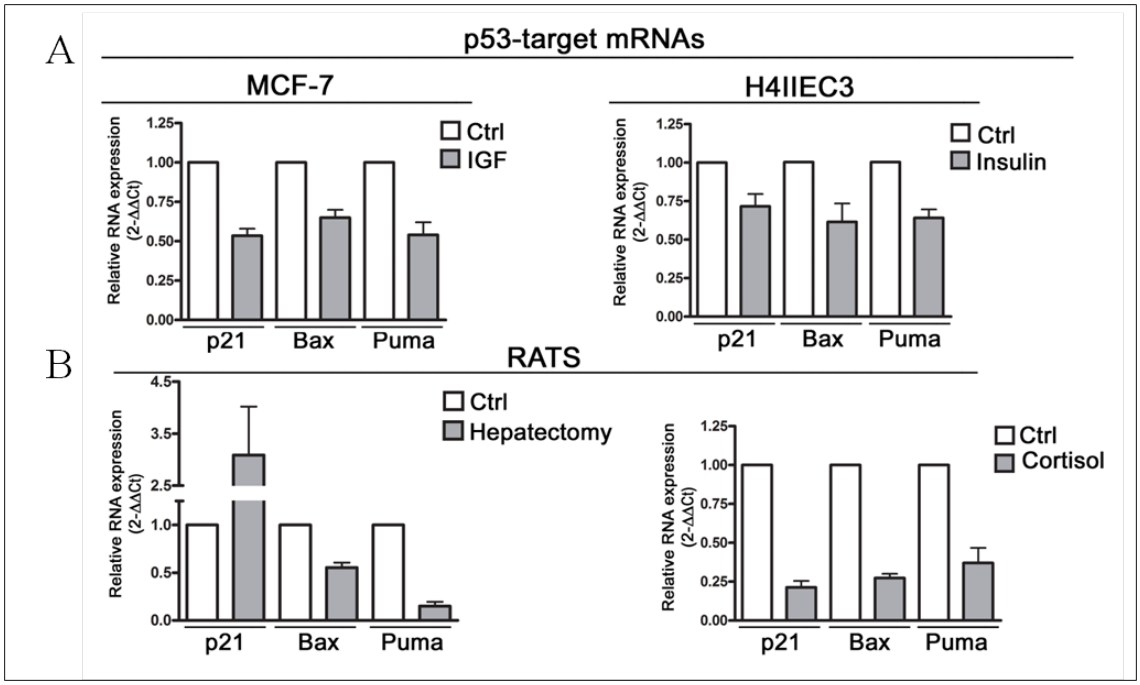

Figure 26 p53 target genes expression is reduced when the rRNA synthesis is up regulated. A, Real-time RT-PCR analysis of the mRNA expression of the three p53-target genes (*p21*, *Bax* and *Puma*) in control and MCF-7 cells exposed to IGF-1 for 12 h; in control and in H4IIEC3 cells stimulated by insulin for 16 h; **B,** in control and in regenerating and cortisol-stimulated rat liver. Histograms show the values (mean ± s.d.) of three experiments.

p53 function after drug-induced stress is impaired when rRNA synthesis is increased

During the normal cell growth p53 function is not required, its activation is necessary only in response to stresses when p53 levels and p53 ability to activate the transcription of its target genes are increased. For this reason we wondered whether the reduced p53 level in cells with increased rRNA synthesis might also be reflected in a reduced p53 stabilization and activity after cytotoxic stress. For this purpose we used IGF-1-stimulated MCF-7 cells treated with either Hydroxyurea or Gemcitabine. These drugs were chosen because they do not directly inhibit ribosome biogenesis (Derenzini et al. 1981; Burger et al. 2010). We found that in IGF-1-stimulated MCF-7 cells both drugs stabilized p53 to a lesser extent than in control cells, as evaluated by Western blot analysis (Fig. 27A). Regarding the expression of the p53-target genes, we observed that the p21, BAX and PUMA mRNA level was markedly lower in IGF-1-stimulated than in control MCF-7 cells after either Hydroxyurea or Gemcitabine treatment (Fig. 27B). Therefore, these results indicated that the up-regulation of rRNA synthesis induced a lowering of the p53 level which was responsible for the down-regulation of the p53-target gene response after exposure to cytotoxic stresses.

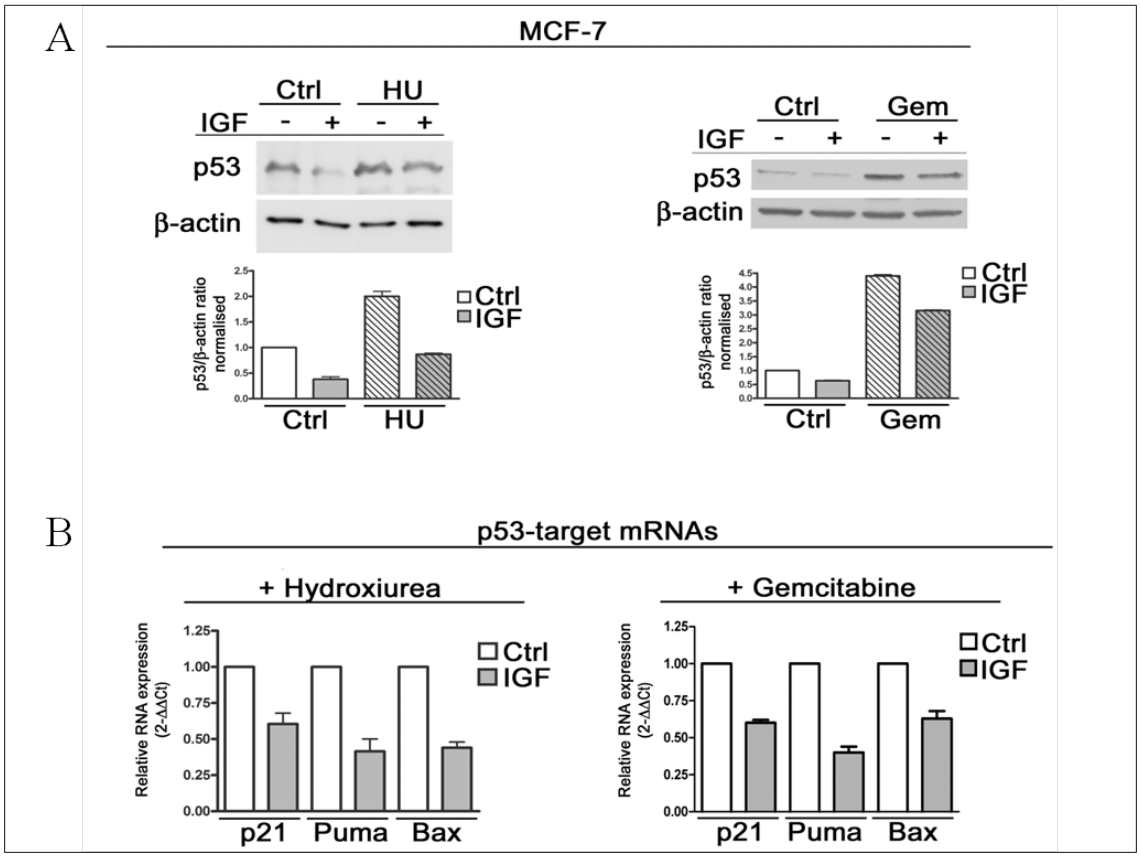

Figure 27 rRNA synthesis up-regulation reduces p53 levels and activity after drug-induced stress. A, Representative Western blot and densitometric analysis of p53 expression in control and MCF-7 cells exposed to IGF-1, treated either with 3.4 μM Hydroxyurea for 3 h or with Gemcitabine at the dose of 10 μg/ml for 4 h. **B** Real-time RT-PCR analysis of the mRNA expression of *p21*, *Puma* and *Bax* in control and MCF-7 cells exposed to IGF-1 treated either with 3.4 μM Hydroxyurea for 3 h or with Gemcitabine at the dose of 10 μg/ml for 4 h. Histograms show the values (mean ± s.d.) of three experiments.

DISCUSSION

This study demonstrated that the control of p53 stabilization and activity in mammalian cells may be due to the balance between the rRNA and ribosomal proteins' synthesis.

p53 activation usually occur after a stress, and according the nucleolar disruption model in order to stabilize and activate p53 a stress must induce nucleolar disruption and compromise nucleolar function (Rubbi and Milner 2003). Our results demonstrated that these severe structural-functional changes of the nucleolus were not a necessary condition for p53 stabilization.

Selective down-regulation of rRNA synthesis did not induce nucleolar disruption while stabilizing p53. Furthermore we showed that up-regulation of ribosomal biogenesis reduced the level of p53. When both the synthesis of rRNA and ribosomal proteins decreased, p53 was not stabilized. The meaning of p53 stabilization and activity were due to change of the balance between rRNA and ribosomal proteins synthesis.

rRNA synthesis down-regulation increases p53 without disrupting nucleolar structure

We demonstrated that, in consequence of a selective reduction of rRNA synthesis, p53 is stabilized even if the nucleolar structure is not altered. In the past, studies about rRNA synthesis alterations were performed mainly by using the Actinomycin D that is able to selectively block the transcription of rRNA genes when used at low doses (Montanaro et al. 2007). However the rRNA synthesis inhibition induced by

Actinomycin D induces the release of nucleolar components in the nucleoplasm that gives rise to p53 stabilization (Rubbi and Milner 2003). For these reasons we looked for an alternative, more specific method to reduce rRNA production. In order to do this we reduced rRNA synthesis by silencing *POLR1A*. This procedure inhibited the expression of POLR1A mRNA and induced a specific inhibition of rRNA synthesis without any evidence of nucleolar disruption and no translocation of nucleolar proteins from the nucleolus to the nucleoplasm. When the rRNA synthesis was down-regulated by *POLR1A* silencing, p53 level was increased and this was the consequence of an increased amount of ribosomal proteins rpL5 and rpL11 bound to MDM2. Therefore, these data were consistent with a ribosomal proteins-MDM2 mediated mechanism of p53 stabilization after rRNA synthesis down-regulation. When both the synthesis of rRNA and ribosomal proteins decreased, such as after either serum deprivation or Rapamycin exposure, p53 was not stabilized. Serum starvation prevented p53 accumulation also in *POLR1A*-silenced cells, thus indicating that p53 accumulation after the inhibition of rRNA synthesis by *POLR1A* interference was strictly dependent upon availability of newly synthesized ribosomal proteins. Therefore, we concluded that p53 stabilization after the reduction of rRNA synthesis by *POLR1A* interference was actually due to a relatively increased availability of ribosomal proteins for MDM2 binding as a consequence of their reduced use in ribosome building.

These results are only partially consistent with previous data suggesting that, in order to stabilize p53, cell stresses should induce a severe perturbation in the nucleolar structure with the release of the nucleolar proteins from the nucleolus, the so-called “nucleolar disruption” (Rubbi and Milner 2003). A lot of data support this theory and indicate that the mechanism involves ribosomal proteins binding with MDM2: in consequence of

necleolar disruption, ribosomal proteins are released from the nucleolus and leak in the nucleoplasm where bind MDM2 thus inhibiting p53 degradation (Zhang and Lu 2009; Deisenroth and Zhang 2010). Our results demonstrated that a selective and specific reduction of rRNA synthesis induces a p53 stabilization through the mechanism involving ribosomal proteins binding to MDM2 but without inducing profound alterations of the nucleolar morphology. The inhibition of new rRNA synthesis halt the progression of ribosome assembly, resulting in an excess of free ribosomal proteins. In turn, without adequate amount of rRNA to bind, ribosomal proteins are available to interact with Mdm2 and stabilize p53.

We described a p53 stabilization model that does not require any severe stress and does not involve any structural alteration of the cell. This is in line with recent evidences that underline how p53 functions can be induced also by mild stress conditions. Indeed p53 functions are not only involved in promoting a tumor suppressive response to acute stress, but p53 has a major role also in regulating diverse aspects of health and disease (Vousden and Lane 2009). In this way mild environmental stresses can activate the so called “every day functions” of p53 that work to adapt the cellular metabolism to the new metabolic necessities without inducing cell death or senescence. Recent studies have painted a more complex picture of p53 as regulator of diverse biological processes, modulating glucose uptake, reducing glycolysis and enhancing mitochondrial respiration (Vousden and Ryan 2009). This mechanism of p53 activation may be also important for the modulation of those p53 functions which depend on basal levels of p53 or the activation of p53 by low levels of constitutive stress, such as the control of stem cell renewal (Meletis et al. 2006; Liu et al. 2009).

rRNA synthesis up-regulation reduces p53 level and activity

The results obtained in cells with a reduced rRNA synthesis, prompted us to investigate what happened when the rRNA synthesis was up-regulated. We used three different experimental conditions of rRNA synthesis stimulation: cancer cells treated with insulin or exposed to the insulin-like growth factor 1, rat liver stimulated by cortisol and regenerating rat liver after partial hepatectomy. In all experimental conditions we observed a reduction of p53 expression level. We are aware that these conditions are not specifically and exclusively characterized by the stimulation of rRNA synthesis. However all of them lead to rRNA synthesis stimulation by different pathways and the consequence is always a reduced p53 level.

The evaluation of p53 transcriptional target genes showed a reduced transcription of p21, BAX and PUMA, demonstrating a failure in the ability of induce a p53 response in all the experimental conditions characterized by an increased ribosome biogenesis. At this point we wondered whether the reduced p53 level in cells with increased rRNA synthesis might also be reflected in a reduced p53 stabilization and activity after cytotoxic stresses. For this purpose we used IGF-1-stimulated MCF-7 cells treated with either Hydroxyurea or Gemcitabine, two drugs that do not directly inhibit ribosome biogenesis. After drug exposure, MCF-7 cells treated with IGF-1 were able to stabilize p53 to a lesser extent than control cells. Also the transcription level of p21, BAX and PUMA was markedly lower in IGF-1-stimulated than un-stimulated MCF-7 cells after either Hydroxyurea or Gemcitabine treatment. These results indicated that the up-regulation of rRNA synthesis induced a lowering of the p53 level which was

responsible for the down-regulation of the p53-target gene response after exposure to cytotoxic stresses.

Regarding the mechanism involved in regulating p53 activation after rRNA synthesis up-regulation we demonstrated the key role played by ribosomal proteins-MDM2 interaction (this mechanism of p53 stabilization is schematically represented in Fig. 28). In homeostatic conditions p53 level is controlled by the steady state balance between rRNA and ribosomal protein synthesis. Ribosomal proteins not utilized for ribosome building bind to MDM2, thus regulating the MDM2-mediated p53 proteasomal degradation. After a down-regulation of rRNA synthesis the ribosomal proteins no longer engaged in ribosome biogenesis bind in larger amount to MDM2, thus reducing the MDM2-mediated p53 proteasomal degradation with the consequent increase of p53 stabilization. When the synthesis of rRNA is up-regulated a greater amount of ribosomal proteins are used for ribosome building, which are therefore no longer available for MDM2 binding. Thus, a greater portion of MDM2 is left free to induce p53 proteasomal degradation.

Interestingly we demonstrated that, when the rRNA synthesis was up-regulated, the ability to activate a p53 response was impaired. These findings can have important implications in tumorigenesis, for example they could explain the increased risk of developing tumors in those non neoplastic, pathological conditions characterized by an up-regulated rRNA synthesis. Among these, chronic liver disease, caused by viral hepatitis, alcohol abuse, and inborn metabolic errors. Eighty percent of all hepatocellular carcinomas (HCCs) worldwide occur when chronic liver disease has reached the cirrhotic stage (Libbrecht et al 2005). The mechanisms underlying the development of chronically diseased liver in HCC are currently being investigated.

Interestingly, there is evidence that, as far as chronic liver diseases from viral infections are concerned, an increased nucleolar size of the hepatocyte is constantly associated with later onset of HCC. Both hepatitis B (HBV) and hepatitis C (HCV) viruses, which are responsible for the development of chronic liver disease, have been shown to up-regulate the activity of the RNA polymerase I and III (Wang et al 1998). A series of studies performed to define whether nucleolar changes might be related to neoplastic transformation in chronic liver disease from viral infection demonstrated that the presence of abnormally enlarged hepatocyte nucleoli represent a very strong risk factor for developing HCC (Trerè et al 2003). In other words, only those chronic liver lesions in which the viral infection induced hypertrophy, and consequently a functional up-regulation of nucleoli, would be susceptible to cancer development (Montanaro et al 2008). Thus, it is likely that the up-regulation of rRNA synthesis could be responsible of the increased cancer susceptibility by impairing p53 function according the mechanism described above.

Furthermore the reported mechanism of p53 regulation consequent to rRNA synthesis up-regulation might explain the increased risk of cancer onset in people with obesity, insulin-resistance and type II diabetes which are characterized by a hyperinsulinemic status with activation of the insulin and IGF-1 pathways (Renehan et al. 2006, 2008; Frasca et al. 2008; Cannata et al. 2010). This mechanism might be also responsible for the worse evolution of cancer in patients with the above-mentioned metabolic features, by reducing the efficacy of the anti-tumour therapies that activate p53 (Renehan et al. 2006, 2008; Frasca et al. 2008; Cannata et al. 2010).

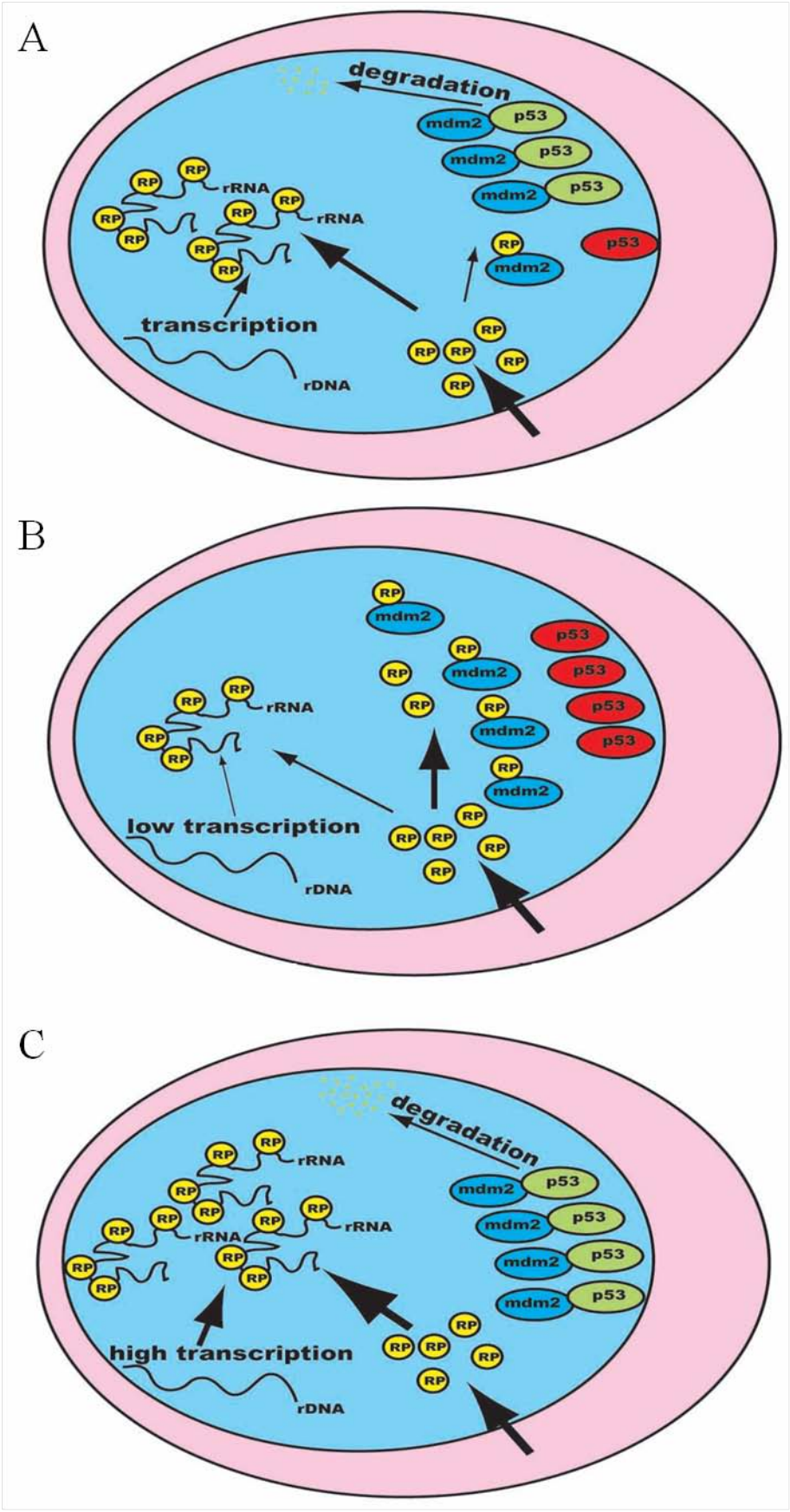
A
degradation
mdm2
p53
RP
rRNA
transcription
rDNA
B
low transcription
C
high transcription

Figure 28 Schematic representation of how the balance between rRNA and ribosomal protein synthesis regulates p53 levels. A Homeostatic rRNA and ribosomal protein synthesis. The p53 level is controlled by the steady-state balance between rRNA and ribosomal protein synthesis. Ribosomal proteins not utilized for ribosome building bind to MDM2, thus regulating the MDM2-mediated p53 proteasomal degradation. **B rRNA synthesis down-regulation.** After a down-regulation of rRNA synthesis the ribosomal proteins no longer engaged in ribosome biogenesis bind in larger amount to MDM2, thus reducing the MDM2-mediated p53 proteasomal degradation with the consequent increase of p53 stabilization. **C rRNA synthesis up-regulation**. After an up-regulation of rRNA synthesis a greater amount of ribosomal proteins are used for ribosome building, which are therefore no longer available for MDM2 binding. Thus, a greater portion of MDM2 is left free to induce p53 proteasomal degradation.

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