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Riassunto

Il gene CDKN1A codifica per p21waf1/cip1 (p21), inibitore delle chinasi ciclina-dipendenti, che è capace di arrestare il ciclo cellulare in fase G1 in situazioni di stress tra cui un danno indotto al DNA. É inoltre coinvolto anche in altre funzioni cellulari come ad esempio la regolazione dell'apoptosi. Il gene CDKN1A è principalmente regolato a livello trascrizionale dalla proteina soppressore del tumore p53, tuttavia è riportata in letteratura una modulazione indipendente da suddetta proteina. Molti dati in letteratura indicano che svariate proteine ribosomali, oltre ad intervenire nella sintesi proteica esercitano dei ruoli extraribosomali.

L'attività da me svolta durante il dottorato è stata indirizzata alla comprensione del ruolo della proteina rpL3 nell'espressione p53-indipendente di p21 ed il significato biologico di tale regolazione.

A tale scopo ho valutato l'attività trascrizionale del gene CDKN1A in condizione di iperespressione di rpL3 attraverso un saggio di luciferasi ed ho valutato tramite RT-PCR e Western Blotting i livelli di mRNA e di proteina p21. Ho condotto, inoltre, esperimenti di immunoprecipitazione della cromatina e di EMSA per valutare l'abilità di rpL3 di interagire con il promotore di p21 e la natura di tale legame. Allo scopo di identificare i partner proteici di rpL3 che mediano questo legame, ho analizzato il ruolo svolto dalle proteine NPM ed Sp1, entrambe coinvolte nella regolazione trascrizionale di p21, attraverso esperimenti di immunoprecipitazione della cromatina e saggi di luciferasi, ed, utilizzando mutanti del promotore di p21, identificato la regione di tale promotore responsabile del legame di rpL3.

Inoltre, ho eseguito esperimenti di analisi del ciclo cellulare e della vitalità cellulare in seguito a danno al DNA indotto dal trattamento con farmaci chemioterapici (5-fluorouracile e oxaliplatino) in condizioni di iperespressione e di silenziamento di rpL3. Successivamente ho indagato la regolazione dell'espressione di p21 mediata da rpL3 in condizioni di danno al DNA ed analizzato il ruolo svolto da rpL3 nell'attivazione dei meccanismi di riparo del DNA in seguito al trattamento con i suddetti farmaci. Questi esperimenti hanno dimostrato che il trattamento con farmaci chemioterapici provoca un aumento della quota proteica di rpL3 non legata ai ribosomi. Questa frazione libera di rpL3, regolando l'espressione di p21, è responsabile del blocco della proliferazione cellulare in risposta ad un danno di lieve entità, o dell'induzione dell'apoptosi per inibizione dei meccanismi di riparo per ricombinazione omologa e non omologa, qualora il danno sia maggiore e non riparabile. Questi esperimenti hanno inoltre dimostrato che quest'azione sembra essere in parte indipendente da p21.

Complessivamente gli esperimenti condotti durante il mio progetto di dottorato hanno dimostrato che rpL3 esercita una funzione extraribosomale in condizioni di stress cellulare, regolando l'espressione di p21 attraverso un meccanismo indipendente da p53. In particolare, rpL3 sembra fungere da mediatore di stress svolgendo una funzione antitumorale attraverso il blocco della proliferazione cellulare o l'induzione dell'apoptosi in dipendenza dall'entità dello stress subito dalla cellula.

Summary

The CDKN1A gene encodes for the protein p21waf/cip1 (p21), inhibitor of chinase cyclin-dependent, that is able to induce G1 cell cycle arrest under stressing conditions such as DNA damages and is also involved in other cell functions as regulation of apoptosis.

CDKN1A is mainly regulated at transcriptional level by the tumor suppressor protein p53, although a p53-independent regulation of p21 expression has been reported. Several ribosomal proteins exerts extraribosomal functions besides their main role in the translation process.

The aim of this study is to understand the role of the ribosomal protein rpL3 in p53-independent regulation of p21 expression and the biological significance of this regulation.

To this aim, I investigated the transcriptional activity of CDKN1A upon rpL3 overexpression by a reporter luciferase assay and analyzed p21 mRNA and protein levels through RT-PCR and Western Blotting. To evaluate the ability of rpL3 to interact with p21 promoter I performed ChIP and EMSA experiments. Then, to identify protein factors that mediate the interaction between rpL3 and p21 promoter, I analyzed the role of NPM and Sp1 proteins, both involved in p21 trancriptional regulation, through ChIP experiments and reporter luciferase assays. Furthermore, using p21 promoter mutants, I was able to identify the promoter region responsible for this interaction.

Next, cell cycle and cell viability analysis were performed after DNA damage induction mediated by chemotherapeutic drugs (5-fluorouracil, oxaliplatin) while altering rpL3 levels.

Then, I investigated the rpL3-mediated regulation of p21 expression upon DNA damage and analyzed the role played by rpL3 in the regulation of DNA repair mechanisms. These data showed that DNA damage induces accumulation of free-ribosome rpL3. This amount of rpL3, regulating p21 expression, is responsible for cell cycle arrest in response to a mild DNA damage, and apoptosis when the damage in higher and not reparable. In addition, these experiments demonstrated that the role of rpL3 is partially independent of p21.

These experiments demonstrated that rpL3 exerts an extraribosomal function upon ribosomal stress, regulating p21 expression through a mechanism independent of p53. In particular, rpL3 seems to act as tumor suppressor inhibiting cell proliferation or triggering apoptosis in dependent of the nature of the stress.

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1. Introduction

1.1 Extraribosomal functions of ribosomal proteins

Ribosomal proteins (RPs) are abundant RNA-binding proteins found in every cell. In addition to their role in the ribosome, it has been reported that some RPs also have other functions. For example, the bacteriophage Qβ encodes a polypeptide that binds to three *E. Coli* host proteins, the translation factors EFTu, EFTs and the rpS1, to serve as the RNA replicase responsible for replication of its genome (Warner and McIntosh, 2009).

Stoichiometry of ribosomal components is essential for ribosome synthesis, requiring equimolar production of ribosomal RNA (rRNA) and some 70 to 80 RPs. In *E. coli,* this is carried out largely by the RPs, which can function extraribosomally as translational repressors of RP synthesis. As an example, the S10 operon encodes 11 different RPs, one of which is rpL4 that can inhibit translation of the entire operon by binding to the 5'UTR of the S10 operon mRNA (Lempiainen and Shore, 2009).

Such autoregulation of RP production is less common in eukaryotic cells, but there are several documented cases of RPs regulating their own synthesis. In our laboratory it has been demonstrated that rpL3 is able to regulate its production. In addition to canonical mRNA isoform normally translated into protein, the rpL3 gene produces an alternative mRNA isoform, containing a PTC (premature termination codon) degraded by NMD (Nonsense mediated-mRNA decay). Overexpression of rpL3 causes an increase in the level of alternative unproductive mRNA isoform, which results in a decrease of rpL3 production (Cuccurese et al., 2005). This is clearly an extraribosomal function, but not in an extraribosomal system. Of

note, the negative feedback loop triggered by the accumulation of rpL3, represents a strategy that finely regulates the amount of ribosomal protein to an appropriate level, that could be relevant especially to other extraribosomal functions of rpL3. Furthermore, recent data suggest a role for rpL3 in tumors and drug resistance. In several malignancies, telomerase activity is upregulated during cancer progression; microarray analyses of gene expression profile in different esophageal carcinoma cell lines showed that rpL3 level positively correlated with telomerase activity (Kondoh et al., 2001). Moreover, proteomic analysis indicated that rpL3 might be involved in the induction of 5-fluorouracil (5-FU) resistance in human colon cancer cells (Tanaka et al., 2008).

1.2 Ribosomal stress

Ribosome biogenesis is the most demanding energetic and metabolic expenditure of the cell. The entire process is estimated to use more than 60% of cellular resources and therefore it is carefully monitored for quality control. Increasing evidence indicates that surveillance of ribosome assembly plays an important role in the cellular self-evaluation, in which defects in ribosome synthesis can lead to cell cycle arrest or apoptosis through extraribosomal functions of RPs (Castro et al., 2008).

Alterations in the expression of RPs and protein factors involved in the ribosome biogenesis seem to correlate with tumorigenesis since many of these proteins are overexpressed in solid tumors and leukemia cells. On the other hand, some data suggest that several RPs might function as tumor suppressors, as described in zebrafish (Amsterdam et al., 2004). A picture of

the complex choreography through which RPs act as sentinels of defects in ribosome assembly is gradually developing.

Recently, an increasing number of RPs have been proposed as p53 activators, leading to cell cycle arrest and apoptosis. In response to nucleolar stress, the ribosomal components rpL11, rpL5, rpL23, and rpS7 are released from the nucleolus into the nucleoplasm where they associate with Mdm2, inhibiting its E3 ubiquitin ligase activity toward p53 and thus promoting p53 stabilization and activation (Deisenroth and Zhang, 2010). In addition, rpL26, which is ubiquitinated by MDM2, binds to the 5′-UTR of p53 mRNA and increases its translation in response to DNA damage. Lastly, rpS3 interacts directly with p53 and protects it from Mdm2-mediated ubiquitination induced by oxidative stress (Ofir-Rosenfeld et al., 2008). It has been shown that rpL23 regulates p21 gene promoter transactivation mediated by nucleophosmin (NPM), a nucleolar protein involved into ribosome maturation, and Myc-interacting zinc finger protein 1 (Miz1) by sequestering NPM into the nucleolus (Wanzel et al., 2008). Thus, both nucleolar and ribosome biogenesis-related proteins are very important regulators of the nucleolar stress signaling cascade that leads to p53 activation.

Very recently, some light has been shed on the presence of p53-independent pathways regulating the relationship between ribosome biogenesis and cell proliferation in mammalian cells. For example, it has been demonstrated that ribosomal stress causes a decrease in the levels of the serine/threonine kinase PIM1. Consequently, the inhibitor of the cell cycle $p27^{Kip1}$, target of PIM1, increases thus blocking cell proliferation (Iadevaia et al., 2010) (Fig. 1.1).

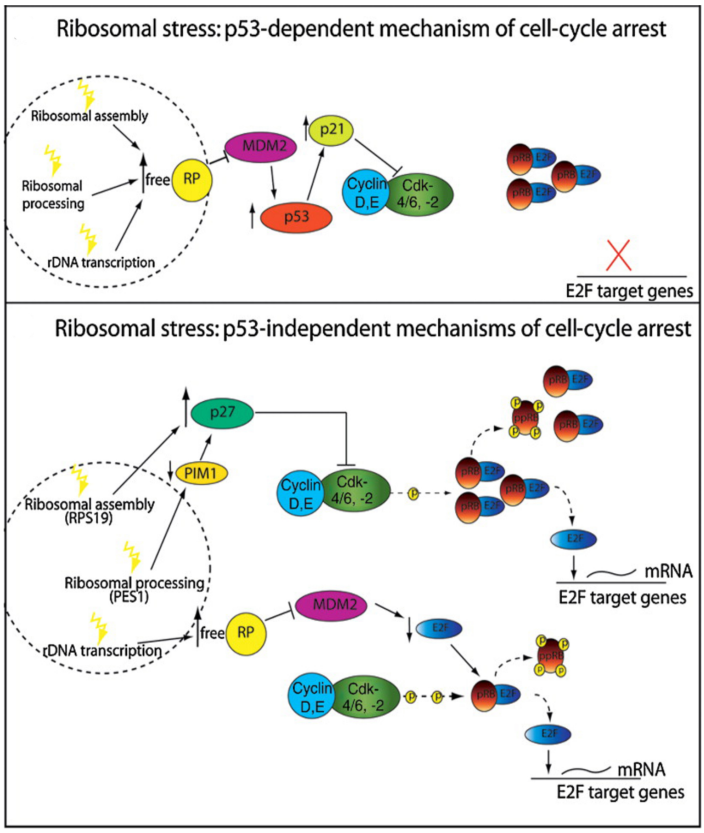

Figure 1.1: Ribosomal stress. Schematic representation of the p53-dependent and independent cell cycle arrest after ribosomal stress.

1.3 p21$^{waf1/cip1}$

p21 (also known as p21$^{waf1/cip1}$) is a key regulator of cell cycle progression involved in a vast array of complex interactions and networks within the cell. Various regulatory mechanisms contribute to finely coordinate multiple biological functions of p21 altering its levels. They include transcriptional regulation, epigenetic silencing, mRNA stability, ubiquitin-dependent and ubiquitin-independent degradation of the protein (Jung et al.).

p21 can function as a tumor suppressor by mediating G1 growth arrest

by inhibiting the kinase activity of cyclin-dependent kinases (CDKs) CDK1 and CDK2. However, in certain cellular contexts p21 may also act as an oncogene since it may display tumorigenic and antiapoptotic activities (Abbas and Dutta, 2009). For example, p21 protects against apoptosis induced by sodium butyrate in U937 cells (Rosato et al., 2004), and by prostaglandin A2 in human colorectal carcinoma (Gorospe et al., 1996). On the other hand, there is evidence that p21 may also be proapoptotic through either p53-dependent or p53-independent mechanisms under certain cellular stresses (Tsao et al., 1999; Yang et al., 2003). However, this effect has been observed mainly associated with the ectopic overexpression of p21. Furthermore, p21 gene overexpression in p53-null human laryngeal squamous carcinoma cells can assume a dual role in the regulation of apoptosis depending on drug treatment; in fact p21 overexpression enhances apoptosis in cisplatin-treated cells whereas it attenuates apoptotic signals in methotrexate-treated cells (Kraljevic Pavelic et al., 2008).

Besides p53, a variety of transcription factors can modulate p21 (Macleod et al., 1995). The majority of p21 gene regulatory proteins interact with the proximal region of p21 promoter. This region includes six GC-rich binding sites that are recognized by Sp1, Sp3 and Kruppel-like trascription factors (Koutsodontis et al., 2002; Wu and Lingrel, 2004). Substantial evidence supports the essential role of Sp1 in the upregulation of p21 gene by several protein factors such as c-Myc (Gartel et al., 2001) or drugs such as 5-FU and Sulforaphane (Chew et al., 2012; Koutsodontis and Kardassis, 2004). Recently, it has been suggested that p21 could also play a role in several pathways involved in the DNA damage response. However, the relationship of p21 activity with these pathways is not yet clear and the role

of p21 remains controversial (Abbas and Dutta, 2009).

1.5 Aim of the study

It is now well documented that ribosomal stress, defined as an alteration of ribosome synthesis owing to a variety of causes including DNA damage, causes the binding of several ribosomal proteins to Mdm2. This event relieves Mdm2 inhibitory activity toward p53, leading to p53 activation that results in a p53-dependent inhibition of cell proliferation (Castro et al., 2008).

A number of reports suggest that perturbations of ribosome biogenesis, can activate specific checkpoints and block cell proliferation also through p53-independent mechanisms, involving ribosome biogenesis-related proteins and regulators of cell proliferation such as p21 (Zhang and Lu, 2009).

We have studied post-transcriptional regulatory strategies of mammalian RPs, and we have demonstrated that rpL3 autoregulates its own expression through the association of alternative splicing and nonsense-mediated mRNA decay (AS-NMD) in order to fine tune the levels of protein that exert extraribosomal functions (Cuccurese et al., 2005).

The aim of my thesis project is to investigate the molecular mechanism by which rpL3 might modulate the intracellular levels of p21 independently of p53 and to understand how this regulation may affect chemotherapy response.

2. Materials and Methods

2.1 Cell cultures, transfections and drug treatment

Human cell lines Calu-6 (Cuccurese et al., 2005) and HCT-116$^{p53-/-}$ were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids (Euroclone, West York, UK), 2mM L-glutamine and penicillin-streptomycin 50 U/ml.

p21ΔCalu-6 cell line, derived from Calu-6 cell line and stably silenced for p21, was grown in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids (Euroclone), 2mM L-glutamine, penicillin-streptomycin 50 U/ml and 0,5 μg/ml puromycin (Sigma-Aldrich).

siRNA transfections were performed in Calu-6 and HCT-116$^{p53-/-}$ cells (2,5 x 10^6 cells, 6 mm-well plate) at a concentration of 150 nM using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids were transfected in Calu-6, HCT-116$^{p53-/-}$ and p21ΔCalu-6 cells (2,5 x 10^6 cells, 60 mm-well plate) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

After DNA transfections, RNA and proteins were extracted by using the Trizol procedure (Invitrogen) for RT–PCR analysis and western blot, respectively.

Transfection efficiency was assessed by cotransfecting a GFP-expressing vector and normalizing RNA levels against GFP mRNA levels (data not shown).

Calu-6 cells (2,5 x 10^6 cells, 60 mm-well plate) were treated with 200 nM Mithramycin A (Sigma-Aldrich) for 16 hours before transfections or chromatin immunoprecipitation. Drug treatments were performed after siRNA or DNA transfection by adding 10 μM or 100 μM of 5-Fluorouracil or OXHliplatin (Sigma-Aldrich, St. Louis, Missouri) to all cell lines.

2.2 Plasmids

The cDNA of NPM was obtained by RT–PCR from Calu-6 cells using the primers 5′-ATGGAAGATTCGATGGAC-3′ (forward) and 5′-TTAAAGAGACTTCCTCCA-3′ (reverse), and cloned into a version of the eukaryotic expression vector pcDNA3.1/His C (Invitrogen) containing the FLAG epitope. The construct was verified by DNA sequencing.

The cDNA of Sp1 was obtained by RT–PCR from Calu-6 cells using the primers 5′- ATGGATGAAATGACAGCT-3′ (forward) and 5′-TCAGAAGCCATTGCCACT-3′ (reverse), and cloned into a version of the eukaryotic expression vector pcDNA3.1/HisMyc A (Invitrogen) containing the His epitope. The construct was verified by DNA sequencing.

The plasmids encoding HA-rpL3, HA-NPM, rpL7a-His and were already available (Russo et al., 2011). The plasmids pWWP, pWP124, pWP101 and pWPdel-Sma I were kindly provided by Dr. Yoshihiro Sowa (Huang et al., 2000).

The I-SceI expression vector pCAGGS-ISceI was a gift from Prof. E. Avvedimento.

2.3 RNA interference

The siRNAs targeting NPM and rpL3 were purchased from Santa Cruz

Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-29771, sc-76400).

To stably silence p21, Calu-6 cells were seeded the day before transfection in a 60 mm plate and 24 hours later they were transfected with 2 μg of a p21 short hairpin RNA (shRNA) expressing pSM2c vector contained in the Expression Arrest™ human shRNA library (Open Biosystems, Huntsville, AL, USA). Transfected cells were selected with 1 μg/ml of puromycin (Sigma-Aldrich) for 7 days and then the p21 depletion was evaluated by western blotting.

The sense strand of the p21 shRNA sequence is: 5'-TGCTGTTGACAGTGAGCGACCAGCCTCTGGCATTAGAATTTAGTGAAGCCACAGATGTAAATTCTAATGCCAGAGGCTGGGTGCCTACTGCCTCGGA-3'.

A short hairpin non-silencing construct was used as control.

2.4 PCR

For Reverse Transcriptase-PCR analysis, 1 μg of total RNA was reverse-transcribed into cDNA with the random primer hexamer technique using 200 U of Superscript II RNase H^- Reverse Transcriptase (Invitrogen). The reaction was carried out at 42°C for 50 min and was terminated by heating to 75°C for 15 min. Ten of the 40 μl of reaction mix were PCR-amplified in a final volume of 50 μl, using 5 μM of each specific primer, 10 mM dNTPs and 0.5 U of Taq DNA polymerase (Invitrogen). Typically, 25–30 cycles of amplification were performed. The primers were: cDNA p21 5'-CGCGGATCCATGTCAGAACCGGCTGGG-3' (forward) and 5'-

CCGGAATTCATTAGGGCTTCCTCTTGGA-3' (reverse); and β-actin 5′-GGCACCACCTTCTACA-3′ (forward) and 5′-CAGGAGGACAATGAT-3′ (reverse).

In separate experiments, we ascertained that the cycle number was within the linear range of amplifications. PCR products were visualized on 1% agarose gel containing the fluorescent Vista Green dye (Amersham Pharmacia Biotech). The labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the PhosphorImager (Bio-Rad, Haercules, California).

Real time PCR was used to analyze DNA in ChIP experiments as described in the sub-section "**Chromatin immunoprecipitation**".

2.5 Western blotting

For western blotting assay, protein samples (30 μg) were resolved by 12% SDS-gel electrophoresis and transferred into nitrocellulose filters. The membranes were blocked in PBS, 0,2% Tween and 5% dry milk for 2h, and then challenged with anti-rpL3, anti-rpL7a (Primm, Milan, Italy), anti-rpS19, anti-FLAG (Sigma-Aldrich, St. Louis, USA; F3165), anti-Sp1 (Millipore, Billerica, USA; 07-645), anti-p21, anti-NPM, anti-HA, anti-His, anti-PARP and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, California; sc-397, sc-47725, sc-57592, sc-803, sc-7150, sc-8035). The proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce, Rockford, Illinois). Loading in the gel lanes was controlled by detection of α-tubulin protein.

2.6 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed starting from 1 x 10^6 Calu-6 cells. DNA and proteins were cross-linked by the addition of 1% formaldehyde for 10 min at room temperature, and cross-linking was stopped by the addition of 125 mM glycine for 5 min at room temperature. Cells were then lysated in a RIPA buffer. Chromatin was fragmented by sonication to an average size of 500–1000 bp in sonication buffer and pre-cleaned with protein A-agarose. Ten microliters of each supernatant was removed as input, and the rest of the supernatant was immunoprecipitated with anti-rpL3, anti-NPM, anti-Sp1 antibodies, or normal mouse IgG overnight at 4°C. After immunoprecipitation, 30 µl of protein A-agarose was added and incubated for 1 h at 4°C to capture the immune complexes. The agarose beads were washed, 30 microliters of DNA-proteins immunocomplexes were resolved by 12% SDS-gel electrophoresis and transferred into nitrocellulose filters analyzed by western blotting. The chromatin was extracted from the remaining aliquots of DNA-proteins immunocomplexes, the protein–DNA crosslink was reversed, and the proteins were digested with proteinase K. DNA was then purified and analyzed by real-time PCR analysis with the primers, p21 promoter (-194/+88) 5'-ACCGGCTGGCCTGCTGGAACT-3' (forward) and reverse 5'- TCTGCCGCCGCTCTCTCACCT-3' (reverse); p21 3'-UTR (+3455/+3771) 5'-ATGTTAGGCAAGTTACTTAACTTA-3' (forward) and 5'-CTCTTGGTAACTTCACACCAAGTT-3' (reverse) using SYBR GreenEr qPCR SuperMix Universal (Invitrogen) following the manufacturer's instructions.

2.7 Dual Luciferase Assay

Luciferase assays were performed with the Dual-Glo Luciferase assay system (Promega, Milan, Italy) following manufacturer's instructions. Samples were read with Turner Luminometer and expressed as relative luciferase, i.e. R_T/R_C, where R_T and R_C are (Firefly luciferase)/(Renilla luciferase).

2.8 Cell cycle analysis

Cells were collected in PBS 1X and fixed in 70% ethanol. Before analysis, fixed cells were washed twice, centrifuged and resuspended in 1 ml of PBS containing 10 μg/ml of RNase and 50 μg/ml of propidium iodide (Sigma-Aldrich). Subsequently fluorescence of propidium iodide was detected in a linear scale using a CyAn ADP Flow Cytometer (DAKOCytomation, Ely, UK) and analysed using Summit Software.

2.9 BrdU incorporation

For BrdU (5'-Bromo-2'-deoxyuridine) incorporation, Calu-6 or HCT-116$^{p53-/-}$ cells were labeled for 40 minutes with 20 μM BrdU (Sigma-Aldrich), harvested and fixed in 70% ethanol. Subsequently, cells were incubated with 20 μl of anti-BrdU-FITC antibody (BD Biosciences, Italy) for 45 min in dark at room temperature. Cells were then washed twice with PBS 1x + Tween 0,1%, centrifuged and resuspended in 1 ml of PBS containing 10 μg/ml of RNase and 5 μg/ml of propidium iodide (Sigma-Aldrich). Samples were analyzed by a CyAn ADP Flow Cytometer

(DAKOCytomation) and quantified using Summit Software.

2.10 Mitochondrial membrane potential measurement

To quantify changes in mitochondrial membrane potential, Calu-6 or HCT-116$^{p53-/-}$ cells were labeled with 0.1 μM of the mitochondrial membrane potential–sensitive fluorescent dye, tetramethylrhodamine ethyl ester (TMRE) (Invitrogen), for 30 min at 37°C, analyzed by a CyAn ADP Flow Cytometer (DAKOCytomation) and quantified using Summit Software.

2.11 Ribosome isolation

Calu-6 cells or HCT-116$^{p53-/-}$ cells were pelleted and resuspended in lysis buffer (10mM NaCl, 10mM MgCl2, 10mM Tris–HCl (pH 7.5), 0.5% NP-40, aprotinin 1 mg/ml, leupeptin 1 mg/ml, pepstatin A 1mg/ml, phenylmethylsulfonyl fluoride 100mg/ml). After incubation in ice for 10 min, the extract was centrifuged for 1 min in a microcentrifuge at a maximum speed at 4°C and the supernatant (total extract) was frozen in liquid nitrogen. To isolate ribosomes and ribosomal subunits, the total extracts were spun at 100 000 g for 2 h on 15% sucrose cushion. After centrifugation, the pellet (which includes polysomes and ribosomal subunits) was resuspended in Loading Buffer (63mM Tris–HCl (pH 6.8), 5% glycerol, 1% SDS, 2.5% bromophenol-blue) for western blot analysis, whereas the supernatant was precipitated with 10% trichloroacetic acid and resuspended in Loading Buffer for western blot analysis.

2.12 γ-H2AX quantification

To analyze the levels of double strand breaks (DSB) of DNA, Calu-6 cells, HCT-116 $^{p53-/-}$ cells or p21DCalu-6 cells were fixed and permeabilized with cold (−20°C) 70% ethanol overnight, washed with 1% BSA-PBS, and further permeabilized with 0.25% Triton in 1% BSA-PBS for 5 min on ice, incubated with anti-γ-H2AX antibody (dilution, 1/37.5) in 10% normal goat serum-1% BSA-PBS, washed with 1% BSA-PBS, and incubated with a secondary antibody conjugated with Alexa Fluor 488. Finally, propidium iodide (final concentration, 0.05 mg/ml) and RNase A (final concentration, 0.5 mg/ml) were added. Fluorescence intensities were determined with a CyAn ADP Flow Cytometer (DAKOCytomation) and quantified using Summit Software.

2.13 Clonogenic assays

For clonogenic assay, Calu-6 cells or HCT-116 $^{p53-/-}$ cells were trypsinized, washed in PBS, and seeded in triplicate at 2,000 cells per well, in six-well plates. Colonies were allowed to grow for 10 days and visualized following washing with PBS, fixation in methanol for 30 min, washing again with PBS, and staining with 0.05% methylene blue for 30 min. Percent survival was normalized to the observed number of colonies generated from untreated cells.

2.14 Analysis of homologous recombination frequency in vivo

HeLa cells carrying a single integrated copy of a green fluorescent protein (GFP) recombination reporter construct were a gift from Prof. E. Avvedimento (Universita` di Napoli ‘‘Federico II,’’ Naples, Italy). This reporter construct contains two GFP genes: one nonfunctional GFP gene

mutated to contain a I-SceI cleavage site and an additional, truncated GFP gene that can correct the I-SceI site mutation. The I-SceI restriction endonuclease is used to introduce a double-strand break (DSB) in the reporter gene. Chromosomal repair of the reporter gene by homologous recombination leads to GFP expression, which is analyzed by flow cytometry. To analyze the DSB-induced homologous recombination, HeLa cells were transiently transfected with siRNA against endogenous rpL3 or pHA-rpL3 and the I-SceI expression vector pCAGGS-ISceI or with a cytomegalovirus (CMV) empty vector. 72 hours after transfection, the percentage of GFP-positive cells was determined with a CyAn ADP Flow Cytometer (DAKOCytomation) and quantified using Summit Software.

2.15 NHEJ assay in vitro

pGL3-control plasmid (Promega) was completely linearized by the restriction endonuclease HindIII or NarI and the linearized DNA was extracted from agarose gel with Gel Extraction kit (Invitrogen). Circular plasmid and linearized DNA was then co-transfected with pRT-RL plasmid into cells. The transfected cells were lysed and assayed for luciferase activity with Dual Luciferase Assay (Promega) (see sub-section "Dual luciferase assay"). Overall NHEJ capacity was calculated by firefly luciferase activity from cells transfected with HindIII or NarI-digested DNA relative to that of the intact plasmid.

2.16 Statistical analysis

The data are obtained from three independent experiments and presented as means ± standard deviation.

3. Results

3.1 rpL3 upregulates p21 expression at transcriptional level independently from p53

To investigate whether rpL3 controls p21 expression in a p53 deficient environment, we analyzed p21 mRNA and protein levels upon rpL3 overexpression in p53-null Calu-6 cells (Cuccurese et al., 2005). The exogenous expression of the HA-tagged rpL3 protein resulted in a dose-dependent increase of p21 protein and mRNA (Fig. 3.1A, B).

To examine the possibility that p21 upregulation was due to rpL3 controlling the p21 promoter, we performed a p21 reporter luciferase assay. The overexpression of rpL3, in contrast to rpL7a (Russo et al., 2005), led to the activation of p21 reporter in a dose-dependent manner (Fig. 3.1C). Control CMV-luciferase reporter activity did not change upon rpL3 nor rpL7a overexpression (Fig. 3.1D).

We next investigated whether rpL3 was able to interact with p21 promoter. Calu-6 cells were collected and ChIP analysis using rpL3 antibody was performed. IgG antibody was used as specificity control.

The presence of rpL3 was investigated by western blotting in the DNA-immunoprecipitated complexes (Figure 1E). Quantitative PCR assay on the samples performed with primers specific for the proximal region of p21 promoter (-194 to +88) (Wu et al., 2003) or control loci (3'UTR) showed the presence of a specific signal for p21 promoter in the DNA-rpL3 immunocomplex. No signal was detected in the IgG control (Fig. 3.1E).

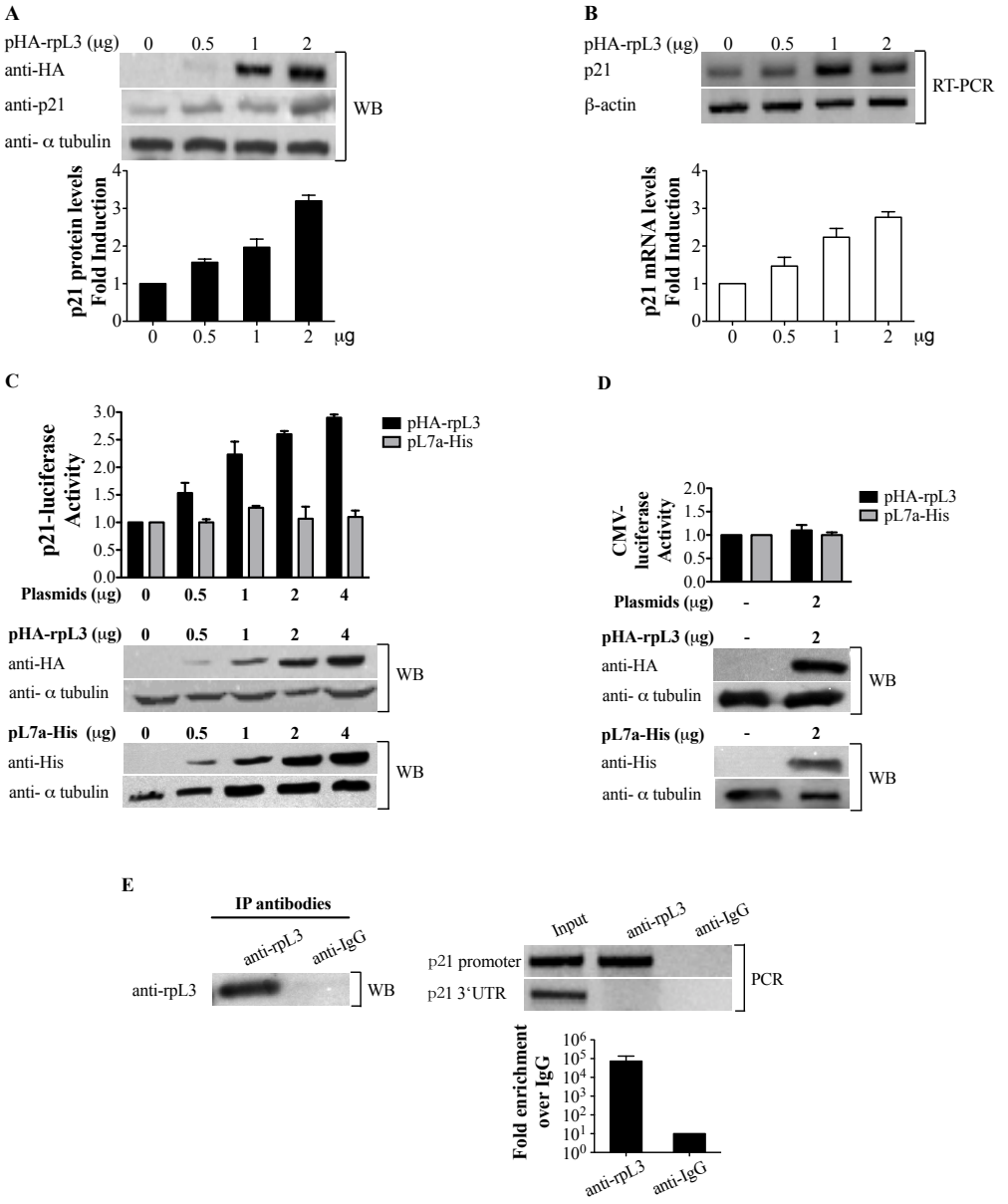

Figure 3.1. Role of rpL3 in the regulation of p21 expression. (A) Western Blot (WB) analysis of protein samples from Calu-6 cells transfected with the indicated amount of pHA-rpL3 with antibodies directed against HA and p21, 24 hours after transfection. **(B)** Representative RT-PCR analysis of total RNA from the same samples. β-actin was used as a control for RNA loading. Quantification of p21 protein and mRNA levels by PhosphorImager (Bio-Rad) is shown. **(C and D)** Analysis of the relative Firefly luciferase

activity, normalized to Renilla luciferase (pRL), in Calu-6 cells transiently transfected with p21-Luc or CMV-Luc plasmid with or without the indicated plasmids, 24 hours after transfection. WB analysis of protein extracts of the same samples with antibodies directed against HA and His. **(E)** WB analysis of DNA-rpL3 or DNA-IgG immunocomplexes with antibodies against rpL3 and IgG. Note the absence of signal in DNA-IgG immunocomplex. The same DNA-immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or control loci.

3.2 NPM and SP1 modulate rpL3-dependent p21 gene promoter transactivation

The absence of a direct binding between rpL3 and p21 promoter, observed in EMSA experiments (not shown), prompted us to search for rpL3-interacting regulatory proteins involved in the rpL3-mediated regulation of p21 transcription. We have previously reported that NPM, in a complex with rpL3, is required for the regulation of the rpL3 gene alternative splicing (Russo et al., 2011). Thus, we explored the possibility that the rpL3-NPM complex was involved in the transactivation of the p21 gene transcription. ChIP experiments by using anti-rpL3, anti-NPM and IgG showed that both rpL3 and NPM bind to the p21 promoter, but not to a control region of the DNA (p21 3'UTR) (Fig. 3.2A).

To investigate whether the interaction between rpL3 and NPM on p21 promoter affects its activity, a reporter luciferase assay was performed while altering intracellular levels of NPM in cells with HA-rpL3 overexpression. The results demonstrated that under both physiological and supra-physiological levels of rpL3, the overexpression of NPM did not affect the activity of p21 gene promoter (Fig. 3.2B). Whereas under physiological conditions of rpL3 expression, the NPM silencing was associated with the inhibition of p21 transcriptional activity as indicated by a significant decrease (40%) in p21 gene promoter activity (Fig. 3.2C). Of interest, the

rpL3 overexpression rescued the reduction of p21 transcriptional activity caused by NPM silencing suggesting that additional factors are required for the rpL3-mediated activity on p21 gene promoter.

In the attempt to identify other proteins involved in the rpL3-mediated activation of p21 transcription, we focused on the possible role of Sp1, a protein factor that regulates p21 transcription (Koutsodontis et al., 2002). ChIP experiments in Calu-6 cells treated with the specific Sp1 inhibitor mythramycin A (MTM) (Koutsodontis and Kardassis, 2004) show that a specific association between Sp1 and rpL3 occurs on the p21 gene promoter in absence of MTM. Of interest, the presence of MTM prevents the binding of both rpL3 and Sp1 on the p21 gene promoter, indicating that rpL3 is able to bind p21 gene promoter only in the presence of Sp1 (Fig. 3.2D).

In order to study the function of Sp1 in rpL3-dependent transactivation of p21 gene promoter, a reporter luciferase assay was performed in Calu-6 cells overexpressing rpL3 and Sp1, with or without MTM treatment. Figure 3.2E shows that in the absence of MTM, rpL3 or Sp1 overexpression increased the activity of p21 gene promoter, as previously observed (Cen et al., 2008). In addition, rpL3 and Sp1 overexpression had a synergistic effect on the transactivation of p21 gene promoter. Interestingly, the ability of both rpL3 and Sp1 to activate the transcription of luciferase was strongly reduced in the presence of MTM. This data suggested that the ability of rpL3 to activate p21 transcription requires the presence of Sp1 on the promoter.

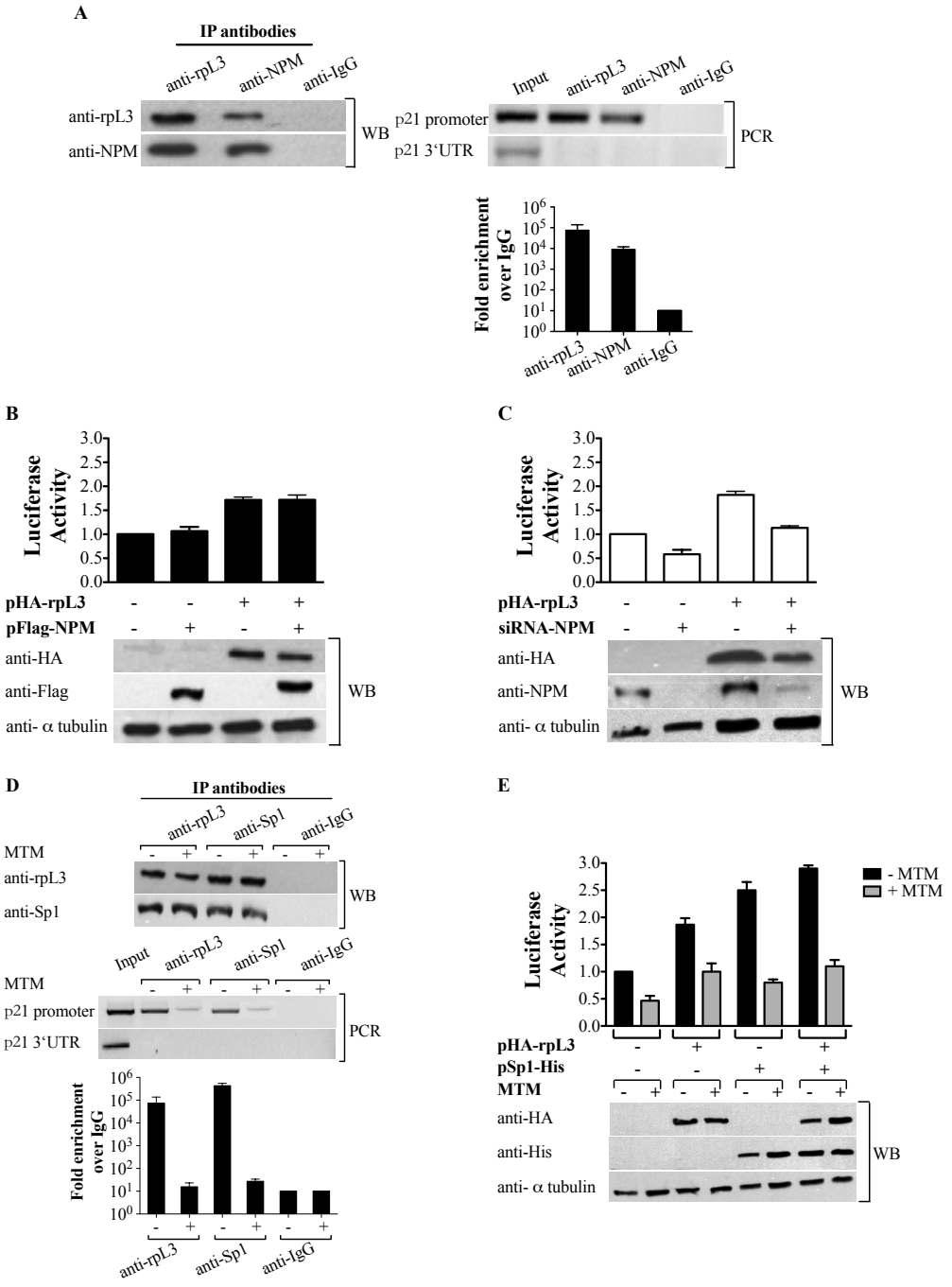

Figure 3.2. **Role of NPM and SP1 in the rpL3-mediatyed p21 transcriptional regulation. (A)** WB analysis of DNA-rpL3, DNA-NPM or DNA-IgG immunocomplexes

with antibodies against the indicated proteins. Note the absence of signal in IgG immunocomplex. The same DNA-immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or 3'UTR control loci. **(B)** The relative Firefly luciferase activity, normalized to Renilla luciferase (pRL), in Calu-6 cells transiently transfected with the indicated plasmids, 24h after transfection. WB analysis of protein extracts from the same samples with antibodies directed against the HA and Flag tags. **(C)** The relative Firefly luciferase activity, normalized to Renilla Luciferase (pRL), in Calu-6 cells transiently transfected with the indicated plasmids, 24h after transfection. WB analysis of protein extracts from the same samples with antibodies directed against the HA and NPM. **(D)** WB analysis of DNA-rpL3, DNA-Sp1or DNA-IgG immunocomplexes from Calu-6 cells untreated or treated with 200 nM of mythramycin A (MTM) for 16 hours with antibodies against rpL3 or Sp1. Note the absence of signal in DNA-IgG immunocomplex. The same DNA-immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or control loci. **(E)** The relative Firefly luciferase activity, normalized to Renilla luciferase (pRL), in Calu-6 cells transiently transfected with the indicated plasmids and treated for 16h or not with 200 nM of mythramycin A (MTM), 24h after transfection. WB analysis of protein extracts from the same samples with antibodies directed against the HA and His tags.

3.3 Sp1-binding boxes 3 and 4 are involved in rpL3-dependent transactivation of p21 gene promoter

The proximal region of the human p21 promoter carries six GC-rich Sp1-binding sites (numbered 1-6 in plasmid pWP124, Fig. 3.3). To determine which of Sp1-binding elements mediate rpL3-responsiveness, Calu-6 cells were transiently transfected with pW124 or truncated plasmids in which two or four Sp1-binding sites were simultaneously deleted (pWP101 and pWPdel-Sma I) (Huang et al., 2000) in the presence or absence of pHA-rpL3 (Fig. 3.3). Upon the overexpression of rpL3, deletion of Sp1-binding sites 1 and 2 (pWP101) lead to an increase in the luciferase activity comparable to that obtained when all the six Sp1 binding motifs are present (pWP124). Additional deletion of Sp1-binding sites 3 and 4 (pWPdel-Sma I) abrogated completely rpL3 effect on p21 gene promoter transactivation (Fig. 3.3). This data suggests that the region spanning from -

60 to -101 upstream from the transcription start site, including Sp1-binding sites 3 and 4, corresponds to rpL3 responsive element and is involved in rpL3-mediated activation of p21 gene promoter.

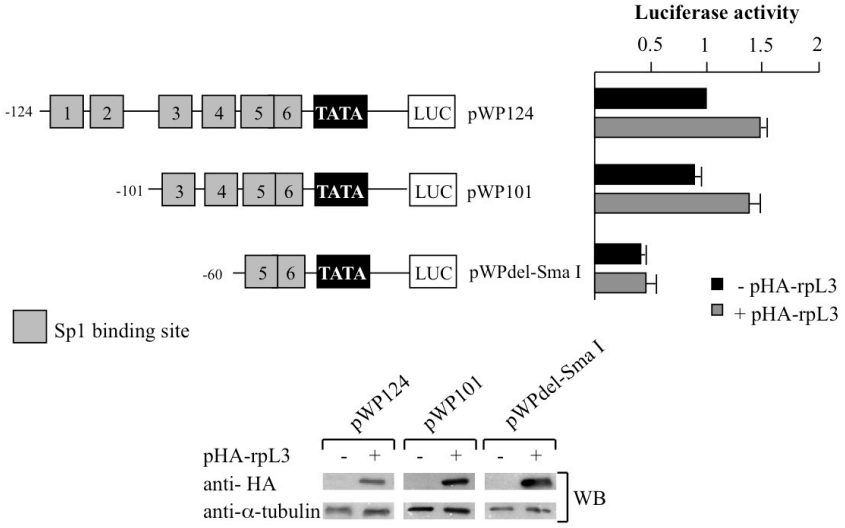

Figure 3.3. Analysis of p21 promoter regions involved in rpL3-mediated p21 transcriptional regulation. On the left, a schematic representation of the deletion constructs of the p21 gene promoter used in the luciferase transfection assay. Analysis of the relative Firefly luciferase activity, normalized to Renilla luciferase (pRL), in Calu-6 cells transiently transfected with the indicated constructs, 24 hours after transfection. Proteins from the same samples were analyzed by WB assay with antibody directed against the HA tag.

3.4 rpL3 overexpression induces cell cycle arrest or apoptosis in Calu-6 cells

In order to investigate the effect of rpL3-mediated upregulation of p21 transcription on cell proliferation, we performed 5-bromodeoxyuridyne (BrdU) incorporation to monitor DNA synthesis and propidium iodide (PI) staining to analyze DNA content of Calu-6 cells transiently transfected with 1 μg or 2 μg of pHA-rpL3. As expected, rpL3 overexpression induced an increase of p21 expression in a dose-dependent manner (Fig. 3.4A). BrdU incorporation in cells transfected with 1 μg of pHA-rpL3 was significantly

decreased versus that observed in control (about 0.7% versus 25%, respectively). This indicated that the enforced expression of rpL3 produced a strong reduction in the percentage of cells in S-phase of the cell cycle. Analysis of cell cycle using the counterstaining with PI indicated that the observed decrease of cells in S-phase was associated with an increase of cell population in the G1-phase of the cell cycle (about 80% versus 50% in the control). Notably, in the presence of higher rpL3 levels (2 μg of pHA-rpL3) we observed a significant increase in the sub-G1 population, indicative of cell death (about 21% versus 2% in the control) (Fig. 3.4A).

To gain further insight into the mechanism by which rpL3 causes cell cycle arrest and apoptosis, we proceeded to perform a cell cycle analysis over 72 hours in Calu-6 cells transiently transfected with 1 μg or 2 μg of pHA-rpL3 (Fig. 3.4B). In cells transfected with 2 μg of pHA-rpL3, an increase in sub-G1 cell population versus control was observed at all time points, although the strongest effect was detected after 48 hours. At this time, the percentage of cells transfected with 2 μg of pHA-rpL3 in the sub-G1 phase of the cell cycle increased approximately 7-fold as compared to control (about 36% versus 5%, respectively). This effect was not observed with 1 μg of pHA-rpL3. Transfection of cells with this amount of pHA-rpL3 did not induce apoptosis even 72 hours after transfection. Furthermore, we observed a G1/S arrest at 24 and 48 hours (as already shown in Fig. 3.4A) and more interestingly at 72 hours the dividing ability of Calu-6 cells transfected with 1 μg of pHA-rpL3 was restored, as evidenced by the presence of a G2/M peak (Fig. 3.4B). These results suggest that rpL3 may

induce G1/S arrest or apoptosis depending on its concentration inside the cells.

To further study the apoptotic effect of rpL3, we analyzed PARP protein cleavage by western blotting and reduction of mitochondrial inner membrane potential (ΔΨm) by tetramethyl-rhodamine ethyl ester (TMRE) staining, a hallmark of mitochondrial apoptosis (Youle and Strasser, 2008), in Calu-6 cells transfected with 2 μg of pHA-rpL3. As expected, we observed increased level of cleaved PARP in cells transfected with 2 μg of pHA-rpL3 (Fig. 3.4C). Furthermore, Figure 3.4C shows that the percentage of Calu-6 cells with a physiological ΔΨm decreased from 88% to 58% (control cells versus 2 μg of pHA-rpL3 transfected cells). Overall these results indicate that higher levels of rpL3 protein obtained with 2 μg of pHA-rpL3 activate apoptosis through mitochondrial pathway in Calu-6 cells.

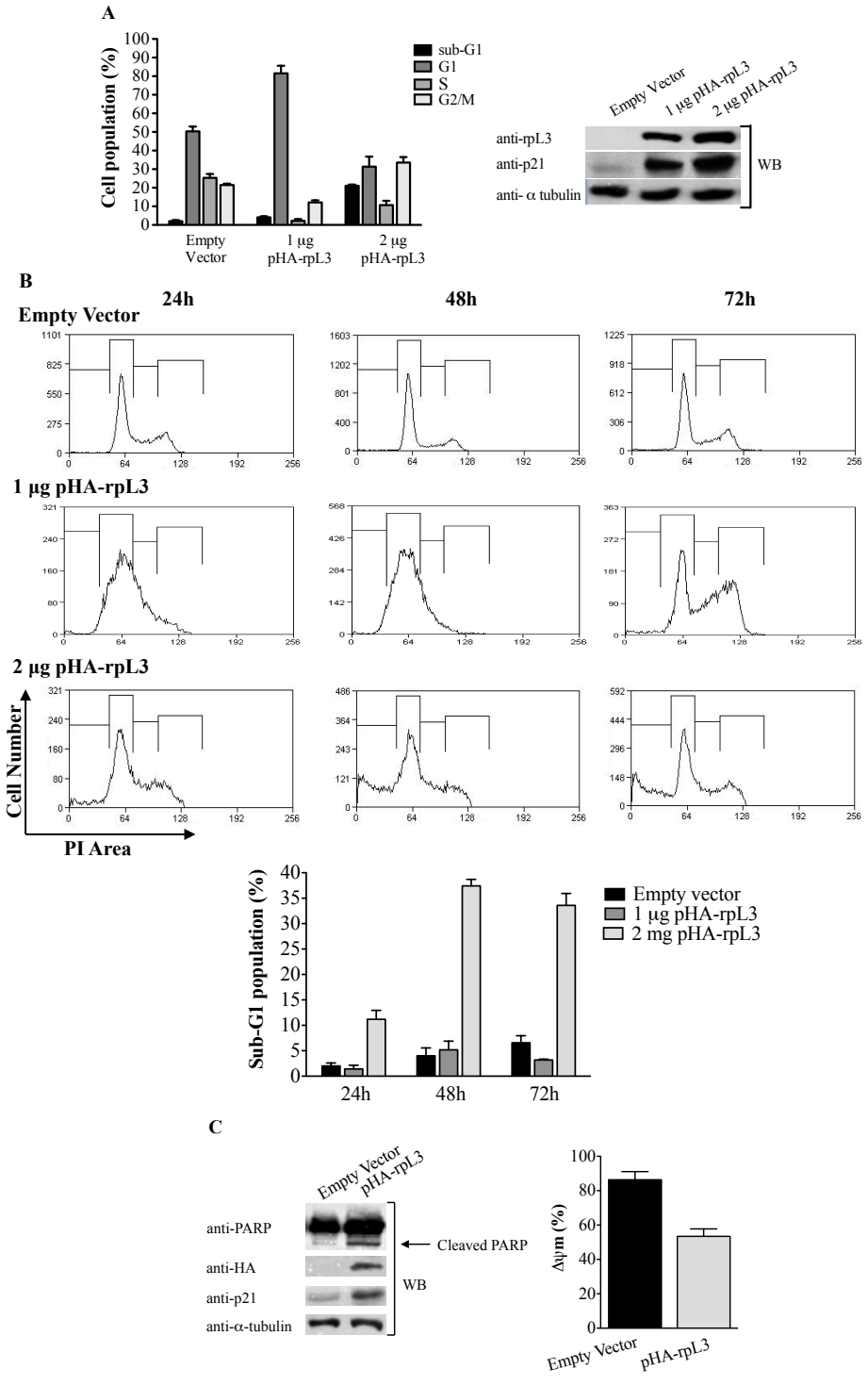
A
Cell population (%)
sub-G1
G1
S
G2/M
Empty Vector
1 µg pHA-rpL3
2 µg pHA-rpL3
Empty Vector
1 µg pHA-rpL3
2 µg pHA-rpL3
anti-rpL3
anti-p21
anti- α tubulin
WB
B
24h
48h
72h
Empty Vector
1 µg pHA-rpL3
2 µg pHA-rpL3
Cell Number
PI Area
Sub-G1 population (%)
Empty vector
1 µg pHA-rpL3
2 mg pHA-rpL3
24h
48h
72h
C
Empty Vector
pHA-rpL3
anti-PARP
anti-HA
anti-p21
anti-α-tubulin
Cleaved PARP
WB
Δψm (%)
Empty Vector
pHA-rpL3

Figure 3.4. Effects of rpL3-mediated upregulation of p21 gene expression on the cell cycle and cell viability. (A) FACS analysis of the Calu-6 cells transfected with the indicated plasmids after staining with FITC conjugated anti-5-bromodeoxyuridine antibody and counterstaining with propidium iodide, 48h after transfection. On the bottom the percentage of cells in different phases of cell cycle is shown. WB analysis of protein samples from the same samples with antibodies directed against the HA tag and p21. **(B)** Analysis of DNA content by propidium iodide staining of Calu-6 cells transfected with the indicated plasmids, 24h, 48h and 72h after transfection. A quantification of percentage of cells in sub-G1 phase is shown. **(C)** WB analysis of protein extracts from Calu-6 cells transfected with the indicated plasmids with antibodies directed against PARP, the HA tag and p21, 48h after transfection. The same samples were analyzed for mitochondrial membrane potential by TMRE staining, and fluorescence was measured by flow cytometry 48h after transfection.

3.5 Cell cycle arrest and apoptosis upon rpL3 overexpression are p21-dependent

To verify that rpL3-induced cell cycle arrest or apoptosis in Calu-6 cells occurs through a specific pathway involving p21, we examined the effects of rpL3 overexpression on cell proliferation and cell death after p21 silencing.

We performed cell cycle analysis in Calu-6 cells and p21-depleted Calu-6 (p21ΔCalu-6) cells transiently transfected with 1 μg or 2 μg of pHA-rpL3. Figure 3.5A shows that rpL3 was unable to induce cell cycle arrest upon p21 depletion. In addition, as shown in Figure 3.5B, the higher dosage of rpL3 failed to mediate the induction of apoptosis in p21-depleted cells, thus demonstrating that the dual effect of rpL3 on cell cycle arrest or apoptosis is dependent from p21. All these results indicate that rpL3 is able to induce cell cycle arrest or activate the intrinsic apoptotic pathway in Calu-6 cells by regulating the intracellular amounts of p21.

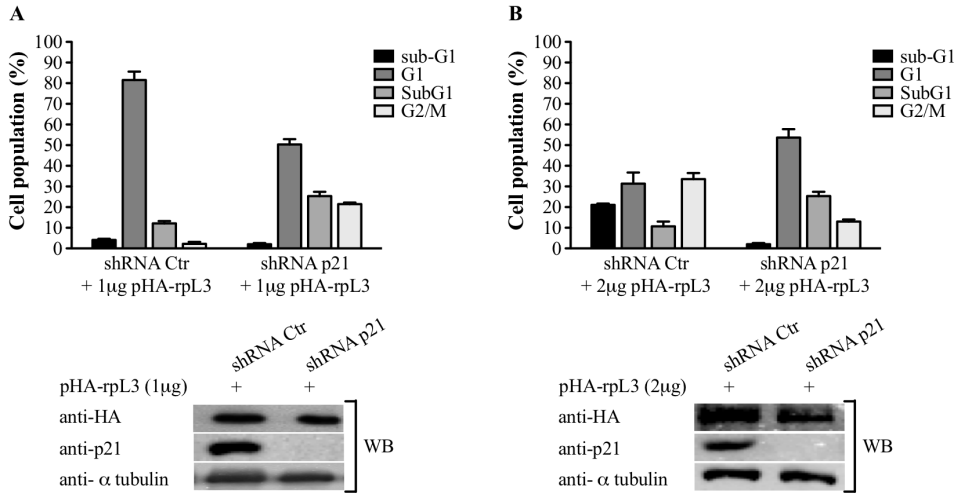

Figure 3.5. Role of p21 on rpL3-mediated cell cycle arrest and apoptosis. (A and B) Analysis of DNA content by propidium iodide staining of Calu-6 cells transfected with the indicated plasmids 24h after transfection. WB analysis of protein extracts from the same samples with the indicated antibodies.

3.6 DNA damage increased the amount of free-ribosome rpL3

Several RPs have been showed to accumulate in response to ribosomal stress triggered by DNA damage (Zhang and Lu, 2009). To address the possibility that rpL3 could accumulate after DNA damage, Calu-6 cells and HCT-116 $^{p53-/-}$ untreated or treated with 5-fluorouracil (5-FU) or Oxaliplatin (OXH) for 24 h were collected and separated by ultracentrifugation into two fractions; pellet, which includes polysome and ribosomal subunits, and supernatant, which includes free-ribosome proteins. Western blotting analysis of the two fractions showed that rpS19 and rpL7a remained exclusively associated with ribosome after exposure to the indicated drugs and in addition drug treatment caused a reduction of the total amount of rpL7a and rpS19. Of note, we observed a detectable amount of ribosome-free rpL3 even in absence of ribosomal stress and these levels were

increased in the ribosome-free fraction in response to both 5-FU or OXH treatment (Fig. 3.6).

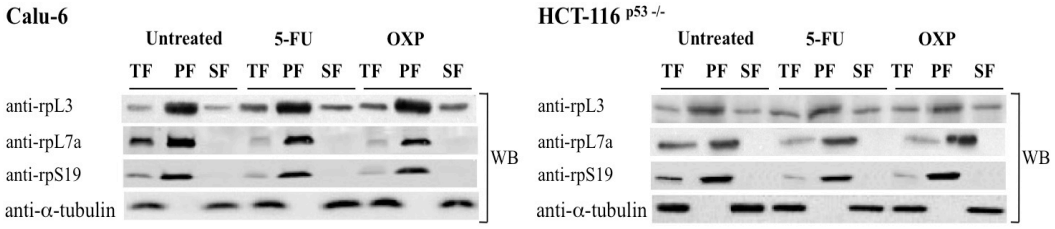

Figure 3.6. Specific accumulation of free-ribosome rpL3 upon DNA damage. Calu-6 or HCT-116$^{p53-/-}$ cells untreated or treated with the indicated drugs for 24 hours were collected and fractionated in ribosomal and non ribosomal fractions. The two fractions were analyzed by WB with the indicated antibodies. The per-sample ratio of the amount of protein loaded onto a gel for TF, Total fraction, PF, Pellet fraction and SF, Supernantant fraction was 1:10:1.

3.7 rpL3 mediates ribosomal stress after DNA damage

In order to investigate whether rpL3 could play a role in ribosomal stress, we analyzed proliferation and viability of Calu-6 cells and HCT-116 $^{p53-/-}$ cells untreated or treated with 5-FU or OXH while silencing rpL3. Fig. 3.7A shows that rpL3 silencing did not cause any changes in the cell phase distribution as compared to control. Treatment with 5-FU or OXH affected differently the DNA synthesis; the incorporation of BrdU in 5-FU-treated cells was significantly increased vs. that observed in control (about 74% vs 32%), whereas OXH caused the lack of BrdU incorporation (2,5% vs control 32%). These results indicate 5-FU treatment causes cells to enter the S-phase but unable to complete DNA synthesis, which is also indicated by the reduction of cells in the G2/M phase (4% vs. control 162%). The exposure to OXH, however, abolished their ability to enter S-phase.

rpL3 silencing prevented the inhibitory effects on cell proliferation of both drugs despite the diverse outcomes observed, suggesting that rpL3 may mediate the sensitivity of the cells to drug treatment.

To further study the rpL3 role in the apoptotic response to 5-FU or OXH, we analyzed the reduction of ΔΨm by TMRE staining, in Calu-6 cells and HCT-116 $^{p53-/-}$ untreated or treated with 5-FU or OXH upon rpL3 silencing. As expected, Figure 3.7B shows that the percentage of cells with impaired ΔΨm increased notably after 5-FU or OXH treatment as compared to control (26% 5-FU-cells, 46% OXH-cells versus 5.5% control). Interestingly, rpL3 silencing resulted in a substantial decrease in apoptotic cells following 5-FU and OXH treatment, respectively. Furthermore, silencing of rpL3 caused a significant increase in colony formation compared to 5-FU or OXH-treated cells (Fig. 3.7C). These results suggest that rpL3 may have a role in mediating cytotoxic effects of DNA-damaging agents in Calu-6 cells and HCT-116 $^{p53-/-}$ cells.

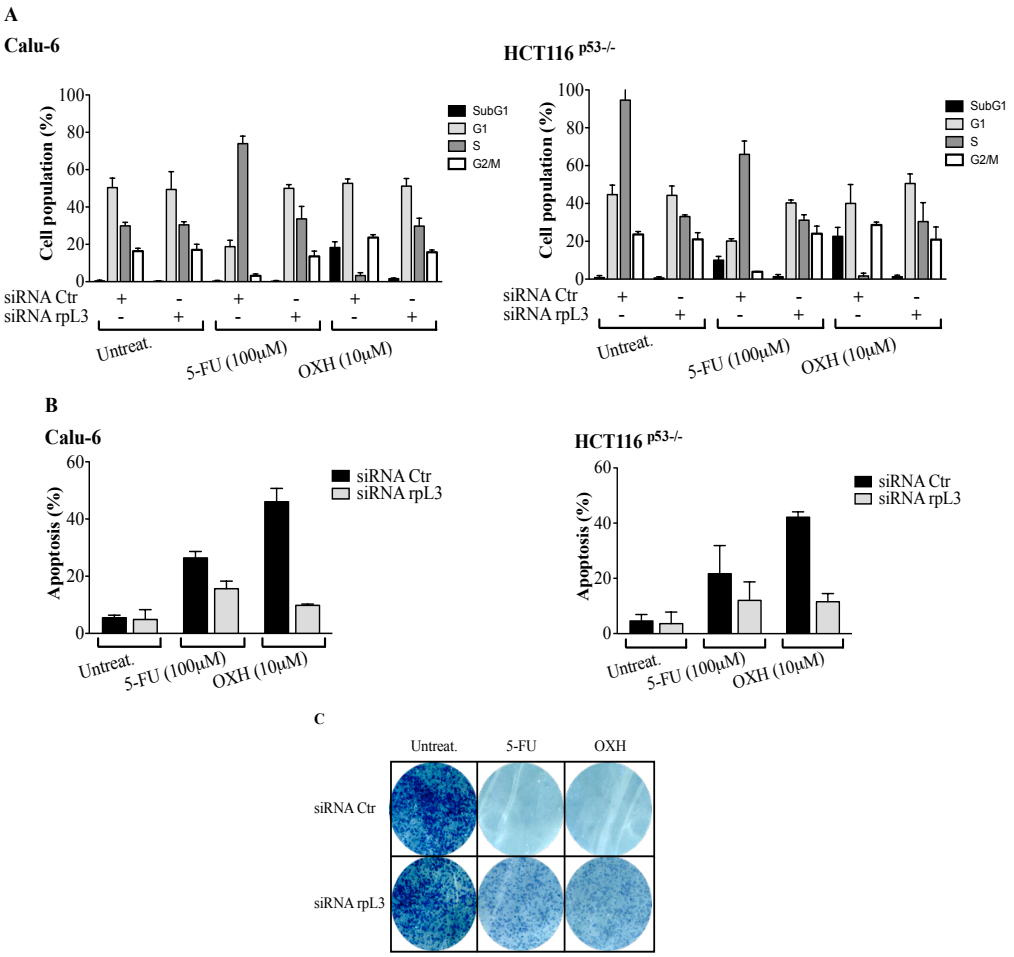

Figure 3.7. Effects of rpL3 on ribosomal stress (A) FACS analysis of Calu-6 cells and HCT-116$^{p53-/-}$ cells transiently transfected with the indicated siRNA and treated with the indicated drugs for 24h after stained with FITC conjugated anti-5-BrdU antibody and counterstained with PI. The percentage of cells in different phases of cell cycle is shown. (**B)** The same samples were analyzed for mitochondrial membrane potential by TMRE staining. **(C)** Staining after 10 days with methylene blue of the same samples replaced in drug-free medium.

3.8 rpL3 inhibits HR and NHEJ pathways

Chemotherapeutic agents cause a marked accumulation of DNA double strand breaks (DSBs). The formation of γ-H2AX foci at damaged site is one of the highly sensitive markers of damage induced by these agents (Kuo and Yang, 2008). Therefore, we performed quantification of γ-H2AX foci in 5-FU or OXH-treated in Calu-6 cells and HCT-116 $^{p53-/-}$ following rpL3 silencing. While the percentage of γ-H2AX foci positive cells increased after 5-FU or OXH exposure in the control, both 5-FU and OXH caused less accumulation of γ-H2AX foci when rpL3 was silenced (Fig. 3.8A). Analysis of homologous recombination frequency *in vivo* and NHEJ assay *in vitro* after 5-FU or OXH exposure showed that both major pathways were upregulated when rpL3 was silenced and downregulated when rpL3 was overexpressed (Fig. 3.8B, C and D). These results suggest that accumulation of rpL3 after exposure to the chemotherapeutics was responsible for the inhibition of both DNA repair pathways.

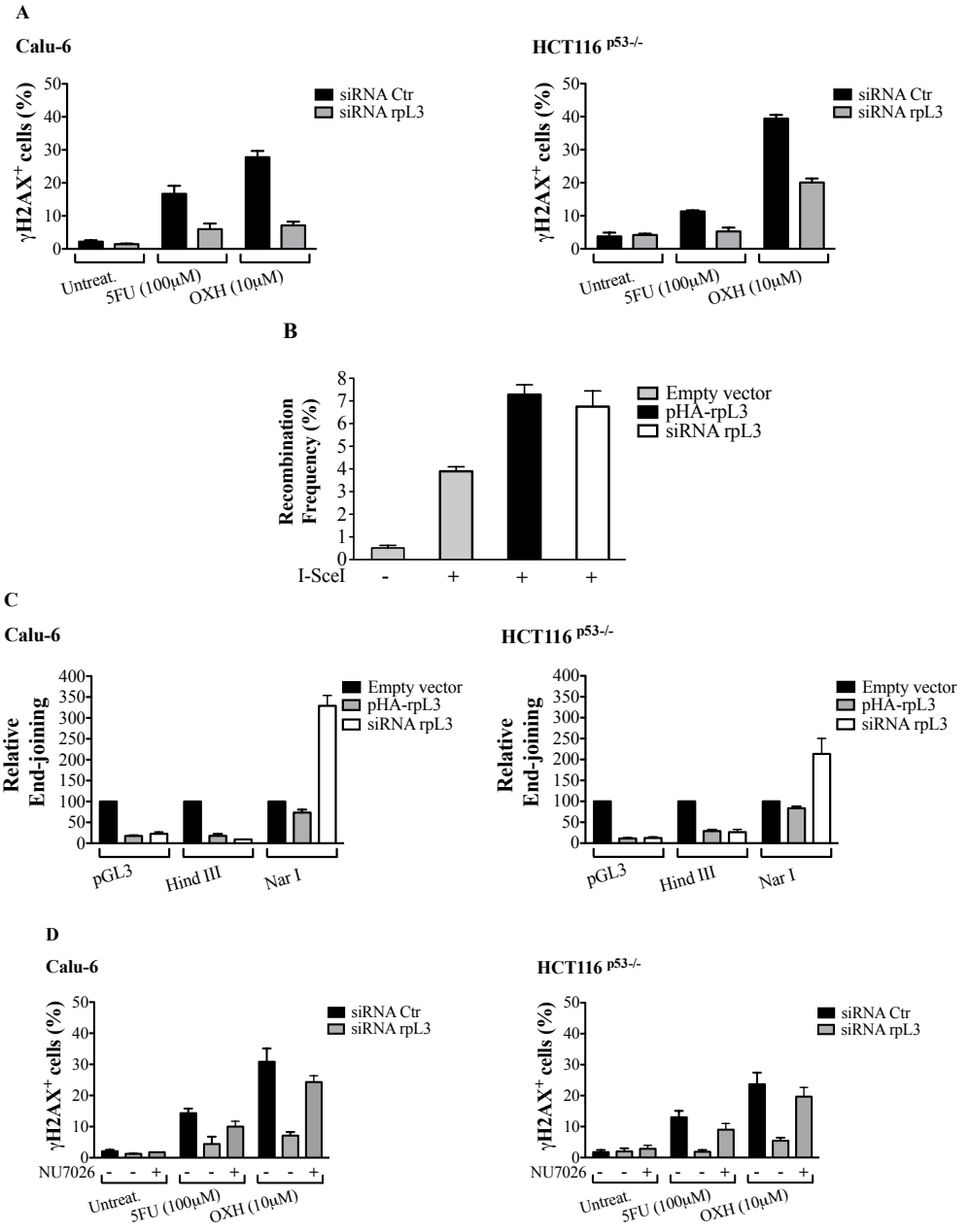

Figure 3.8. Effect of rpL3 silencing on HR and NHEJ pathways (A) γH2AX quantification in Calu-6 cells or HCT-116$^{p53-/-}$ cells untreated or treated with the indicated drugs and transiently transfected with the indicated siRNAs by staining with an anti-γH2AX monoclonal antibody followed by secondary fluorescein-conjugate antibodies. **(B)**

HeLa cells containing the DR-GFP reporter constructs integrated into the genome were transiently transfected with a plasmid expressing the I-SceI enzyme and the indicated siRNAs. 48h later, cells were assessed for GFP expression by flow cytometry. (**C)** Calu-6 cells transiently transfected with pGL3-luciferase linearized by the restriction endonuclease HindIII or NarI with the indicated siRNAs for 48h were analyzed for the relative luciferase activity, normalized against Renilla Luciferase (pRL). **(D)** γH2AX quantification in Calu-6 cells or HCT-116$^{p53-/-}$ cells untreated or treated with the indicated drugs and transiently transfected with the indicated siRNAs by staining with an anti-γH2AX monoclonal antibody followed by secondary fluorescein-conjugate antibodies.

3.9 p21 partially mediates rpL3 effects on drugs sensitivity

p21 plays a significant role in several aspects of the DNA damage response. It has been reported that p21 is upregulated after low levels of DNA damage. However, it is known that p21 downregulation is required to induce apoptosis when the extent of DNA damage is not reparable (Cazzalini et al., 2010). Having shown that overexpressed pL3 was able to regulate p21 expression independently of p53, we next investigated whether this regulation also occurs after treatment with DNA damaging drugs. ChIP experiments showed that rpL3 was able to bind p21 promoter following both 5-FU and OXH treatment (Fig. 3.9A and B). Reporter luciferase assay shows that treatment with 10 μM of 5-FU or OXH caused an activation of p21 promoter and increased p21 protein levels, restored upon rpL3 silencing, suggesting a positive role of rpL3 in the transcriptional activation of p21 upon mild DNA damage. In contrast, 100 μM of 5-FU or OXH diminished p21 gene promoter transactivation and protein levels, whereas silencing of rpL3 attenuated the p21 transcriptional repression (Fig. 9C). Analogous experiments were performed in HCT-116$^{p53-/-}$ with the same results (Fig. 3.9D).

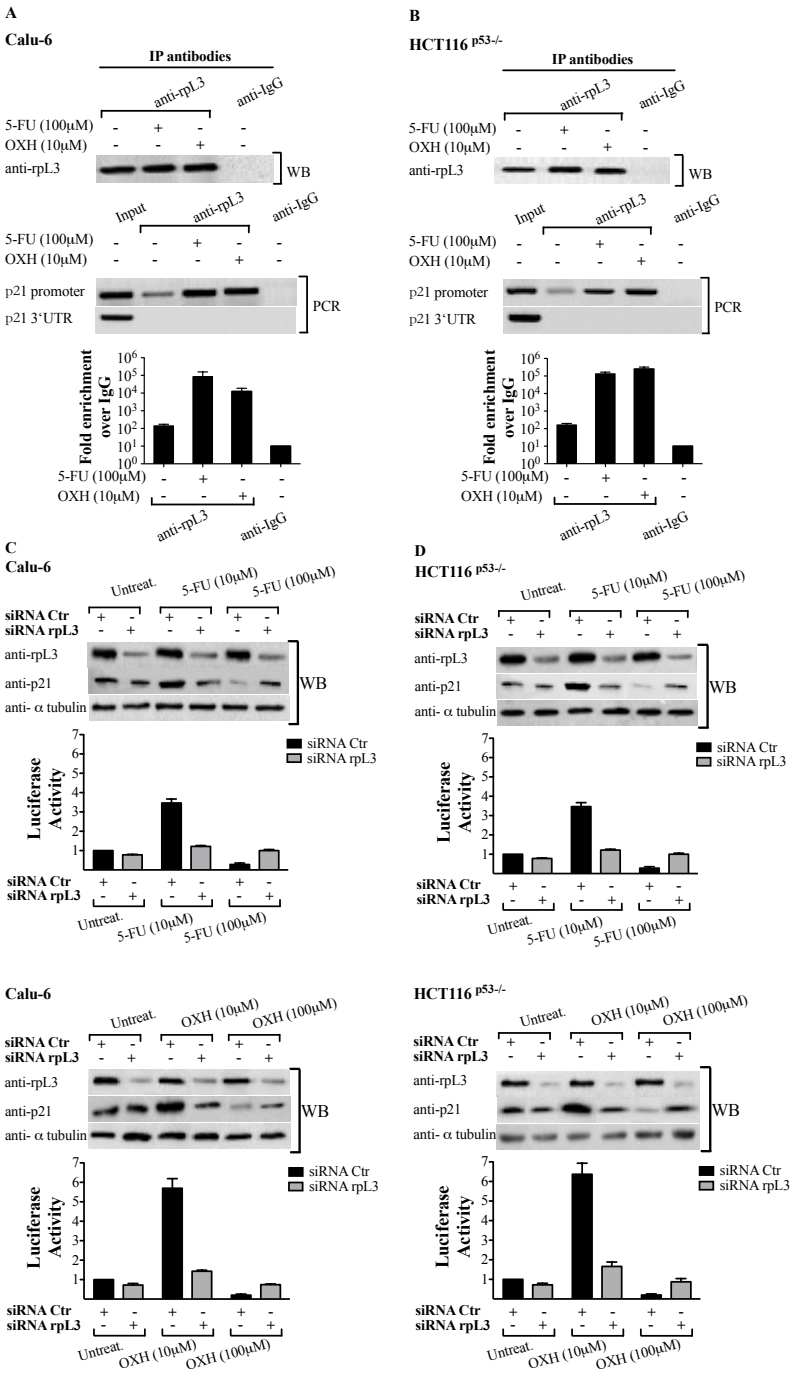

Figure 3.9. Role of rpL3 in the regulation of p21 expression in response to drugs treatment. (A and B) WB analysis of protein samples from DNA-rpL3 or DNA-IgG

immunocomplexes from Calu-6 cells or HCT-116$^{p53-/-}$ cells treated with the indicated drugs for 24h with antibody against rpL3. Note the absence of signal in DNA-IgG immunocomplex. The same DNA-immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or control loci. **(C and D)** WB analysis of protein extracts from Calu-6 or HCT-116$^{p53-/-}$ cells transiently transfected with the indicated plasmids or siRNAs and treated or not with the indicated drugs for 24h with the indicated antibodies. Analysis of the relative Firefly luciferase activity, normalized to Renilla Luciferase (pRL) in the same samples.

To investigate whether the mechanism by which rpL3 sensitizes cells to chemotherapeutic treatment occurs through a pathway involving p21, we measured DSBs accumulation after 5-FU or OXH exposure in p21-depleted Calu-6 cells while altering rpL3 levels. Figure 3.10A shows that rpL3 silencing prevented accumulation of DSBs after drugs treatment in the presence of physiological levels of p21, but not when p21 was silenced. Of note, rpL3 overexpression was responsible for increased percentage of cells with DSBs regardless of p21 status, and exerted an additive effect in combination with 5-FU and OXH treatment (Fig. 3.10B). These results indicate that rpL3 is able to modulate DNA damage repair in part by regulating the intracellular amount of p21. However additional mechanisms that sensitize cells to chemotherapeutic drugs are also required.

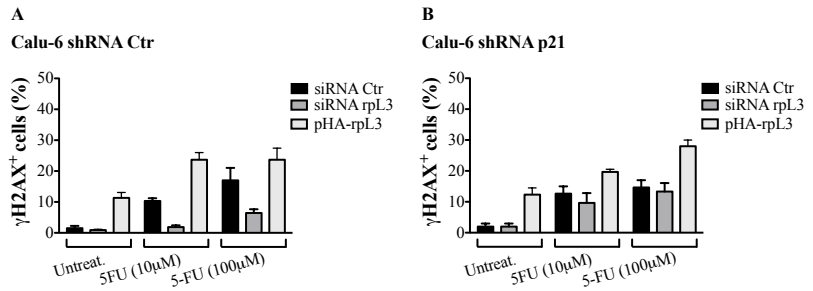

Figure 3.10. Role of p21 in rpL3-mediated regulation of drugs sensitivity. (A and B) γH2AX quantification in Calu-6 shRNA Ctr cells or Calu-6 shRNA p21 cells transiently transfected with the indicated plasmid and siRNAs by staining with an anti-γH2AX monoclonal antibody followed by secondary fluorescein-conjugate antibodies.

4. Discussion

The nucleolus appears to provide a link between ribosome subunit biosynthesis, cell cycle progression and stress signaling.

In eukaryotes, ribosome biogenesis is a complex process that requires a number of coordinated events prior to nuclear export of the mature subunits to the cytoplasm (Fromont-Racine et al., 2003; Kressler et al., 2009) Interestingly, the protein content of the nucleolus is dynamic and alters under stress condition, revealing a complex reorganization of the nucleolus under the stress response. Recent studies have shed new light on the mechanisms underlying the regulation of these events and have revealed new connections between ribosome biogenesis and cell cycle. In fact, perturbation of ribosome assembly has recently emerged as a relevant cell cycle arrest or apoptosis-promoting pathway. The signaling pathways able to connect these two processes have long been relatively unknown. However, more recently the role of the RPs-Mdm2-p53 stress response pathway in the coregulation of these events has been widely demonstrated (Zhang and Lu, 2009). Alterations in processes such as rRNAs synthesis, rRNAs modification and RPs imbalance produce alterations in ribosome biogenesis and result in ribosomal stress. In response to this event several RPs translocate to the nucleoplasma and bind to Mdm2, thus promoting p53 stabilization and subsequent p53-mediated cell cycle arrest or apoptosis (Amsterdam et al., 2004).

Several p53-independent pathways that require nucleolar proteins, such as ARF, NPM or free RPs have been descrive (Donati et al., 2012; Sherr, 2006). These findings provide a rational basis for the use of drugs that specifically impact ribosome biogenesis for the treatment of cancers lacking

functional p53 and extend the scenario of mechanisms involved in the relationship between cell growth and cell proliferation.

In this thesis, I unraveled a new extraribosomal function for human rpL3 in addition to its role in the regulation of the alternative splicing of its own gene. Specifically, I demonstrated that rpL3, when overexpressed, is able to induce cell cycle arrest or apoptosis in Calu-6 cells by modulating p21 level thorugh a p53-independent pathway. To our knowledge the present study demonstrates for the first time a direct role of an RP, specifically rpL3, in the regulation of p21 transcription.

I started my project with the observation that rpL3, when overexpressed, was able to positively modulate the activity of p21 promoter. Moreover, ChIP analysis on Calu-6 cells showed that rpL3 interacts with p21 proximal promoter (-194 to +88) (Figure 3.1). In an attempt to understand the mechanism of the rpL3-mediated upregulation of p21 expression, I focused on the identification of protein partners of rpL3 protein involved in this activity. It is known that NPM is able to indirectly bind to p21 proximal promoter and activate its transcription (Wanzel et al., 2008). Furthermore, our previous results demonstrated that NPM is able to interact directly with rpL3, and that this interaction is functional to the regulation of the rpL3 protein levels inside the cells (Russo et al., 2011). Starting from this knowledge, a possible involvement of this complex in the rpL3-induced p21 transactivation has been hypothesized. ChIP experiments established that NPM and rpL3 are present in a reciprocal DNA-immunoprecipitate indicating that both proteins are recruited on p21 proximal promoter (Figure 3.2A). Data from protein overexpression indicated that NPM alone or in combination with rpL3 did not affect the

activity of p21 promoter, while NPM silencing markedly reduced the constitutive and rpL3 induced transactivation of p21 promoter (Figures 3.2B and C). Taken together, these results clearly demonstrate that NPM represents a positive regulator of rpL3-mediated transactivation of p21 gene, although it is not a crucial player in this event.

It is well known that p21 proximal promoter contains six Sp1 binding sites and that Sp1 is able to transcriptionally regulate the activity of different genes including those involved in the regulation of the cell cycle (Koutsodontis et al., 2002). Specifically, Sp factors have been proposed to be essential for transactivation of the p21 promoter by members of the p53 family proteins. I demonstrated that rpL3 associates *in vivo* with Sp1 on the p21 proximal promoter only in absence of MTM. Although treatment with MTM did not prevent the formation of a complex between rpL3 and Sp1 into the cells, it hindered its recruitment on p21 promoter (Figure 3.2D). Data from rpL3 and Sp1 overexpression in Calu-6 cells in the absence of MTM indicated that expression of Sp1 in combination with rpL3 strongly increased rpL3-mediated p21 transactivation. These findings together with the observation that rpL3, even in excess, was unable to transactivate p21 promoter in the presence of MTM strongly indicated that the binding of Sp1 to the proximal p21 promoter region is essential for the rpL3 mediated transactivation of p21 promoter. Thus, we propose that Sp1 is the key component of rpL3-mediated p21 transactivation. In fact, Sp1 binding to GC boxes 3 and 4 is a prerequisite for rpL3 recruitment on p21 proximal promoter (Figure 3.3).

In light of these results, we propose a working model in which overexpression of rpL3 leads to a formation of a multiprotein complex

containing at least rpL3, Sp1 and NPM on the proximal region of p21 promoter with consequent upregulation of p21 expression. This upregulation leads to a dual outcome, either arresting the cell cycle progression at G1/S phase or promoting apoptosis. The cells fate depends on the levels of rpL3 and p21 protein (Figure 3.4A). To better understand the dynamics of these events, time-course analysis of the DNA content in Calu-6 cells transfected with different amounts of pHA-rpL3 was performed. Interestingly, we observed that 1 μg of pHA-rpL3 induced a reversible G1/S arrest of the cell cycle; in fact, Calu-6 cells restored their capability to progress through the G1/S transition at 72h after transfection. Instead, transfection with 2 μg of pHA-rpL3 triggered mitochondrial apoptosis even at 24h after transfection (Figures 3.4B and C). Moreover, we showed that p21 is essential for both rpL3-induced cell cycle arrest and mitochondrial apoptosis, since stably depletion of p21 in Calu-6 cells completely abolished both events (Figure 3.5). Taken together, these data demonstrate that rpL3 plays an important role in the activation, through modulation of p21 levels, of two independent pathways; on one hand, reversible arrest of cell cycle, and on the other hand, mitochondrial apoptosis. In light of these observations, our working model predicts that the intracellular levels of rpL3 could be crucial in regulating the hierarchy of interactions of p21 with multiple factors, leading to the assembly of specific protein complexes which could trigger two distinct effects (Russo et al., 2013).

These data are in complete agreement with recent findings demonstrating a much more complex scenario than had been expected regarding p21 role in the regulation of cell cycle and DNA damage repair. In

particular, p21 has been proven to also exert a proapoptotic function under certain conditions in specific systems beyond its well-known role in growth inhibition. Notably, in most of these cases, the cellular systems used express a non-functional p53 isoform. The mechanism by which p21 may promote apoptosis is poorly understood and different working models have been proposed. However, the common assumption is that the cellular activities of p21 are tightly regulated by multiple factors that seem to be specific for each function in different systems. For example, the interaction of p21 with PCNA is implicated in sodium butyrate-induced apoptosis (Chopin et al., 2004), other studies indicate that the modulation of proapoptotic or antiapoptotic genes is responsible for p21-induced apoptosis (Hsu et al., 1999; Wu et al., 2002).

Next, I evaluated whether rpL3 could have a role in the ribosomal stress in response to DNA damage, as reported for other RPs. Analysis of ribosome and free-ribosome fractions after exposure to chemotherapeutic agents 5-FU and OXH revealed an extraribosomal accumulation of rpL3 (Fig. 3.6). Evaluation of their effects on proliferation and viability upon rpL3 silencing showed that both chemotherapeutic agents require the presence of rpL3 to exert their cytotoxic effects (Fig. 3.7 and 3.8). Two main DNA repair pathways, homologous recombination and NHEJ, cooperate to repair DNA DSBs, induced by 5-FU and OSH. Then, I investigated the ability of rpL3 to modulate these two processes in drug-treated cells. My data show that rpL3 overexpression significantly decreased the activity of both pathways, while rpL3 silencing improved their effectiveness (Fig. 3.8). The results described above indicate that rpL3, by hindering both homologous recombination and NHEJ repair processes, has

the potential to inhibit recovery of mildly and seriously damaged p53-null tumor cells after drug treatment and to increase the susceptibility of tumor cells to chemotherapy.

p21 exerts a key role in the response of cells to genotoxic stress. Our understanding of p21 functions has greatly improved. In addition, it has also elucidated the dual role of p21 in DNA damage repair. Although the role of p21 in this context is still controversial, recent lines of evidence support a positive role for p21 in DNA repair. p21 function in response to DNA damage is modulated by the extent of genotoxic lesions, through either upregulation of downregulation of the protein: low levels of DNA lesions cause increasing of p21, and induce cell cycle arrest, in contrast, after extensive DNA damage, p21 downregulation allow cells to undergo apoptosis. Data from ChIP experiments and reporter luciferase assays showed that rpL3 is critical for regulating both upregulation and downregulation of p21 at transcriptional level following different extent of DNA damage (Fig. 3.9).

Moreover, I showed that while p21 is essential for DNA repair, since stable depletion of p21 in Calu-6 cells completely abolished the rescue of γH2AX accumulation after drugs exposure, overexpression of rpL3 increased the DNA damage suggesting a p21-independent role in the inhibition of DNA DSBs repair as a novel mechanism of action by which rpL3 affects tumor cells (Fig. 3.10).

Data reported in this thesis indicate that rpL3 is a critical regulator of p21 expression in response to ribosomal stress. rpL3 is able to both upregulate and downregulate p21 at transcriptional level dependent on the extent of DNA damage, triggering two distinct pathways; cell cycle arrest or

mitochondrial apoptosis in a p53-independent manner. Although the molecular details underlining these mechanisms remain to be clarified, I speculate that determinant of cell fate after DNA damage are the molecular partners of rpL3 in mediating regulation of p21 expression.

At the present, the challenge is to identify rpL3 molecular partners involved in the cellular response to rpL3 accumulation. This will help to elucidate the role of RPs in the control of cell cycle and cell death through p21-dependent and independent molecular pathways.

It is worth noting that the rpL3 gene is located on chromosome 22q13. Allelic loss on 22q13 is a common somatic alteration in several neoplastic processes including breast and colorectal cancer. However, no tumor suppressor gene on this chromosome region involved in these neoplasms has been identified thus far (Castells et al., 2000). The identification of the cellular targets of chemoterapeutic drugs is an important challenge for the improvement of therapy regimens in the future.

Understanding the mechanism by which cytotoxic agents cause cell death and by which tumors become resistant to chemotherapy is an essential step towards predicting or even overcoming that resistance and improving the overall response rate.

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Autoregulatory circuit of human rpL3 expression requires hnRNP H1, NPM and KHSRP

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ABSTRACT

Alternative pre-mRNA splicing (AS) is a major mechanism that allows proteomic variability in eukaryotic cells. However, many AS events result in mRNAs containing a premature termination codon, which are degraded by nonsense-mediated mRNA decay (NMD) pathway. We have previously demonstrated that human rpL3 autoregulates its expression through the association of AS with NMD. In fact, overexpression of rpL3 promotes downregulation of canonical splicing and upregulation of alternative splicing that produces an NMD-targeted mRNA isoform. The result of these events is a decreased production of rpL3. We have also identified heterogeneous nuclear ribonucleoprotein (hnRNP) H1 as a splicing factor involved in the regulation of rpL3 alternative splicing and identified its regulatory *cis*-elements within intron 3 transcript. Here, we report that NPM and KHSRP are two newly identified proteins involved in the regulation of rpL3 gene expression via AS-NMD. We demonstrate that hnRNP H1, KHSRP and NPM can be found associated, and present also in ribonucleoproteins (RNPs) including rpL3 and intron 3 RNA *in vivo*, and describe protein–protein and RNA–protein interactions. Moreover, our data provide an insight on the crucial role of hnRNP H1 in the regulation of the alternative splicing of the rpL3 gene.

INTRODUCTION

Alternative splicing (AS) is an important mechanism of gene expression control, through which an individual gene gives rise to different mRNAs encoding distinct proteins, thus allowing functional and genetic variability (1). In addition to the canonical splice elements, auxiliary non-splice site RNA sequences have been identified and are located in both introns and exons. These additional elements are needed for a proper splicing and regulation of the process. They may function as enhancers or silencers of the splicing reaction, and may influence recognition and usage of the splice sites by the splicing apparatus through the binding of specific regulatory proteins (2). These proteins include members of the serine–arginine-rich (SR) proteins, the heterogeneous nuclear ribonucleoprotein (hnRNP) family and other RNA binding proteins (3). The SR proteins represent a family of highly conserved *trans*-acting factors that usually induce splicing, whereas the hnRNP proteins can affect the splicing depending on the interacting cis-elements and on the gene context (4,5). Moreover, in some case, the result of a splicing reaction is determined by the antagonistic action of hnRNP and SR proteins since they are able to recognize and bind to a composite regulatory element generated by the overlapping of enhancer and silencer elements (6).

AS can also control gene expression quantitatively by generating unproductive mRNAs that are targeted for degradation by nonsense-mediated decay (NMD) (7). NMD is a surveillance pathway that detects and selectively degrades aberrant mRNAs harboring a premature termination codon (PTC), thus preventing the production of truncated polypeptides potentially deleterious to the cell. NMD is highly conserved in eukaryotes; many studies have dissected machinery components, and the issue of its localization in a cell compartment has been thoroughly discussed (8,9 and references therein). The process regulating gene expression through AS-NMD association has been defined Regulated Unproductive Splicing and Translation (RUST) (10).

Although the mechanisms of the AS have been extensively studied, the complex regulation of such process is still an issue. The complexity of the regulation pathways is due to an intricate and dynamic network of protein–protein and RNA–protein interactions. Changes in the

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activity, number and association of splicing factors in the network can modify the selection of the splice site and the activity of the splicing machinery, and therefore the splicing pattern of the target pre-mRNA.

The regulation of pre-mRNA splicing plays an important role in human pathologies (11). Impairment of the AS is closely related to some disease mechanisms as in the case of spinal muscular atrophy and myotonic distrophy. Recently, new therapies aimed to correct defects arising in pre-mRNA splicing, the so-called 'splice-correction' or 'splice-modulation' therapies, have been developed (12).

The accuracy of the AS depends on the stoichiometry and interactions of positive and negative regulatory proteins. Consequently, the identification of the proteins participating in the modulation of the AS is an essential step to study the many aspects of gene expression.

Data from several laboratories demonstrate that some alternative isoforms of mRNA-encoding ribosomal proteins (rp) are NMD substrates. In *Caenorhabditis elegans*, it has been shown that the AS of genes rpL3, rpL12, rpL10 and rpL7 gives rise to aberrant mRNA isoforms, which contain a PTC resulting from an incomplete intron removal, and are natural substrate of NMD (13). The AS event appears conserved in humans and in other mammals on rpL3 and rpL12 genes. In fact, we have previously demonstrated that human rpL3 gene transcript gives rise to a canonical mRNA and to an alternative mRNA isoform containing a PTC targeted to decay by NMD. rpL3 is able to modulate its own production via a negative feedback loop. In fact, rpL3 overexpression results in a decreased level of the canonically spliced mRNA, and an increased production of the alternatively spliced isoform (14). We have also identified the hnRNP H1, as a transacting factor able to interact *in vitro* and *in vivo* with rpL3 and with intron 3 transcript of the rpL3 gene. Our data demonstrated that hnRNP H1 is involved in promoting the AS of human rpL3 pre-mRNA. In addition, we have identified and characterized the *cis*-acting regulatory elements, G runs, involved in hnRNP H1-mediated regulation of splicing (15). In the present study, we analyze the role of hnRNP H1 in the rpL3 autoregulatory loop, and we report the identification of two new regulatory proteins, KHSRP (K-homology splicing regulatory protein, also known as KSRP) and NPM (Nucleophosmin, also known as B23, numatrin or NO38), which exhibit opposite effects on the splicing reaction of rpL3 pre-mRNA. Our data contribute to shed light on protein–protein and RNA–protein interactions within putative RNP complexes involved in the modulation of splicing of the rpL3 gene.

MATERIALS AND METHODS

Cell cultures, transfections and drug treatment

Human cell line HeLa was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin–streptomycin 50 U/ml.

L3–8 cell line, derived from rat PC12 Tet-Off cell line and conditionally overexpressing rpL3 upon doxycyclin removal (14), was grown in DMEM supplemented with 5% fetal calf serum (FCS), 10% horse serum, 2 mM L-glutamine and 100 μg/ml G418 (Invitrogen), hygromycin 200 μg/ml (USB, Santa Clara, CA, USA) and doxycyclin 10 ng/ml (Sigma, St Louis, MO, USA). The expression of HA-rpL3 was induced upon removal of doxycyclin. siRNA transfections were performed in HeLa cells (1×10^6 cells, 6 mm well plate) at a concentration of 150 nM by using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids were transfected in L3–8 cell line or in HeLa cells (2.5×10^6 cells, 6 mm well plate) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after DNA or siRNA transfections, cells were treated with 100 μg/ml cycloheximide (CHX) for 4 h to block NMD. Then, RNA and proteins were extracted by using the Trizol procedure (Invitrogen) for RT–PCR analysis and western blot, respectively. Transfection efficiency was assessed by cotransfecting a GFP-expressing vector and normalizing RNA levels against GFP mRNA levels (data not shown).

DNA constructs and production of recombinant proteins

The cDNA of NPM was obtained by RT–PCR from HeLa cells using the primers 5′-ATGGAAGATTCGATGGAC-3′ (forward) and 5′-TTAAAGAGACTTCCTCCA-3′ (reverse), and cloned into a version of the eukaryotic expression vector pcDNA4/HisMax C (Invitrogen) containing the HA epitope, into the prokaryotic expression vector pRSET-A (Invitrogen) containing the Histidine tag, and in the prokaryotic expression vector pGEX4T3 (GE Healthcare, Waukesha, WI, USA). The cDNA of hnRNP H1 was obtained by RT–PCR from HeLa cells using the primers 5′-ATGATGTTGGGCACGGAA-3′ (forward) and 5′-CTATGCAATGTTTGATTGAAAA-3′ (reverse), and cloned into the prokaryotic expression vector pRSET-A. The plasmids encoding GST-hnRNP H1, GST-rpL3, HA-hnRNP H1, Flag-KHSRP and pGEM4Z-Int3 were already available (15,16).

The recombinant proteins GST-hnRNP H1, GST-rpL3, GST-rpL7a and GST were expressed in *Escherichia coli* and purified by using glutathione Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare). The recombinant proteins His-NPM, His-hnRNP H1 and His-rpL7a were expressed in *E. coli* and purified by the nickel–nitrilotriacetic acid (Ni–NTA)-Agarose chromatography according to the manufacturer's instructions (Qiagen, Valencia, California). His-tagged KHSRP was expressed in Sf9 cells using the Baculovirus system (Baculogold, BD Biosciences) and purified by Ni–NTA-Agarose chromatography (16).

RNA interference

The target sequences of small interfering RNAs (siRNA) in hnRNP H1 were: 5′-GGAAATAGCTGAAAAGGCT-3′ and 5′-CCACGAAAGCTTATGGCCA-3′ (Ambion, Foster City, CA, USA). The siRNAs targeting NPM and KHSRP were purchased from Santa Cruz

Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-29771, sc-44831).

GST pull down

For GST pull-down assay, 50 μg of the fusion protein or GST control, as bait were immobilized on glutathione-Sepharose beads and incubated with 20 μg of the recombinant protein of interest in pull-down buffer (50 mM Tris–HCl, pH 7.5, 0.4 mM EDTA, 150 mM NaCl, 10% glicerol, 1% NP-40, 1 mM sodium-ortovanadate, 50 mM NaF, 5 mM DTT and Protease Inhibitor Mix 1X) at 4°C for 1.5 h. The beads were washed extensively and boiled in SDS sample buffer. The eluted proteins were loaded on 12% SDS–PAGE and analyzed by western blotting.

Immunoprecipitation and western blotting

For immunoprecipitation assay, 1 mg of HeLa whole-cell lysate was incubated with 30 μl of protein A/G agarose beads coated with 5 μg of anti-NPM or anti-KHSRP (Santa Cruz Biotechnology sc-47725, sc-33031) at 4°C for 12 h. The beads were washed and boiled in the SDS sample buffer. The eluted proteins were loaded on 12% SDS–PAGE and detected by western blotting. Aliquots of protein samples (30 μg) were resolved by 12% SDS–gel electrophoresis and transferred into nitrocellulose filters. The membranes were blocked in PBS, 0.1% Triton and 5% dry milk for 2 h, and then challenged with anti-NPM, anti-KHSRP, anti-hnRNP H1, anti-HA, anti-Flag (Santa Cruz Biotechnology sc-10042, sc-57592 and sc-807) and anti-rpL3 (Primm, Milan, Italy). The proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

RNA pull-down assay

RNA pull-down assay was carried out by using adipic acid dehydrazide beads. Briefly, 20 μg of intron 3 RNA, transcribed *in vitro* from pGEM4Z-Int3, were placed in a 400 μl reaction mixture containing 100 mM NaOAc pH 5.2 and 5 mM sodium m-periodate (Sigma), incubated for 1 h in the dark at room temperature, ethanol precipitated and resuspended in 100 μl of 100 mM NaOAc, pH 5.2. Then, 300 μl of adipic acid dehydrazide agarose beads 50% slurry (Sigma) equilibrated in 100 mM NaOAc pH 5.2 were added to this mixture, which was then incubated for 12 h at 4°C on a rotator. The beads with the bound RNA were pelletted, washed twice with 1 ml of 2 M NaCl and equilibrated in washing buffer (5 mM HEPES pH 7.9, 1 mM $MgCl_2$, 0.8 mM magnesium acetate). The intron 3 RNA was then incubated with 50 μg of each recombinant protein for 30 min at room temperature in a final volume of 0.6 ml. The beads were then washed four times in 1.5 ml of washing buffer. Bound proteins were eluted in SDS sample buffer loaded on a 12% gel for SDS–PAGE and analyzed by western blotting.

RNP immunoprecipitation assay

For RNP immunoprecipitation assay (RIPA), HeLa cells (2×10^6 cells) were lysed in 600 μl RIPA buffer 1× (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM Na ortovanadate, 0.05 M NaF, 0.5% NP-40) with protease inhibitors mix 1× (Roche, Basel, Switzerland) for 60 min on ice, and then centrifuged at 10 000 *g* at 4°C for 15 min. The supernatant was subjected to a pre-clearing step in which it was incubated with 50 μl of protein A/G plus agarose for 1 h at 4°C. The pre-cleared cell extracts were then incubated with antibodies specific for each protein (Santa Cruz Biotechnology) overnight at 4°C. Protein A/G plus agarose beads (50 μl of 50% slurry) were then added and the mix was incubated for 1 h at 4°C with gentle shaking and centrifuged. The immunoprecipitates were suspended in 100 μl TES buffer (10 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 0.5% SDS), incubated at 65°C for 10 min and centrifuged for 1 min at 10 000*g*. Ten microliters of the supernatant were stored as immunoprecipitated samples and subsequently fractionated by SDS–PAGE to be analyzed by western blotting. RNA was extracted from 90 μl using the Trizol procedure (Invitrogen).

RT–PCR

For RT–PCR analysis, 1 μg of total RNA was reverse transcribed into cDNA with the random hexamers technique using 200 U of Superscript II RNAse H^- Reverse Trancriptase (Invitrogen). The reaction was carried out at 42°C for 50 min and was terminated by heating to 75°C for 15 min. Ten of the 40 μl of reaction mix were PCR amplified in a final volume of 50 μl, using 5 μM of each specific primer, 10 mM dNTPs and 0.5 U of Taq DNA polymerase (Invitrogen). Typically, 25–30 cycles of amplification were performed. The primers were: rpL3-a 5′-CT CCGCTGGGCTCTGCCC-3′ (forward) and 5′-CTTCAG GAGCAGAGCAGA-3′ (reverse); rpL3-c 5′-GGGCATT GTGGGCTACGT 3′ (forward) and 5′-GTAAAGGCCT TCTTCTTAG-3′ (reverse); and β-actin 5′-GGCACCACC TTCTACA-3′ (forward) and 5′-CAGGAGGACAATGA T-3′ (reverse).

In separate experiments, we ascertained that the cycle number was within the linear range of amplifications. PCR products were visualized on 1% agarose gel containing the fluorescent Vistra Green dye (15). The labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the PhosphorImager (Bio-Rad, Haercules, CA, USA).

RESULTS

Interactions between NPM, KHSRP, hnRNP H1 and rpL3 *in vivo* and *in vitro*

We have identified, by a proteomic analysis in a previous study, proteins associated to rpL3 and/or to intron 3 transcript of the rpL3 gene, and we focused our studies on proteins involved in RNA processing as putative components of a RNP complex including rpL3 and mediating

alternative splicing of the rpL3 pre-mRNA. Mass spectrometry results from GST pull-down experiments using GST-rpL3 as bait demonstrated that NPM is one of the proteins able to interact with rpL3 *in vitro*. Conversely, KHSRP was not found associated to rpL3. However, data obtained from RNA pull-down experiments indicate that KHSRP is able to interact with the intron 3 transcript of the rpL3 gene (15). Thus, we asked whether NPM and KHSRP could be part of a RNP complex including rpL3 through a direct or indirect interaction *in vivo*, by performing co-immunoprecipitation assays.

Figure 1 shows the results of the experiments in which NPM and KHSRP were specifically immunoprecipitated from HeLa cells extracts by using antibodies against the endogenous proteins. Immunoprecipitated proteins were separated by SDS–PAGE and the presence of NPM and KHSRP was investigated by western blotting in the reciprocally immunoprecipitated complexes. The results of these experiments showed that NPM and KHSRP were coimmunoprecipitated, thus indicating that they can associate *in vivo*. Furthermore, a specific signal for rpL3 appeared both in NPM as well as in KHSRP immunocomplexes. These data suggest that rpL3 is able to interact *in vivo* with both proteins. Since we have previously demonstrated that hnRNP H1 is found included in a complex with rpL3 and is involved in the regulation of rpL3 gene alternative splicing (15), we looked for hnRNP H1 in NPM and KHSRP immunoprecipitated complexes. The presence of signal for hnRNP H1 in both immunoprecipitates was consistent with a specific association between NPM, KHSRP and hnRNP H1. A control immunoprecipitate obtained with anti-IgG antibodies did not give any signal, when probed with anti-NPM, anti-KHSRP, anti-rpL3 or anti-hnRNP H1 (Figure 1); for a further control, see Supplementary Figure S1. In the light of these results, it is plausible to hypothesize that NPM, KHSRP, hnRNP H1 and rpL3 are associated in a single quaternary complex; alternatively, NPM and KHSRP could be part of independent protein complexes, including or not rpL3, to which they are recruited through different combination of protein–protein interactions.

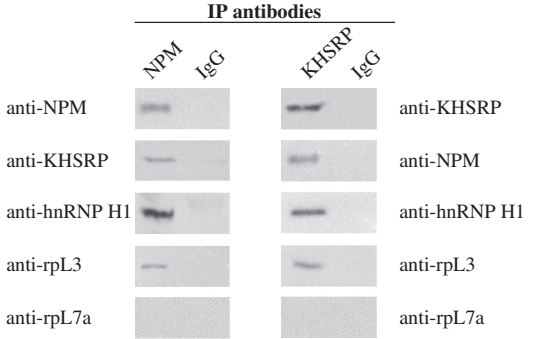

Figure 1. *In vivo* binding of NPM, KHSRP, hnRNP H1 and rpL3. NPM or KHSRP were specifically immunoprecipitated from HeLa cells extracts with antibodies against the endogenous NPM and KHSRP. Immunoprecipitates were separated by SDS–PAGE and immunoblotted with antibodies versus the indicated proteins. Note the absence of signal in IgG immunocomplex. Results illustrated in Figures 1–6 are representative of three independently performed experiments.

In an attempt to define a RNP complex including rpL3, we investigated protein–protein interactions that rpL3 protein partners may establish within the complex. To this aim, we performed GST pull-down assays by using purified recombinant proteins. His-tagged proteins were tested for their ability to bind to immobilized GST-tagged proteins. GST-tagged proteins, and GST as control, were immobilized using GSH-sepharose beads and incubated with purified His-tagged proteins. Western blot analysis was performed on each pull-down sample. Figure 2 shows that His-hnRNP H1 was present in the pull-down preparation of GST-NPM, but not in those of GST-rpL3 or GST; His-NPM was detected in the pull-down preparations of GST-rpL3, GST-hnRNP H1, but not in that of GST; and His-KHSRP was revealed in the pull-down preparations of GST-rpL3 and GST-hnRNP H1, but not in those of GST-NPM or GST. These results indicate that although a direct interaction between rpL3 and hnRNP H1 has not been observed, rpL3 and hnRNP H1 are able to interact directly with NPM and KHSRP, while NPM and KHSRP are not able to interact directly in the *in vitro* assay. No interaction whatsoever was detected with GST-rpL7a used as a further control (data not shown).

Interactions between KHSRP, NPM, hnRNP H1 and the intron 3 transcript *in vivo* and *in vitro*

Mass spectral analysis of our previous RNA pull-down experiments revealed that KHSRP was able to interact with intron 3 transcript of rpL3 gene *in vitro* (15). To confirm this interaction *in vivo*, we performed RNA affinity immunoprecipitation experiments. We specifically immunoprecipitated KHSRP, and hnRNP H1 as control, from HeLa cell extracts by using monoclonal antibodies against the endogenous proteins and searched for the intron 3 transcript in the RNA–protein immunoprecipitate complex (Figure 3A). Amplification of the signal corresponding to the intron 3 of rpL3 transcript, but not of rpL7a gene transcript, by RT–PCR indicated that KHSRP was able to bind with rpL3 pre-mRNA. The absence of signal in the immunoprecipitate with anti-IgG confirmed the validity of this assay.

Although NPM was not found associated to the intron 3 transcript *in vitro* previously (15), we wondered whether this interaction can occur *in vivo*. To this aim, we specifically immunoprecipitated NPM from HeLa cell extracts by using monoclonal antibodies against the endogenous NPM. Analysis of RNA extracted from the immunoprecipitate complex demonstrates that NPM is able to precipitate the intron 3 transcript (Figure 3A).

Next, in order to establish whether one or more among the identified proteins was able to interact directly with intron 3 RNA, or whether additional factors were required, we performed RNA pull-down experiments by

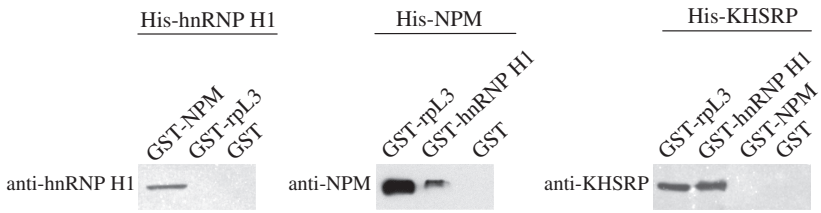

Figure 2. Analysis of the interactions between hnRNP H1, NPM, KHSRP and rpL3. Western blotting (WB) of GST pull-down experiments. Fifty microgram of GST-tagged proteins (GST-NPM, GST-rpL3 and GST-hnRNP H1) or GST (control) were immobilized on glutathione-sepharose beads and incubated with 20 µg of His-hnRNP H1, His-NPM or His-KHSRP. The eluted proteins were then analyzed by immunoblot with antibodies anti-hnRNP H1, anti-NPM and anti-KHSRP. Note the absence of signal in control GST pull-down preparations.

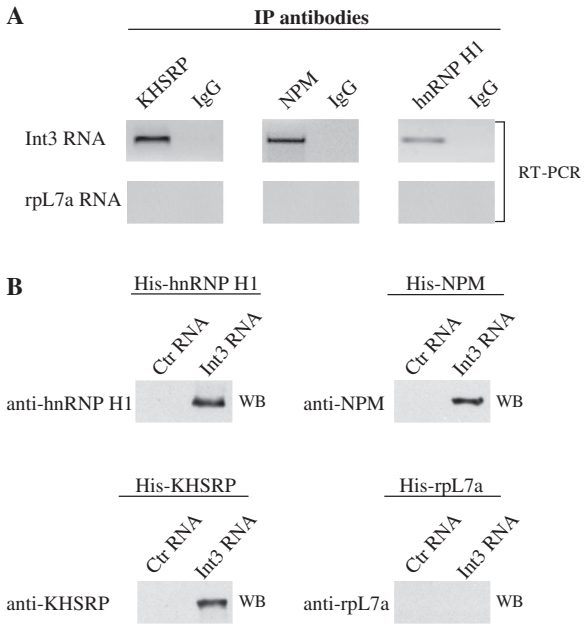

Figure 3. Analysis of the interactions of intron 3 of rpL3 pre-mRNA with NPM and KHSRP, *in vivo* and *in vitro*. (**A**) *In vivo* binding of intron 3 transcript to NPM and KHSRP. RT–PCR analysis, by using primers against rpL3 intron 3 transcript and rpL7a RNA transcript, of RNA extracted from the NPM, KHSRP, hnRNP H1 (control) and IgG immunocomplexes. Note the absence of signal in IgG immunocomplex. (**B**) *In vitro* binding of intron 3 transcript to hnRNP H1, NPM, KHSRP and rpL7a. WB of RNA pull-down experiments, using adipic acid dehydrazide agarose beads coated with intron 3 transcript or unrelated RNA (Ctr RNA) incubated with purified proteins His-hnRNP H1, His-NPM, His-KHSRP and His-rpL7a.

using purified recombinant proteins. For this purpose, a transcript corresponding to the entire intron 3 of the rpL3 gene was used as bait and incubated with the purified recombinant proteins His-hnRNP H1 (control), His-NPM or His-KHSRP and His-rpL7a as a control RNA binding protein (17). Then, RNA-associated proteins were eluted and analyzed by western blotting with antibodies against the endogenous hnRNP H1, NPM, KHSRP and rpL7a. The presence of signals specific for NPM and KHSRP indicated that these two proteins are able to recognize and bind to sequences in the rpL3 intron 3 transcript (Figure 3B).

KHSRP regulates the alternative splicing of rpL3 pre-mRNA

The identification of a specific interaction of KHSRP with rpL3 and intron 3 transcript prompted us to investigate a possible role of KHSRP in the rpL3 gene splicing. To this aim, we analyzed the effects of the alteration in the expression levels of KHSRP on the rpL3 RNA splicing pattern. Increasing amounts of a DNA construct expressing Flag-KHSRP were transiently transfected in HeLa cells. Twenty-four hours after transfection, cells were treated with CHX to stabilize the alternatively spliced isoform of rpL3 mRNA and lysed. Cells extracts were tested for the detection of Flag-KHSRP levels by western blotting with anti-Flag antibodies (Supplementary Figure S2A). Total RNA from the same cell extract was analyzed by RT–PCR using specific primers to amplify canonical (rpL3-c) or alternative (rpL3-a) isoform of rpL3 mRNA. We observed an increase in the rpL3-a mRNA level correlated, in a dose-dependent mode, with increasing amount of KHSRP (Figure 4A). These data indicate that KHSRP positively affects the selection of the 3′-cryptic splicing site within the intron 3 transcript of rpL3 gene. Thus, we investigated the possibility that KHSRP could act as a component of rpL3 autoregulatory loop. We used L3–8 cells, a PC12 Tet-Off cell line stably transfected with a vector containing the human rpL3-coding sequence fused to the hemagglutinin (HA) epitope-coding sequence (14). L3–8 cells were transiently transfected with the DNA construct expressing Flag-KHSRP. We chose a dose of Flag-KHSRP that would result in about a 50% increase of the rpL3 alternative isoform mRNA. Twenty-four hours after transfection, we treated cells with CHX and induced the expression of HA-rpL3 by removing doxycycline from medium. Cells were then lysed and analyzed for the production of Flag-KHSRP and HA-rpL3 by western blotting using anti-Flag and anti-HA antibodies, respectively (Supplementary Figure S2B). Total RNA was analyzed by RT–PCR using specific primers to amplify rpL3-c or rpL3-a mRNAs (Figure 4B). As previously demonstrated the expression of the HA-rpL3 protein resulted in an increase of the alternative isoform mRNA (14). The expression of exogenous KHSRP also caused an increase in rpL3-a mRNA level. Of interest, the increase in the rpL3-a mRNA caused by overexpression of KHSRP was greater after the induction of HA-rpL3 (Figure 4B).

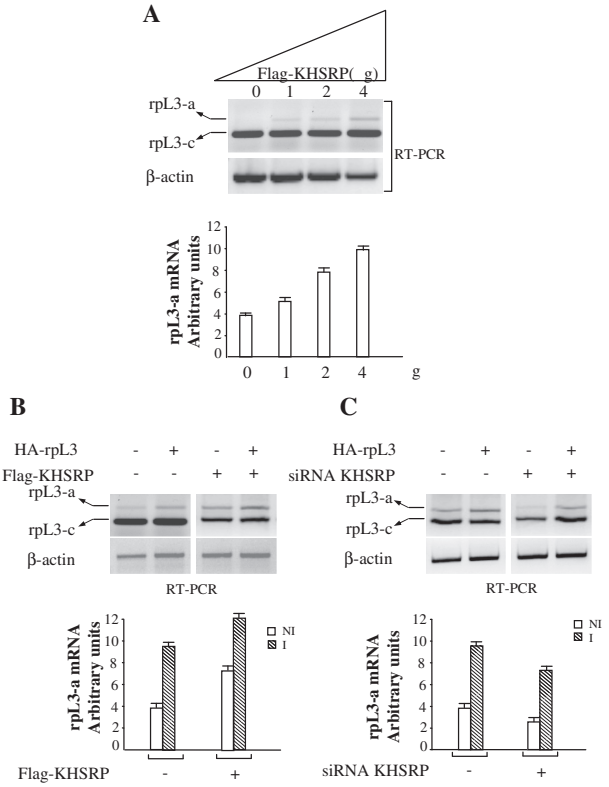

Figure 4. (**A**) Effects of KHSRP overexpression on rpL3-a mRNA levels in HeLa cells. Representative RT–PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of Flag-KHSRP. (**B**) Effects of KHSRP overexpression on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with Flag-KHSRP, non-induced (NI) or induced (I) for HA-rpL3 expression. (**C**) Effects of RNAi-mediated depletion of KHSRP on rpL3-a mRNA levels in L3–8 cells. Representative RT-PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with KHSRP-siRNA, NI or induced I for HA-rpL3 expression from the same samples. The levels of rpL3-a mRNA were quantified by PhosphorImager (Bio-Rad) and normalized to β-actin levels.

The effects of KHSRP on rpL3 splicing were also investigated in condition of mRNA silencing. L3–8 cells were treated with siRNA specific for KHSRP and, 24 h after transfection, cells were treated with CHX and HA-rpL3 production was induced by removing doxycycline from culture medium. Cells were then lysed and protein extracts were analyzed for the expression of KHSRP and HA-rpL3 by western blotting using anti-KHSRP and anti-HA antibodies, respectively. As shown in Supplementary Figure S2C, the decrease of KHSRP level was ~70% compared to the protein amount detected in the control lysates. To examine the effects of the reduced production of KHSRP on the splicing pattern of rpL3 pre-mRNA, we analyzed total RNA extracted from the same samples by RT–PCR using specific primers to amplify rpL3-c and rpL3-a mRNAs. As expected, in cells overexpressing rpL3, we observed an increased level of rpL3-a mRNA isoform. However, in cells, in which KHSRP had been silenced, rpL3 induced overexpression was less effective in promoting the production of rpL3-a mRNA isoform (Figure 4C).

hnRNP H1 role in KHSRP-mediated activity

Since hnRNP H1 (15) and KHSRP have a role in promoting the alternative splicing of rpL3 gene, we asked whether a cooperation between these two proteins would occur and favor the selection of 3′-cryptic splicing site, or whether their functions were redundant. To clarify this issue, we switched off the expression of the gene encoding hnRNP H1 by using RNA interference. To this purpose, siRNAs against hnRNP H1 were transiently transfected in L3–8 cells. Twenty-four hours after transfection, cells were treated with CHX, induced for HA-rpL3 production and harvested; RNA and proteins were extracted. Lysates from cells transfected with siRNA or untransfected were probed with hnRNP H1 antibodies. As shown in Supplementary Figure S3A, the residual level of hnRNP H1 was ~20% of the protein detected in the control lysates. To investigate the effects of the reduced production of hnRNP H1 on the splicing pattern of rpL3 pre-mRNA, we examined the level of rpL3 mRNA isoforms using RT–PCR. Under normal conditions of rpL3 expression, the depletion of hnRNP H1 resulted in a relevant decrease (~80%) of the alternative mRNA (rpL3-a) level compared to controls. In conditions of hnRNP H1 removal, rpL3, although overexpressed, failed to activate the selection of the 3′-cryptic splicing site (Figure 5A). These data indicate clearly that hnRNP H1 plays a crucial role in triggering the alternative splicing reaction of rpL3 pre-mRNA.

Next, we investigated a possible involvement of hnRNP H1 in KHSRP-mediated activity. To this aim, we analyzed the effects of hnRNP H1 depletion and KHSRP overexpression on rpL3 splicing reaction. L3–8 cells were transiently co-transfected with siRNA specific for hnRNP H1 and a DNA construct expressing Flag-KHSRP. Twenty-four hours after transfection, cells were treated with CHX, induced for HA-rpL3 expression, and harvested. hnRNP H1 and KHSRP protein levels were detected by western blotting (Supplementary Figure S3B). RNA extracted from the same lysates was analyzed by RT–PCR. The Figure 5B shows that when hnRNP H1 was depleted, the enforced expression of KHSRP did not cause an increase of rpL3-a mRNA level, either in normal conditions of rpL3 expression, or in rpL3 overproduction. These findings indicate that the ability of KHSRP to influence the splicing of rpL3 pre-mRNA is mediated by hnRNP H1.

NPM controls the alternative splicing of rpL3 gene

To study the functional relevance of the interactions of NPM with rpL3 and intron 3 RNA in the control of the rpL3 pre-mRNA splicing, we overexpressed NPM in HeLa cells. Increasing amounts of a DNA construct expressing HA-NPM were transiently transfected in HeLa cells. Twenty-four hours after transfection, cells were treated with CHX and lysed. The expression levels of

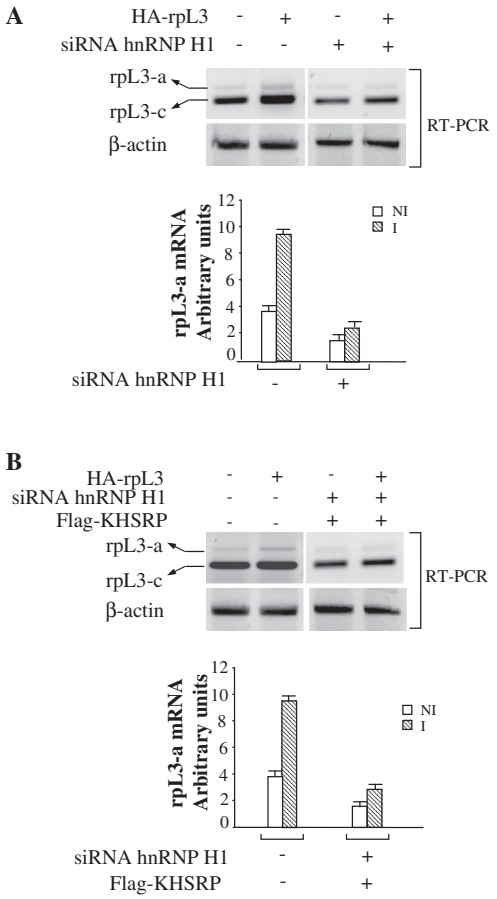

Figure 5. (**A**) Effects of RNAi-mediated depletion of hnRNP H1 on rpL3-a mRNA levels in L3–8 cells. Representative RT-PCR analysis of total RNA from CHX-treated L3–8 cells, untransfected or transfected with hnRNP H1-siRNA, NI or induced I for HA-rpL3 expression. (**B**) Effects of RNAi-mediated depletion of hnRNP H1 and KHSRP overexpression on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or cotransfected with hnRNP H1-siRNA and Flag-KHSRP, NI or induced I for HA-rpL3 expression. The levels of rpL3-a mRNA were quantified by PhosphorImager (Bio-Rad) and normalized to β-actin levels.

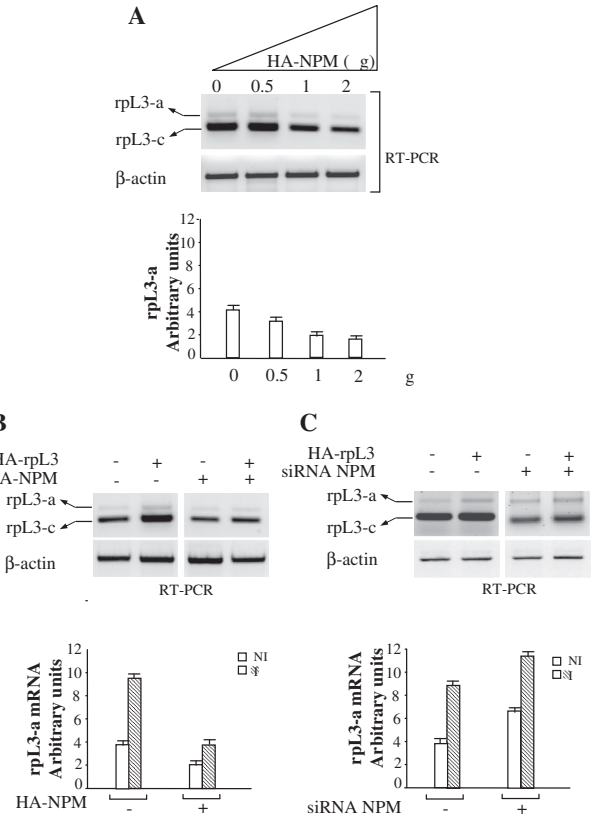

Figure 6. (**A**) Effects of NPM overexpression on rpL3-a mRNA levels in HeLa cells. Representative RT–PCR analysis of total RNA from CHX-treated HeLa cells untransfected or transfected with increasing amounts of HA-NPM. (**B**) Effects of overexpression of NPM on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with HA-NPM, NI or I for HA-rpL3 expression. (**C**) Effects of silencing of NPM on rpL3-a mRNA levels in L3–8 cells. Representative RT–PCR analysis of total RNA from CHX-treated L3–8 cells untransfected or transfected with siRNA against NPM, NI or I for HA-rpL3 expression. The levels of rpL3-a mRNA were quantified by PhosphorImager (Bio-Rad) and normalized to β-actin levels.

the recombinant fusion protein were detected by western blotting using anti-HA antibodies (Supplementary Figure S4A). The pattern of the spliced rpL3 gene transcript was monitored by RT–PCR by using specific primers to amplify rpL3-c and rpL3-a isoforms of rpL3 mRNA. As shown in Figure 6A, NPM overexpression caused a dose-dependent decrease of rpL3-a mRNA amounts. Next, to investigate the role of NPM in the context of rpL3 autoregolatory loop, we performed experiments of overexpression and RNAi-mediated silencing of NPM in L3–8 cell line. Cells were transiently transfected with a DNA vector expressing HA-NPM. We chose a dose of HA-NPM that would result in about 50% decrease of the rpL3-a mRNA isoform. Twenty-four hours after transfection, cells were treated with CHX and induced to express HA-rpL3. Cells were then lysed and analyzed for the production of HA-NPM and HA-rpL3 by western blotting by using anti-HA antibody (Supplementary Figure S4B). Total RNA from the same samples was analyzed by RT–PCR by using specific primers to amplify rpL3-c and rpL3-a isoforms of rpL3 mRNA. The overexpression of NPM resulted in a reduction in the rpL3-a mRNA amount in normal conditions of rpL3 expression, and prevented the increasing of rpL3-a mRNA level when rpL3 protein was overexpressed (Figure 6B).

To investigate the effects of NPM removal on rpL3 gene splicing, NPM expression levels were reduced by transfecting L3–8 cells with specific siRNA. Twenty-four hours after transfection, cells were treated with CHX and induced to express HA-rpL3. Protein extracts from cells were tested for the production of HA-NPM and HA-rpL3 by western blotting (Supplementary Figure S4C). Total RNA was analyzed by RT–PCR by using specific primers to amplify rpL3-c and rpL3-a mRNA isoforms.

In physiological conditions of rpL3 expression, following NPM depletion, the production of rpL3-a mRNA appeared restored above the control expressing NPM. Thus, as expected, HA-rpL3 expression upon NPM silencing caused a significant increase of rpL3-a mRNA levels (Figure 6C).

DISCUSSION

AS of mRNA is mainly responsible for a variety of gene products much larger than expected from the number of genes. It has been estimated that >90% of human genes are alternatively spliced (18,19). However, a number of splicing events give rise to mRNA isoforms containing a PTC that are targeted for decay by NMD. Accumulating data indicate that association of AS and NMD (AS-NMD) may result in a quantitative post-transcriptional regulation of gene expression (7).

In eukaryotes rp expression is regulated by multiple control mechanisms, mostly at post-transcriptional and translational level in order to maintain ribosome biosynthesis at the level appropriate to growth conditions and requirements of the cell (14,15,20,21). However, several reports indicate that rp, in addition to the role as components of the translation machinery, exert a variety of extraribosomal functions, for which additional, specific regulatory strategies are required (22,23). Autoregulation may represent an efficient mechanism to control the level of a single protein; we have demonstrated previously a role of AS-NMD in the regulation of the rpL3 gene expression, promoted by rpL3 protein itself as part of an autoregulatory negative-feedback loop (14,15).

In an attempt to understand the mechanism of the regulatory strategy, we focused our studies on the identification of protein partners of the rpL3 protein, and to the analysis of their contribution to the process. Our previous results demonstrated that rpL3 protein, within a RNP complex including the constitutive splicing factor hnRNP H1, promotes the alternative splicing reaction of its own gene (15). In most cases, the choice of a splicing site is made by a dynamic and complex combination of different splicing regulators; in fact, the function of an individual splicing factor may be different depending on interacting partners present in the regulatory network. The identification of new regulatory proteins and the mapping of protein–protein interactions within the RNP complex including rpL3 are crucial steps to understand the molecular mechanism involved in the selection of the cryptic 3′-splice site within intron 3 transcript of the rpL3 gene. To this aim, we analyzed occurrence of interaction between rpL3 and putative protein partners previously identified through a proteomic analysis (15), and the role of these proteins in the autoregulatory network of rpL3 expression. Our data provide an insight in putative RNP complexes including hnRNP H1, rpL3 protein, pre-mRNA of rpL3 gene, and the newly identified protein factors, NPM and KHSRP, involved in the regulation of rpL3 gene expression via AS-NMD.

NPM is a nucleolar, ubiquitous and multifunctional phosphoprotein. NPM is involved in multiple biological functions including the ribosome biogenesis, the control of cell cycle progression and centrosome duplication. The cellular activities of NPM are tightly regulated by multiple factors that seem to be specific for each function. Post-translational modifications, oligomerization and hetero-oligomerization strongly influence the cellular functions of NPM (24). Emerging evidences indicate a functional correlation between NPM and some rp, independent from ribosome biogenesis or its assembly (25,26). It is known that NPM is involved in both positive and negative regulation of transcription, and a possible role of NPM in the splicing process has been suggested (27). To our knowledge, the present study demonstrates for the first time a role of NPM as splicing factor.

KHSRP is a multifunctional RNA-binding protein that has been mainly implicated in post-transcriptional regulation, mRNA decay and maturation of microRNA precursors (28,29). There are few data supporting an involvement of KHSRP in the splicing control (30,31).

Analysis of immunoprecipitate of NPM and KHSRP in HeLa cell extracts showed that NPM and KHSRP coimmunoprecipitate together with rpL3 and hnRNP H1, indicating that these proteins associate *in vivo*, although the occurrence of multiple complexes including different combination of protein interactions cannot be excluded (Figure 1). In addition, *in vitro* GST pull-down experiments (Figure 2) demonstrated that NPM and KHSRP are able to recognize and bind rpL3 and hnRNPH1, whereas a direct binding between NPM and KHSRP, or rpL3 and hnRNPH1 has not been observed.

On the other hand, RNA immunoprecipitation assays indicated the presence of intron 3 RNA in the immunoprecipitate of NPM and KHSRP, and RNA pull-down experiments showed that NPM and KHSRP are able to contact directly the intron 3 RNA (Figure 3) as previously shown for hnRNP H1 (15). Taken together, these data strongly suggest that NPM and KHSRP are involved in the control of the splicing of rpL3.

Our analysis of the effects of individual expression of NPM, hnRNP H1 or KHSRP provided an insight also on the specific role of each protein factor on the rpL3 gene splicing. In the context of rpL3 autoregulatory circuit, protein overexpression data indicated that KHSRP represents a positive regulator of the alternative splicing that cooperates with rpL3 and hnRNP H1 in the activation of 3′-cryptic splice site (Figure 4). However, silencing of KHSRP caused only ~20% decrease of the alternative mRNA isoform produced when rpL3 is overexpressed, suggesting that KHSRP is not a crucial player in the rpL3 autoregulatory loop (Figure 4). Instead, when hnRNPH1 expression was silenced, rpL3 overexpression failed to result in the activation of cryptic the 3′-splice site (Figure 5A). These findings together with the observation that KHSRP, even in excess, was unable to increase the alternative mRNA isoform in the absence of hnRNP H1 strongly indicate that hnRNP H1 is the key component of rpL3 autoregulatory loop, while KHSRP might play a role as an enhancer of hnRNP H1-mediated activation of rpL3 gene alternative splicing (Figure 5B).

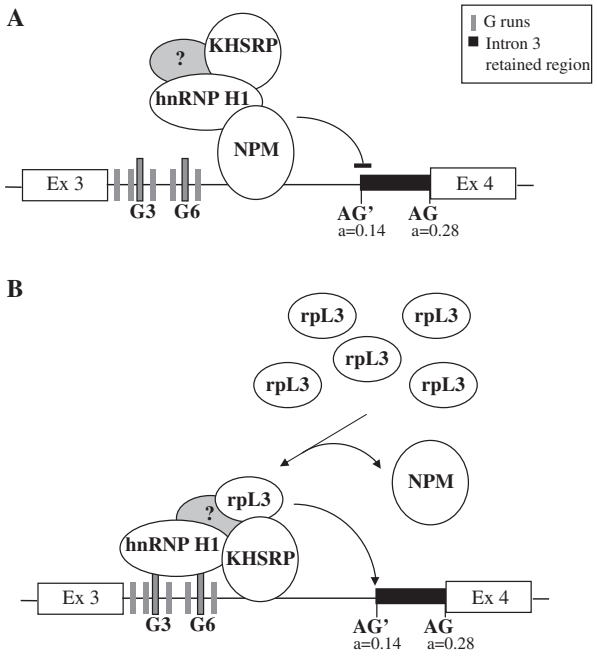

Figure 7. Schematic representation of proposed rpL3 feedback regulation. (**A**) In response to cell requirement for an efficient production of rpL3, NPM represses the alternative splicing of rpL3 gene. NPM, by interacting with hnRNP H1 and intron 3 pre-mRNA, prevents the binding of hnRNP H1 with G3 and G6 motifs. (**B**) Upon accumulation, rpL3 interacts with NPM. As consequence of this interaction, NPM is released from the RNP complex. The association of rpL3 with KHSRP and other transacting proteins induces a rearrangement of the interactions within the RNP complex that favor the interaction of hnRNP H1 with enhancer unit G3 + G6 within intron 3 transcript. Finally, the reassembled RNP complex promotes the selection of the 3′-splice site in intron 3 transcript.

It is known that the RNA binding activity of KHSRP is mediated by four KH domains. Among these, KH1 and KH3 domains recognize and bind specifically to a G-rich target (UGGG and GGGU, respectively) (32). The analysis of the human intron 3 transcript sequence showed that G6 motif, an essential element for the splicing regulatory activity of hnRNP H1, could represent also a binding site for KH1 or KH3. In addition, the UGCAUG element, essential to the binding of KHRSP to src-DCS (Downstream Control Sequence), is also present in the sequence of intron 3 RNA and it is located close to the hnRNP H1 binding site G3 and G6 motifs (31). Consequently, it seems plausible to speculate that KHSRP might interact with these sequences within intron 3 transcript as well as with hnRNP H1 making more stable and efficient the association of hnRNP H1 with intron 3 RNA.

Unlike hnRNP H1 and KHSRP, NPM behaves as a negative regulatory factor of rpL3 gene alternative splicing (Figure 6). It is possible that the inhibitory effect of NPM on alternative splicing might be due to a 'bind and block' mechanism. In fact, NPM could interact with sequences within intron 3 pre-mRNA, perhaps in the vicinity of hnRNP H1 binding sites, and sterically block the access of hnRNP H1, thus preventing its interaction with G3 and G6 elements. Since binding of hnRNP H1 to G-runs site has been demonstrated crucial, the alternative splicing of rpL3 gene would be negatively affected.

Alternatively, in the light of results demonstrating an interplay among different protein factors, possibly assembled in multiple RNP complexes, we propose a working model, which overcomes the model proposed previously (15). The model predicts the existence of, at least, two complexes whose protein composition depends on rpL3 protein levels. When cell functions require an efficient production of rpL3, the interaction of NPM with intron 3 pre-mRNA might affect its secondary structure and mask the binding sites of hnRNP H1. At the same time, repression of AS might be enforced by a concomitant direct binding of NPM to the positive regulator factor hnRNP H1 (Figure 7A).

When rpL3 levels exceed cell requirements for rpL3 ribosomal as well as extraribosomal functions, the protein could interact with NPM; consequently, NPM would be released from the RNP complex to which rpL3 could be associated by interacting with KHSRP. The removal of NPM, and the rearrangement of the interactions within the RNP complex, could make available the enhancer unit G3 + G6 within intron 3 transcript and, at the same time, the presence of rpL3 in the remodeled complex could induce conformational changes that favor the interaction of hnRNP H1 with G3 + G6. Finally, such complex could promote the activation of the alternative mode of splicing reaction (Figure 7B).

At the present, the challenge is to determine the hierarchy of interactions leading to the assembly of RNP complexes, and the dynamic nature of protein–protein interactions causing the remodeling of complexes responsible for the modulation of the splicing of the rpL3 gene.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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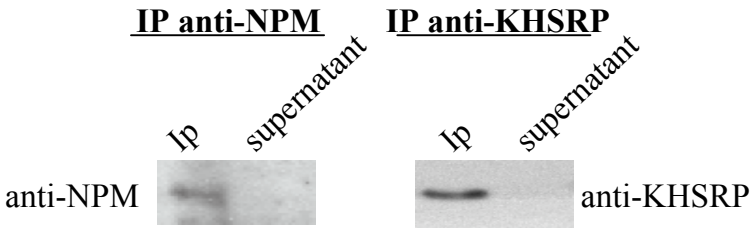
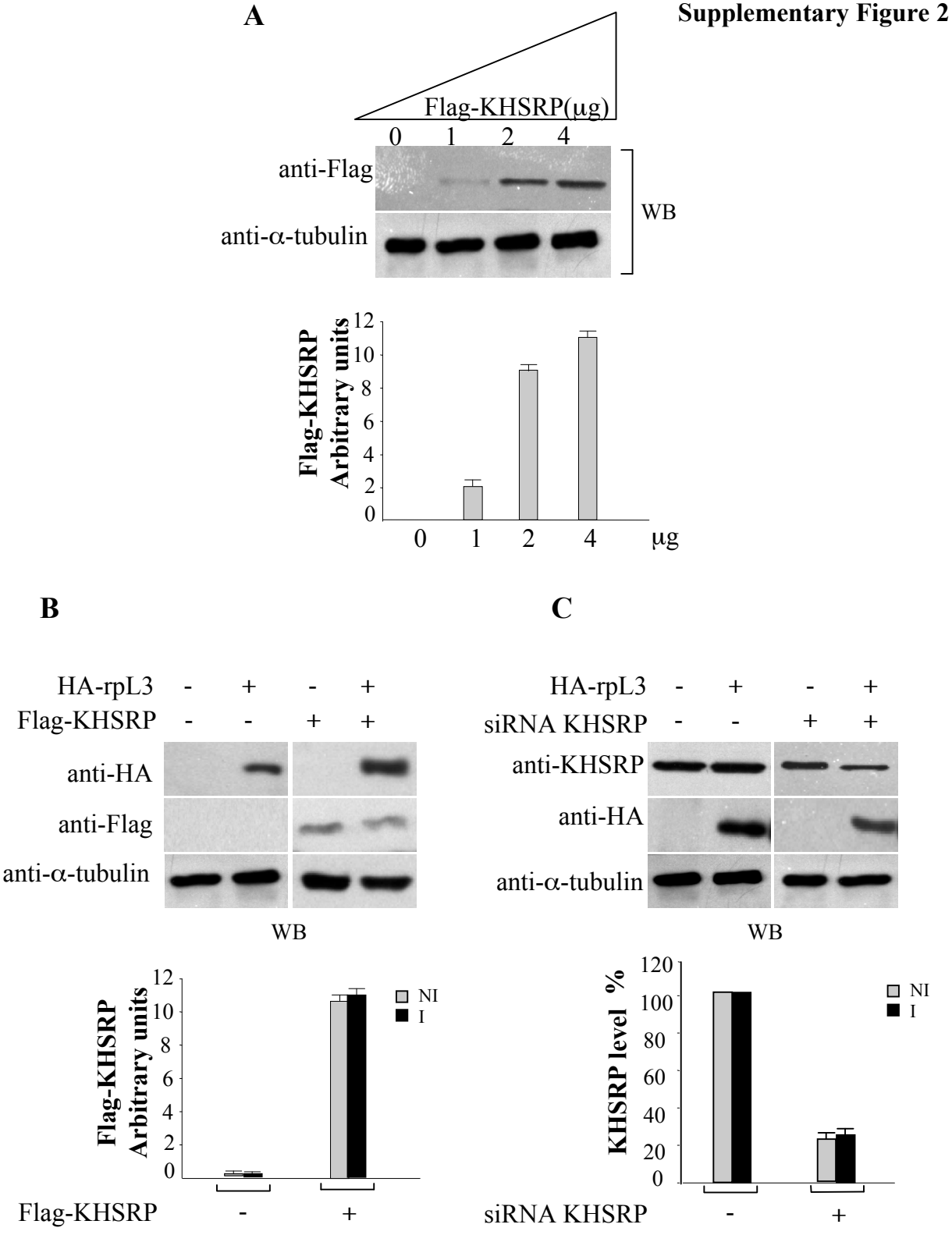
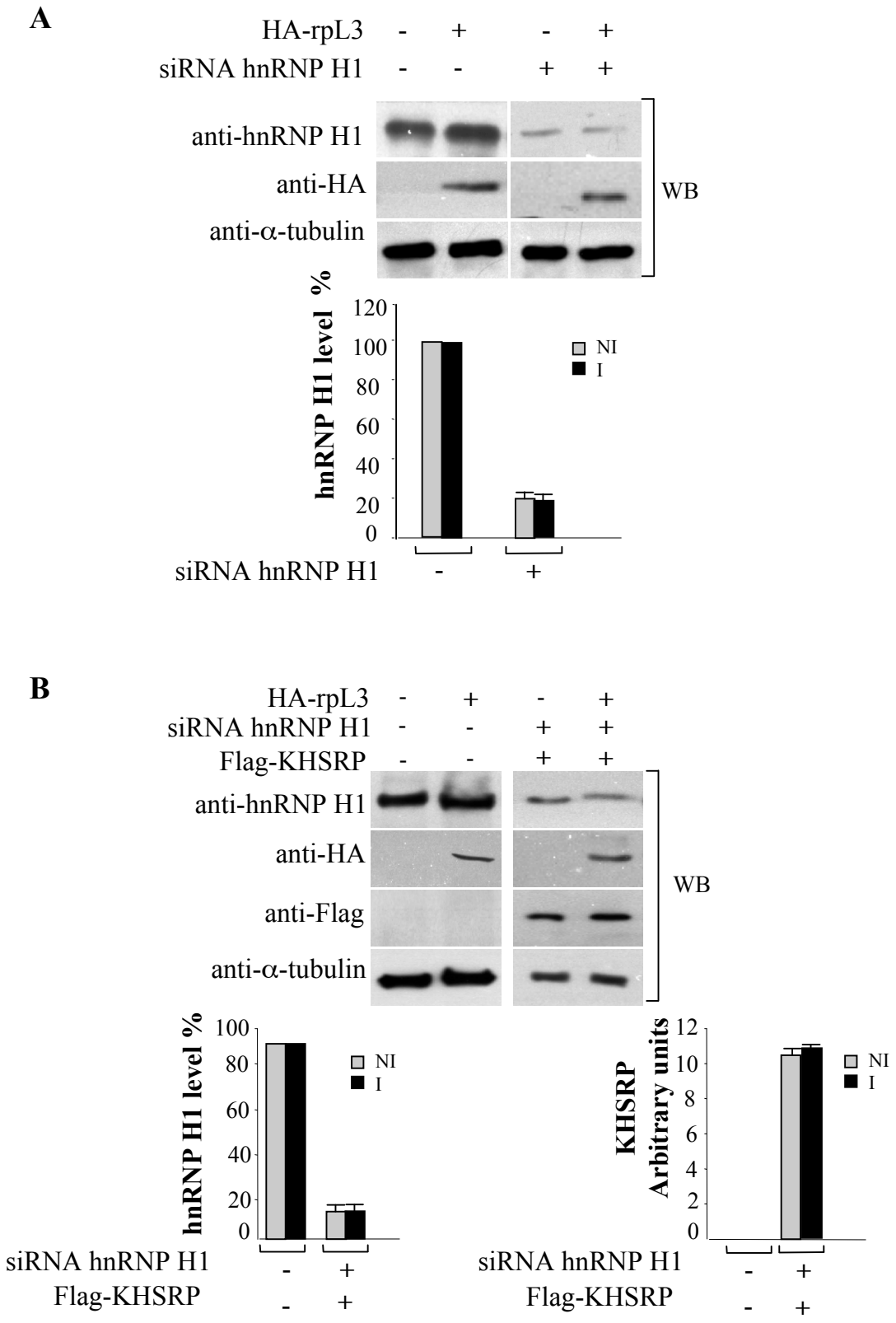
Supplementary Figure 1



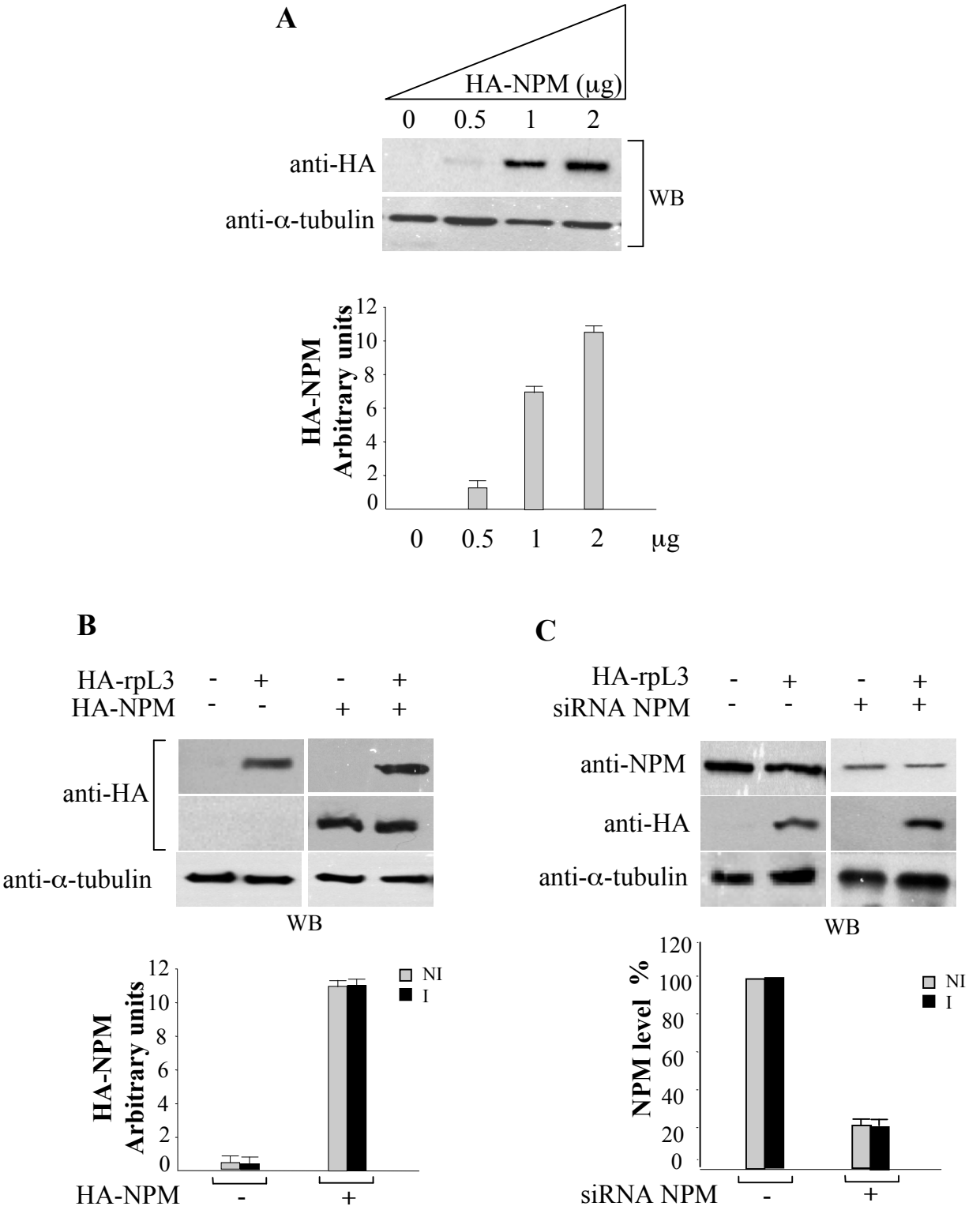
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



SUPPLEMENTARY FIGURE LEGENDS

Figure S1. NPM or KHSRP were specifically immunoprecipitated from HeLa cells extracts with antibodies against the endogenous NPM and KHSRP. Immunoprecipitates and supernatants were separated by SDS-PAGE and immunoblotted for NPM and KHSRP.

Figure S2. (A) Effects of KHSRP overexpression on rpL3-a mRNA levels in HeLa cells. Protein samples from CHX-treated HeLa cells untransfected or transfected with increasing amount of Flag-KHSRP were analyzed by western blotting (WB) with an antibody directed against the Flag tag. (B) Effects of KHSRP overexpression on rpL3-a mRNA levels in L3-8 cells. Protein samples from CHX-treated L3-8 cells untransfected or transfected with Flag-KHSRP, non induced (NI) or induced (I) for HA-rpL3 expression were analyzed by western blotting (WB) with antibodies directed against the HA and the Flag tags. (C) Effects of RNAi-mediated depletion of KHSRP on rpL3-a mRNA levels in L3-8 cells. Protein samples from CHX-treated L3-8 cells untransfected or transfected with KHSRP-siRNA, non induced (NI) or induced (I) for HA-rpL3 expression were analyzed by western blotting (WB) with antibody specific for endogenous KHSRP and HA tag.
The levels of proteins were quantified by PhosphorImager (Bio-Rad) and normalized to tubulin levels.

Figure S3. (A) Effects of RNAi-mediated depletion of hnRNP H1 on rpL3-a mRNA levels in L3-8 cells. Protein samples from CHX-treated L3-8 cells, untransfected or transfected with hnRNP H1-siRNA, non induced (NI) or induced (I) for HA-rpL3 expression were analyzed by western blotting (WB) with antibody specific for the endogenous hnRNP H1 and HA tag. (B) Effects of RNAi-mediated depletion of hnRNP H1 and KHSRP overexpression on rpL3-a mRNA levels in L3-8 cells. Protein samples from CHX-treated L3-8 cells untransfected or cotransfected with hnRNP H1-siRNA and Flag-KHSRP, non induced (NI) or induced (I) for HA-rpL3 expression were analyzed by western blotting (WB) with antibody specific for endogenous hnRNP H1 and with antibody directed against the HA and Flag tags.
The levels of proteins were quantified by PhosphorImager (Bio-Rad) and normalized to tubulin levels.

Figure S4. (A) Effects of NPM overexpression on rpL3-a mRNA levels in HeLa cells. Protein samples from CHX-treated HeLa cells untransfected or transfected with increasing amount of HA-NPM were analyzed by western blotting (WB) with an antibody directed against the HA tag. (B) Effects of overexpression of NPM on rpL3-a mRNA levels in L3-8 cells. Protein samples from CHX-treated L3-8 cells untransfected or transfected with HA-NPM, non induced (NI) or induced (I) for HA-rpL3 expression were analyzed by western blotting (WB) with antibody directed against the HA tag. (C) Effects of silencing of NPM on rpL3-a mRNA levels in L3-8 cells. Protein samples from CHX-treated L3-8 cells untransfected or transfected with siRNA against NPM, non induced (NI) or induced (I) for HA-rpL3 expression were analyzed by western blotting (WB) with antibody directed against the HA tag and NPM.
The levels of proteins were quantified by PhosphorImager (Bio-Rad) and normalized to tubulin levels.

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Human rpL3 induces G_1/S arrest or apoptosis by modulating $p21^{waf1/cip1}$ levels in a p53-independent manner

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Keywords: apoptosis, cell cycle, extraribosomal function, p21, ribosomal protein, rpL3, SP1

It is now largely accepted that ribosomal proteins may be implicated in a variety of biological functions besides that of components of the translation machinery. Many evidences show that a subset of ribosomal proteins are involved in the regulation of the cell cycle and apoptosis through modulation of p53 activity. In addition, p53-independent mechanisms of cell cycle arrest in response to alterations of ribosomal proteins availability have been described. Here, we identify human rpL3 as a new regulator of cell cycle and apoptosis through positive regulation of p21 expression in a p53-independent system. We demonstrate that the rpL3-mediated p21 upregulation requires the specific interaction between rpL3 and Sp1. Furthermore, in our experimental system, p21 overexpression leads to a dual outcome, activating the G_1/S arrest of the cell cycle or the apoptotic pathway through mitochondria, depending on its intracellular levels. It is noteworthy that depletion of p21 abrogates both effects. Taken together, our findings unravel a novel extraribosomal function of rpL3 and reinforce the proapoptotic role of p21 in addition to its widely reported ability as an inhibitor of cell proliferation.

Introduction

p21 (also known as $p21^{waf1/cip1}$), a main inhibitor of cyclin-dependent kinases (CDKs), is a negative regulator of cell cycle progression; various regulatory mechanisms contribute to finely coordinate multiple biological functions of p21 and determine its intracellular levels. They include transcriptional regulation, epigenetic silencing, mRNA translation and stability and ubiquitin-dependent as well as ubiquitin-independent degradation of the protein.[1,2]

In addition to growth-inhibiting activities, p21 can induce or repress p53-dependent or p53-independent apoptosis depending on the cellular system or the nature of the stress.[3-5] In fact, p21 gene overexpression in human laryngeal squamous carcinoma cells lacking p53 protein expression enhances apoptosis in cisplatin-treated cells, whereas it attenuates apoptotic signals in methotrexate-treated cells.[6]

A variety of transcription factors can modulate p21 in addition to p53.[7] The p21 promoter consists of proximal and distal regulatory elements. The majority of p21 gene regulatory proteins interact with the proximal region of p21 promoter that includes six GC-rich binding sites recognized by Sp1, Sp3 and Kruppel-like transcription factors.[8,9] Numerous evidences support the essential role of Sp1 in the upregulation of p21 gene by several protein factors such as c-Myc[10] or drugs, such as Mithramycin A, Sulforaphane and 5-FU.[11,12]

In eukaryotes, many data indicate that r-proteins exert a variety of extra-ribosomal functions. Alteration in the expression of r-proteins and protein factors involved in the ribosome biogenesis correlate with tumorigenesis, since many of these proteins are overexpressed in solid tumors and leukemia cells.[13,14] On the other hand, some data suggest that several r-proteins might have a role as tumor suppressors, as described in zebrafish.[15] Recently, an increasing number of r-proteins have been proposed as additional important components of the tumor suppressor p53's autoregulatory feedback loop, activating p53 and triggering cell cycle arrest and apoptosis.[16] In fact, either drug treatment or enforced expression of rpL11, rpL5, rpL23 causes p53-dependent cell-cycle arrest through HDM2 (human double minute 2) interaction.[17-19] In addition to r-proteins, several nucleolar proteins such as NPM (Nucleophosmin, also known as B23, numatrin or NO38) and Nucleostemin are able to modulate p53 activity.[20,21] Furthermore, emerging evidences indicate a functional correlation between NPM and some r-proteins, independent from ribosome biogenesis. It has been shown that rpL23 regulates NPM and Miz1-dependent p21 gene promoter transactivation by sequestering NPM into the nucleolus.[22] We have studied in recent years post-transcriptional regulatory strategies of mammalian r-proteins,[23] and we demonstrated that human r-protein L3 (rpL3) autoregulates, in a complex with the transacting factors hnRNP H1, NPM and KHSRP, its own expression through the association of alternative splicing and nonsense-mediated mRNA

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Table 1. Primers used in PCR reactions

Primers	Forward (5'-3')	Reverse (5'-3')
NPM cDNA	ATG GAA GAT TCG ATG GAC	TTA AAG AGA CTT CCT CCA
Sp1 cDNA	ATG GAT GAA ATG ACA GCT	TCA GAA GCC ATT GCC ACT
p21 cDNA	ATG TCA GAA CCG GCT GGG	TTA GGG CTT CCT CTT GGA
β-actin	GGC ACC ACC TTC TAC A	CAG GAG GAC AAT GAT
p21 promoter -194/+88	ACC GGC TGG CCT GCT GGA ACT	TCT GCC GCC GCT CTC TCA CCT
p21 3'-UTR +3455/+3771	ATG TTA GGC AAG TTA CTT AAC TTA	CTC TTG GTA ACT TCA CAC CAA GTT

decay (AS-NMD).[24,25] In this paper, we demonstrate that rpL3 is a positive regulator of p21 expression. We show that rpL3 is able to modulate the protein amounts of p21 through a novel pathway independent from p53 involving Sp1 and NPM. Furthermore, we observed that rpL3-mediated p21 upregulation results in cell cycle arrest or apoptosis depending on the intracellular levels of p21 protein.

Results

rpL3 upregulates p21 expression at transcriptional levels independently from p53. In order to investigate whether rpL3 could control p21 expression in a cell environment devoid of p53, we analyzed p21 mRNA and protein levels by western blotting and semiquantitative RT-PCR upon rpL3 overexpression in Calu-6 cells lacking p53 protein expression.[23] The enforced expression of the HA-rpL3 protein resulted in a dose-dependent increase of p21 protein and mRNA (**Fig. 1A and B**).

To investigate the possibility that p21 upregulation is due to the ability of rpL3 to control the activity of p21 promoter, we performed a reporter luciferase assay in conditions of rpL3 overexpression.

For the specificity of the assay, a plasmid expressing rpL7a-His (pL7a-His), an arbitrary ribosomal protein, was used as control.[26] As shown in **Figure 1C**, the enforced expression of rpL3, in contrast to rpL7a, is associated to the upregulation of p21 gene promoter transactivation in a dose-dependent manner.

In addition, we performed analogous experiments by using pCMV, a plasmid containing the CMV promoter fused to luciferase DNA, as a further control (**Fig. 1D**). The results showed that rpL3, as rpL7a, failed to enhance reporter gene expression driven by CMV promoter.

rpL3 binds the p21 gene promoter. We investigated whether rpL3 was able to interact with p21 gene promoter. To this aim, Calu-6 cells were collected and subjected to ChIP by using anti-rpL3 and anti-IgG as specificity control.

The presence of rpL3 was investigated by western blotting in the DNA-immunoprecipitated complexes (**Fig. 2**). Quantitative PCR assay on the samples was performed by using primers specific for proximal region of p21 promoter (-194–+88),[27] which is the region interacting with the majority of p21 regulatory proteins, and primers for control loci.

The presence of a specific signal for p21 promoter in the DNA-rpL3 immunocomplex, absent in the control challenged with anti-IgG antibodies, indicated that rpL3 is able to bind p21 promoter (**Fig. 2**).

Next, in order to evaluate whether rpL3 was able to interact directly with p21 proximal promoter region, an EMSA experiment was performed using purified recombinant GST-tagged rpL3 and GST as control. No mobility shift was observed when recombinant GST-rpL3 was challenged with the probe, suggesting that the binding of rpL3 to the p21 gene promoter is indirect, and it may require additional protein factors (data not shown).

NPM modulates rpL3-dependent p21 gene promoter transactivation. The absence of a direct binding between rpL3 and p21 promoter prompted us to search for rpL3-interacting regulatory proteins involved in the rpL3-mediated regulation of p21 transcription. We have previously reported a direct protein-protein interaction between rpL3 and NPM and demonstrated that this interaction is functional to the regulation of the rpL3 gene alternative splicing.[24]

Here, we explored the possibility that the rpL3-NPM complex was involved in the transactivation of the p21 gene transcription.

To this purpose, we performed analogous ChIp experiments by using anti-rpL3, anti-NPM and anti-IgG as control. As shown in **Figure 3A**, the presence of NPM and rpL3 proteins (WB), and a signal for p21 gene promoter (qPCR) in the reciprocally DNA-immunoprecipitated complexes, absent in the control obtained with anti-IgG antibodies, were consistent with the presence of rpL3 and NPM on the p21 gene promoter.

To investigate whether the interaction between rpL3 and NPM on p21 promoter affects its activity, a reporter luciferase assay was performed by altering intracellular levels of NPM in conditions of HA-rpL3 overexpression. The results demonstrated that either in rpL3 physiological conditions or rpL3 overexpression, the enforced expression of NPM did not affect the activity of p21 gene promoter (**Fig. 3B**). Whereas, in physiological conditions of rpL3 expression, the NPM silencing was associated with the inhibition of p21 transcriptional activity as indicated by a significant decrease (about 40%) in p21 gene promoter activity (**Fig. 3C**). Of interest, in rpL3 overexpression conditions, NPM silencing resulted in a decrease of rpL3-dependent transactivation of p21 gene promoter. These data suggest a positive role of NPM in rpL3-mediated activity on p21 gene promoter.

rpL3-dependent p21 gene promoter transactivation requires Sp1. In the attempt to identify other proteins involved in the rpL3-mediated activation of p21 transcription, we focused our study on the possible role of Sp1, a protein factor that regulates p21 transcription.[8] We first determined whether Sp1 was present in the rpL3-p21 gene promoter immunocomplex. To this purpose, a ChIp experiment in condition of mythramycin A (MTM)

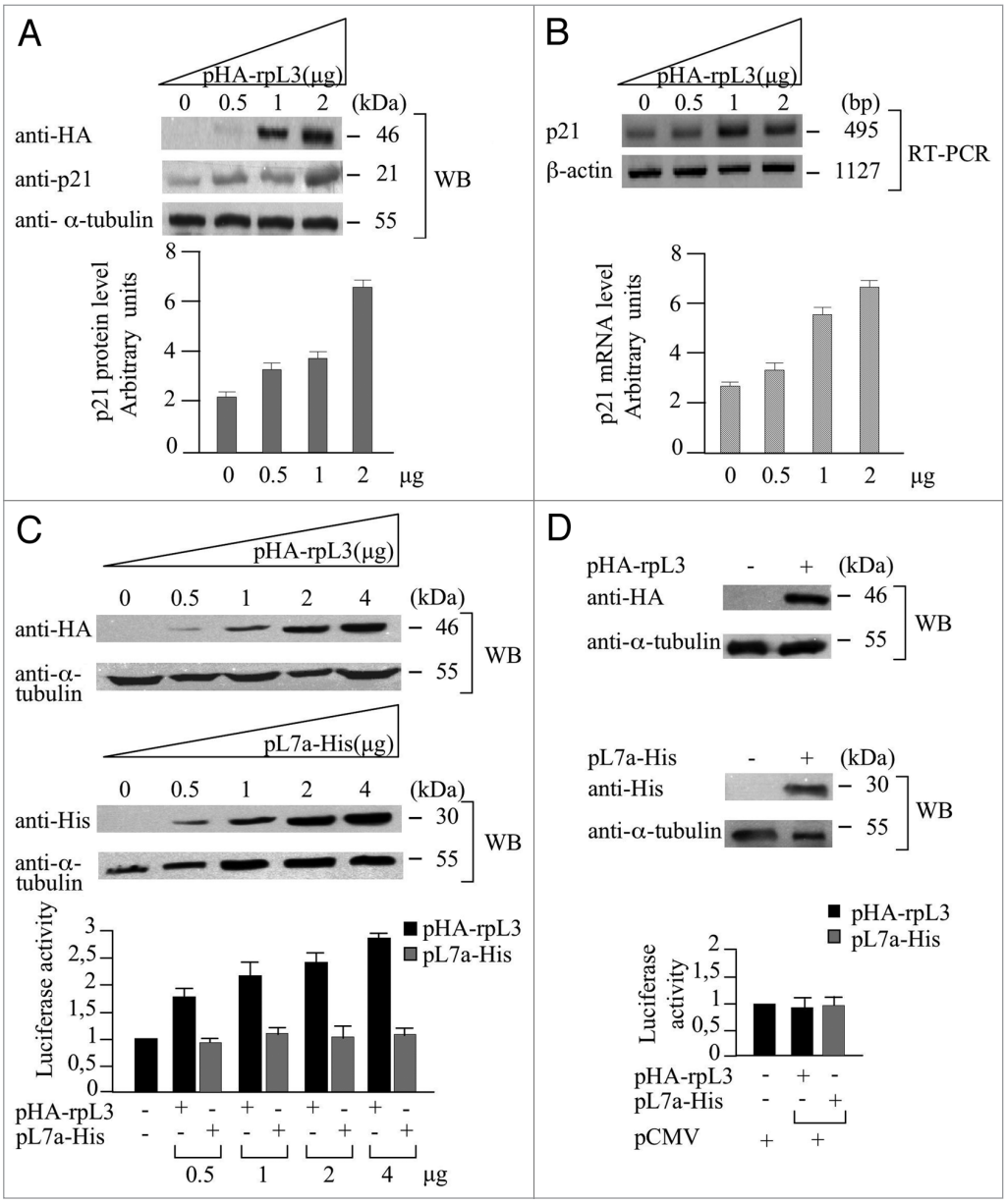

Figure 1. Role of rpL3 in the regulation of p21 expression. (**A**) Protein samples from Calu-6 cells untransfected or transiently transfected with increasing amounts (0, 0.5, 1, 2 μg) of a plasmid expressing rpL3 fused to HA tag (pHA-rpL3) were analyzed by WB assay, with antibodies directed against the HA tag and p21, 24 h after transfection. Loading in the gel lanes was controlled by detection of α-tubulin protein. (**B**) Representative RT-PCR analysis of total RNA from the same samples. β-actin was used as a control of RNA loading. Quantification of p21 protein and mRNA levels by PhosphorImager (Bio-Rad) is shown. (**C**) Protein extracts from Calu-6 cells transiently transfected with the full-length p21 promoter luciferase reporter plasmid (pWWP) alone and cotransfected with pWWP and increasing amounts of pHA-rpL3 or pL7a-His (0, 0.5, 1, 2 and 4 μg) were analyzed by WB assay with antibodies directed against the HA and His tags, 24 h after transfection. Loading in the gel lanes was controlled by detection of α-tubulin protein. Analysis of the relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, in the same samples. (**D**) Protein extracts from Calu-6 cells transiently transfected with CMV-Luc plasmid alone, or cotransfected with pCMV-Luc and 2 μg of pHA-rpL3 or pL7a-His, were analyzed by WB assay with antibodies directed against the HA and His tags. Loading in the gel lanes was controlled by detection of α-tubulin protein. The relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, in the same samples was detected. Results illustrated in **Figures 1–9** are representative of three independently performed experiments.

treatment was performed. MTM is a specific Sp1 inhibitor that is able to displace Sp1 from its binding sites in numerous genes.[11] The **Figure 4** shows the presence of Sp1 and rpL3 proteins (WB), and a signal for p21 gene promoter (qPCR), in the reciprocally DNA-immunoprecipitated complexes, not detected in the control obtained with anti-IgG antibodies, in absence of MTM,

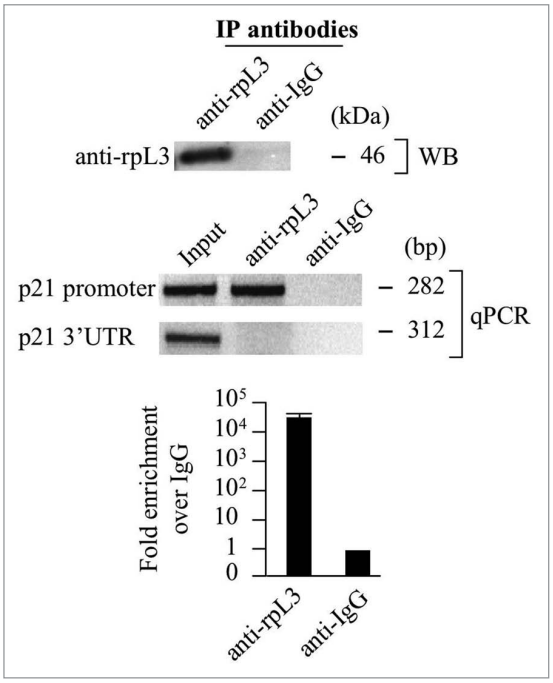

Figure 2. Analysis of the interaction between rpL3 and p21 gene promoter. Protein samples from DNA-rpL3 or DNA-IgG immunocomplexes were analyzed through WB assay with antibody against rpL3. Note the absence of signal in DNA-IgG immunocomplex. The same DNA-immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or control loci.

indicating that a specific association between Sp1 and rpL3 occurs on the p21 gene promoter.

Of interest, the presence of MTM prevents the binding of both rpL3 and Sp1 on the p21 gene promoter, indicating that rpL3 is able to bind p21 gene promoter only in the presence of Sp1.

In order to study the function of Sp1 in rpL3-dependent transactivation of p21 gene promoter, a reporter luciferase assay was performed in conditions of rpL3 and Sp1 overexpression, with or without MTM. **Figure 5A** shows that in absence of MTM, rpL3 or Sp1 overexpression increased the activity of p21 gene promoter, as previously observed (see sub-section "rpL3 upregulates p21 expression at transcriptional level independently from p53" in this paper and ref. 28). In addition, rpL3 and Sp1 had a synergistic effect on the transactivation of p21 gene promoter. Interestingly, the ability of both rpL3 and Sp1 to activate the transcription of luciferase was strongly reduced in the presence of MTM. These data suggest that the ability of rpL3 to activate p21 transcription requires the presence of Sp1 on the promoter.

Sp1-binding boxes 3 and 4 are involved in rpL3-dependent transactivation of p21 gene promoter. The proximal region of the human p21 promoter carries six GC-rich Sp1-binding sites (numbered 1–6 in plasmid pWP124, **Fig. 5B**). To determine which of Sp1-binding elements mediate rpL3 responsiveness, Calu-6 cells were transiently transfected with pW124 or mutant plasmids in which two or four Sp1-binding sites were simultaneously deleted (pWP101 and pWPdel-SmaI)[29] in presence or absence of pHA-rpL3 (**Fig. 5B**). As shown in **Figure 5B**, upon the overexpression of rpL3, deletion of Sp1-binding sites 1 and 2 (pWP101) led to an increase in the luciferase activity comparable to that obtained when all the six Sp1 binding motifs were present (pWP124), whereas additional deletion of Sp1-binding sites 3 and 4 (pWPdel-SmaI) abrogated completely rpL3's effect on p21 gene promoter transactivation. These data suggest that the region spanning from -60–-101 upstream from the transcription start site, including Sp1-binding sites 3 and 4, corresponds to rpL3-responsive element, and it is involved in rpL3-mediated activation of p21 gene promoter.

rpL3 overexpression induces cell cycle arrest or apoptosis in Calu-6 cells. In order to investigate the effect of rpL3-mediated upregulation of p21 transcription on cell proliferation, we performed 5-Bromodeoxyuridine (BrdU) incorporation to monitor DNA synthesis during cell division and propidium iodide (PI) staining to analyze DNA content of Calu-6 cells transiently transfected with 1 μg or 2 μg of pHA-rpL3. As expected, rpL3 overexpression induced an increase of p21 expression in a dose-dependent manner. **Figure 6** shows that BrdU incorporation in cells transfected with 1 μg of pHA-rpL3 was significantly decreased vs. that observed in control (about 0.7% vs. 25%, respectively). This indicated that the enforced expression of rpL3 produced a strong reduction in the percentage of cells in S-phase of the cell cycle. Analysis of cell cycle using the counterstaining with PI indicated that the observed decrease of cells in S-phase was associated with an increase of cell population in the G_1-phase of the cell cycle (about 80% vs. control 50%). Surprisingly, in the presence of higher rpL3 levels (2 μg of pHA-rpL3), we observed only a mild effect on the cell cycle, but rather the presence of a significant sub-G_1 population, indicative of apoptosis (about 21% vs. control 2%).

Intracellular levels of rpL3 affect the choice between G_1/S arrest or mitochondrial apoptosis in Calu-6 cells. To gain further insight into the mechanism by which rpL3 causes cell cycle arrest and apoptosis, we proceeded to perform a time-course analysis of the nuclei DNA content of Calu-6 cells transiently transfected with 1 μg or 2 μg of pHA-rpL3 by flow cytometry (**Fig. 7**).

In cells transfected with 2 μg of pHA-rpL3, an increase in sub-G_1 cell population vs. control was observed at all time points, although the strongest effect was obtained after 48 h. At this time point, the percentage of cells transfected with 2 μg of pHA-rpL3 in the sub-G_1 phase of the cell cycle increased approximately 7-fold as compared with control (about 36% vs. 5%, respectively). This effect was not observed with 1 μg of pHA-rpL3. Transfection of cells with this amount of pHA-rpL3 did not induce apoptosis even 72 h after transfection. Furthermore, we observed a G_1/S arrest at 24 and 48 h (as already shown), and more interestingly, at 72 h, the dividing ability of Calu-6 cells transfected with 1 μg pHA-rpL3 was restored, as evidenced by the presence of a G_2/M peak (**Fig. 7**).

These results suggest that rpL3 may induce G_1/S arrest or apoptosis depending on its concentration inside the cells.

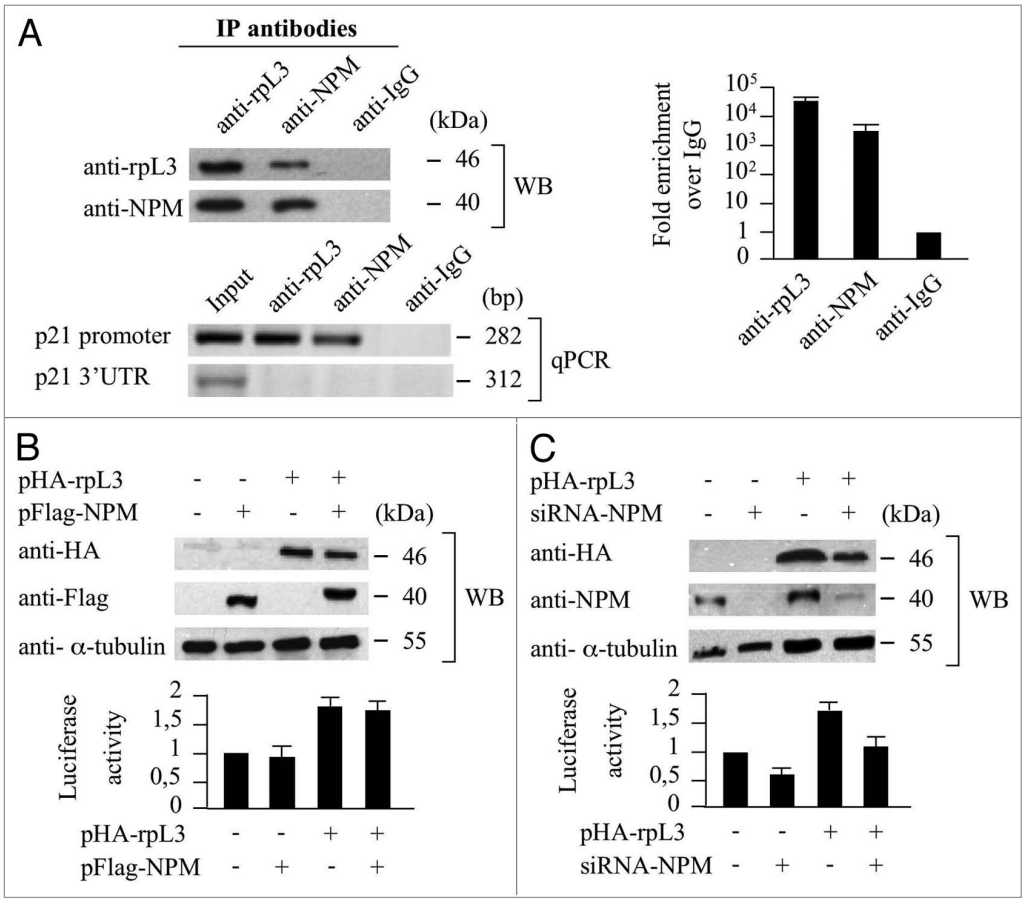

Figure 3. Role of NPM in the rpL3-mediated p21 gene transcriptional transactivation. (**A**) Analysis of the interaction between NPM and rpL3 on p21 gene promoter. Protein samples from DNA-rpL3, DNA-NPM or DNA-IgG immunocomplexes were analyzed through WB assay with antibodies against the indicated proteins. Note the absence of signal in IgG immunocomplex. The same DNA-immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or control loci. (**B**) Effect of NPM overexpression on rpL3-mediated p21 gene promoter activity. Protein extracts from Calu-6 cells transiently transfected with pWWP alone or cotrasfected with pWWP, and the plasmids expressing the recombinant Flag-NPM (pFlag-NPM), with or without pHA-rpL3, were analyzed by WB assay with antibodies directed against the HA and Flag tags. Loading in the gel lanes was controlled by detection of α-tubulin protein. The relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, in the same samples was detected. (**C**) Effects of RNAi-mediated depletion of NPM on rpL3-mediated p21 gene promoter activity. Protein extracts from Calu-6 cells transiently transfected with pWWP alone or cotrasfected with pWWP and siRNA directed against endogenous NPM mRNA (siRNA-NPM) in presence or absence of pHA-rpL3 were analyzed by WB assay with antibodies directed against the HA tag and NPM. Loading in the gel lanes was controlled by detection of α-tubulin protein. The relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, in the same samples was detected.

To further study the apoptotic effect of rpL3, we analyzed PARP protein cleavage by western blotting analysis and reduction of mitochondrial inner membrane potential ($\Delta\Psi m$) by tetramethyl-rhodamine ethyl ester (TMRE) staining, hallmarks of mitochondrial apoptosis,[30,31] in Calu-6 cells transfected with 2 μg of pHA-rpL3. As expected, in these cells, western blot for PARP revealed an increased amount of cleaved PARP (**Fig. 8A**). Furthermore, **Figure 8B** shows that the percentage of Calu-6 cells with a physiological $\Delta\Psi m$ decreased from 88% to 58% (control cells vs. 2 μg of pHA-rpL3 transfected cells). Overall, these results indicate that higher levels of rpL3 protein obtained with 2 μg of pHA-rpL3 activate apoptosis through mitochondrial pathway in Calu-6 cells.

Cell cycle arrest and apoptosis upon rpL3 overexpression are p21-dependent. To verify that rpL3-induced cell cycle arrest or apoptosis in Calu-6 cells occurs through a specific pathway involving p21, we examined the effects of rpL3 overexpression on cell proliferation and cell death after p21 silencing.

We performed analysis of the nuclei DNA content of Calu-6 cells and p21-depleted Calu-6 (p21ΔCalu-6) cells transiently transfected with 1 μg or 2 μg of pHA-rpL3 by flow cytometry. **Figure 9A** (lower panels) shows that rpL3 was unable to induce cell cycle arrest in condition of p21 depletion. In addition, as shown in **Figure 9B** (lower panels), the higher dosage of rpL3 failed to mediate the induction of apoptosis in p21-depleted cells, thus demonstrating that the dual effect of rpL3 on cell cycle arrest or apoptosis is dependent from p21. All these results indicate that

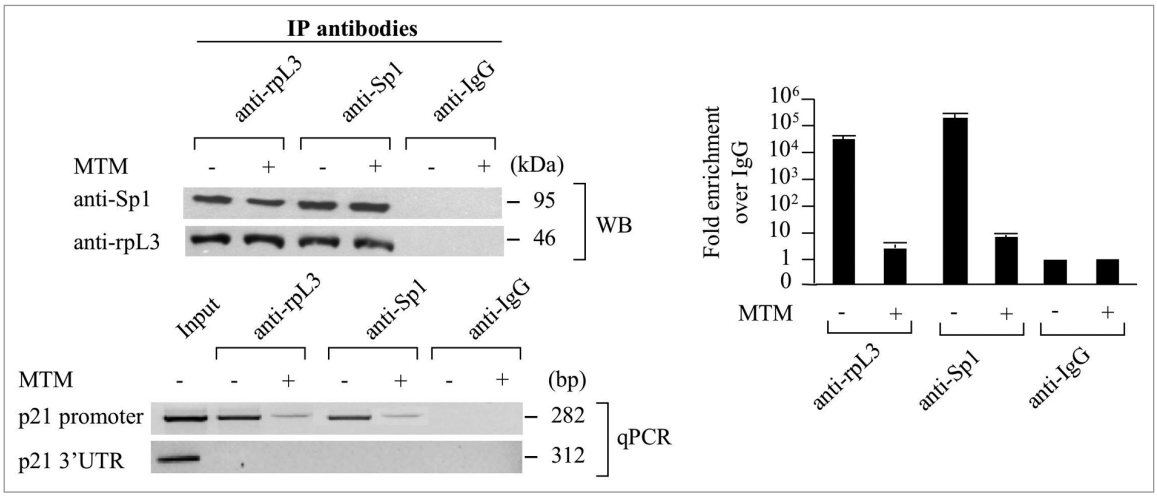

Figure 4. Analysis of the interaction between rpL3 and Sp1 on p21 gene promoter. Protein samples from DNA-rpL3, DNA-Sp1 or DNA-IgG immunocomplexes from Calu-6 cells untreated or treated with 200 nM of mythramycin A (MTM) for 16 h were analyzed through WB assay with antibodies against rpL3 or Sp1. Note the absence of signal in DNA-IgG immunocomplex. The same DNA immunoprecipitates were subjected to quantitative PCR (qPCR) with primers specific for the proximal region of p21 gene promoter or control loci.

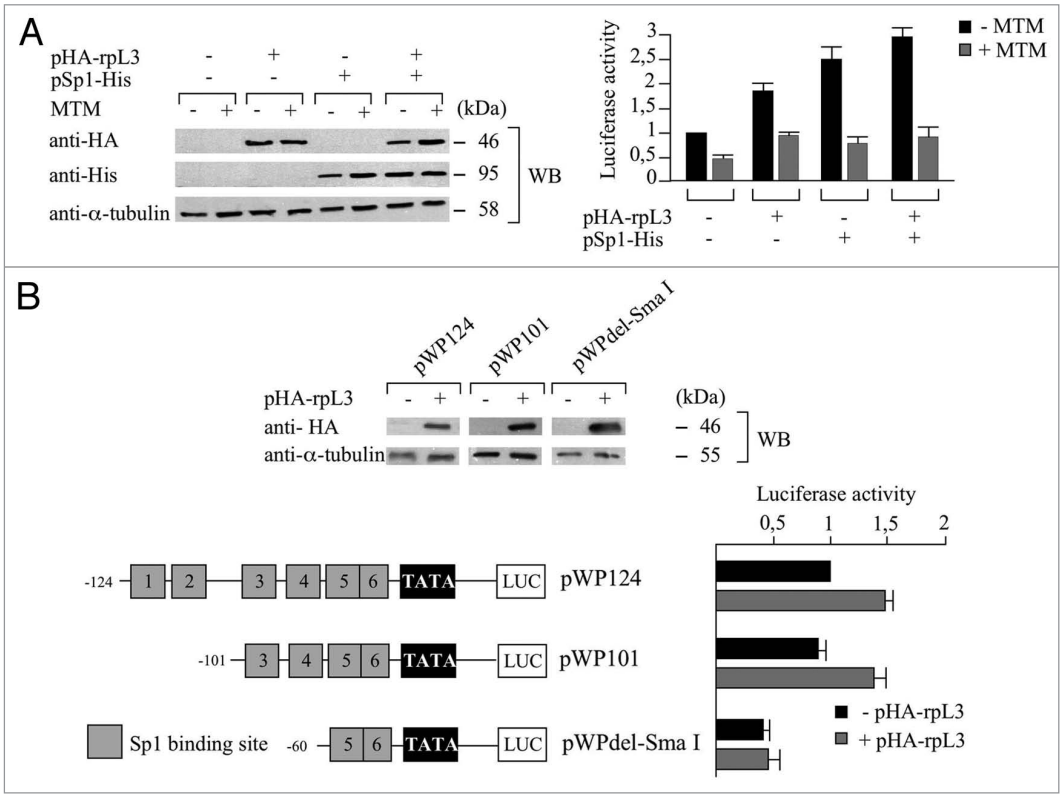

Figure 5. Role of Sp1 in the rpL3-mediated p21 gene transcriptional transactivation. (**A**) Protein extracts from Calu-6 cells untreated or treated with 200 nM of mythramycin A (MTM) and transiently transfected with pWWP alone, or in the presence of pHA-rpL3 or the plasmid expressing the recombinant Sp1-His (pSp1-His) or pHA-rpL3 and pSp1-His were analyzed by WB assay with antibodies directed against the HA and His tags, 24 h after transfection. Loading in the gel lanes was controlled by detection of α-tubulin protein. The relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, in the same samples was detected. (**B**) Analysis of p21 promoter regions involved in rpL3-mediated p21 gene transcriptional activation. On the left, schematic representation of the deletion constructs of the p21 gene promoter used in the luciferase transfection assay. Calu-6 cells were transiently transfected with the pWP124, pWP101 or pWPdel-Smal in presence or absence of pHA-rpL3. Proteins were analyzed by WB assay with antibody directed against the HA tag 24 h after transfection. Loading in the gel lanes was controlled by detection of α-tubulin protein. Analysis of the relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, in the same samples is shown.

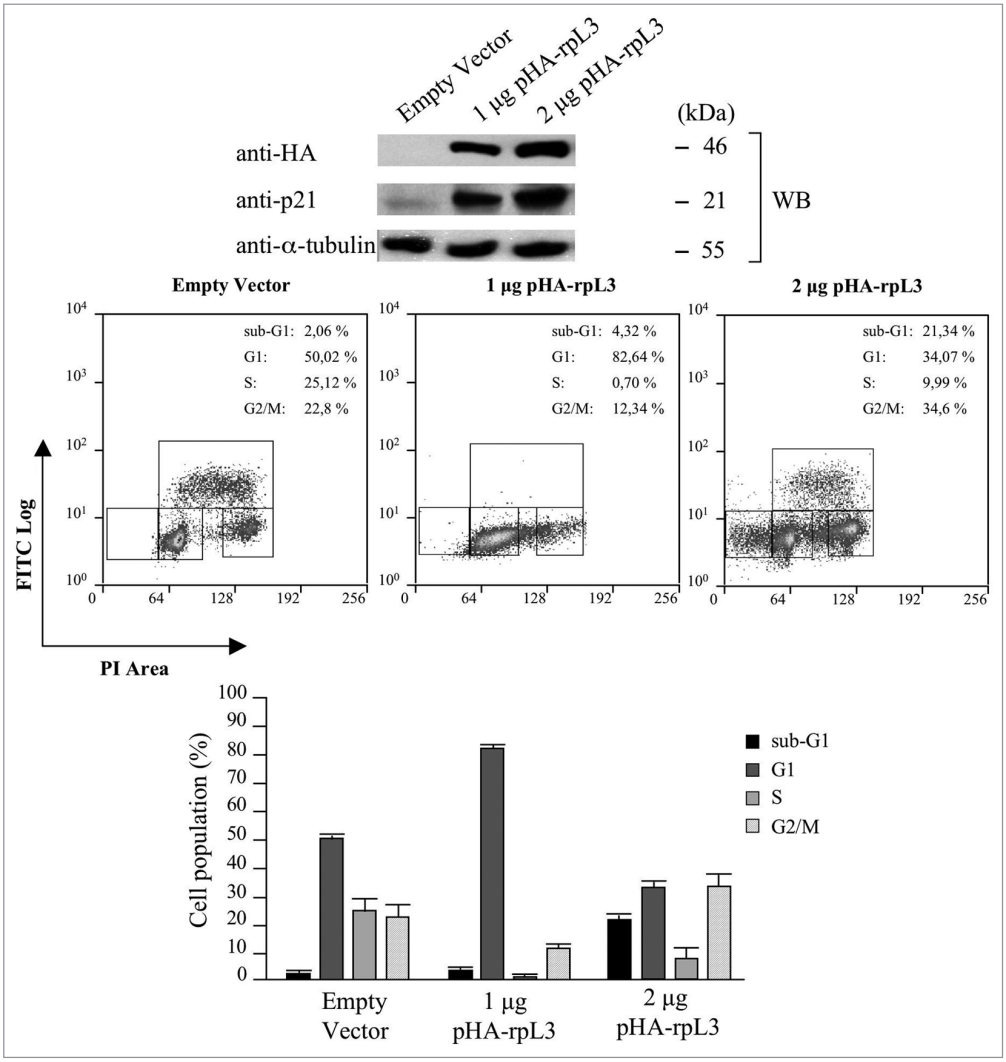

Figure 6. Effects of rpL3-mediated upregulation of p21 gene expression on the cell cycle. (**A**) Protein samples from Calu-6 cells transfected with 2 µg of pcDNAHAV3 (empty vector), 1 µg or 2 µg of pHA-rpL3 were analyzed by WB assay with antibodies directed against the HA tag and p21. Loading in the gel lanes was controlled by detection of α-tubulin protein. (**B**) The same samples were stained with FITC conjugated anti-5-bromodeoxyuridine antibody and counterstained with propidium iodide and analyzed for DNA synthesis and for DNA content by flow cytometry 48 h after transfection. On the bottom, the percentage of cells in different phases of cell cycle is shown.

rpL3 is able to induce cell cycle arrest or activate the intrinsic apoptotic pathway in Calu-6 cells by regulating the intracellular amounts of p21.

Discussion

In eukaryotes, ribosome biogenesis is a complex process that requires a number of coordinated events prior to nuclear export of the mature subunits to the cytoplasm.[32-34] Recent studies have shed new light on the mechanisms underlying the regulation of these events and revealed new connections between ribosome biogenesis and cell cycle.[35] The signaling pathways able to connect these two processes have long been relatively unknown; however, more recently, the role of r-proteins-Mdm2-p53 stress response pathway in the coregulation of these events has been widely demonstrated.[36,37] Alterations in processes such as rRNAs synthesis, rRNAs modification as well as r-proteins imbalance produce alterations in ribosome biogenesis and result in nucleolar stress. In response to this event, several r-proteins translocate to the nucleoplasm and bind to Mdm2, thus promoting p53 stabilization and subsequent p53-mediated cell cycle arrest or apoptosis.[38] Several p53-independent pathways that require nucleolar proteins, such as ARF, NPM or free r-proteins, have also been well-studied.[39,40]

Data reported in this paper allow us to define a new extraribosomal function for human rpL3 in addition to its role in the regulation of the alternative splicing of its own gene.[23] Specifically, we demonstrate that rpL3 is able to induce cell cycle arrest or

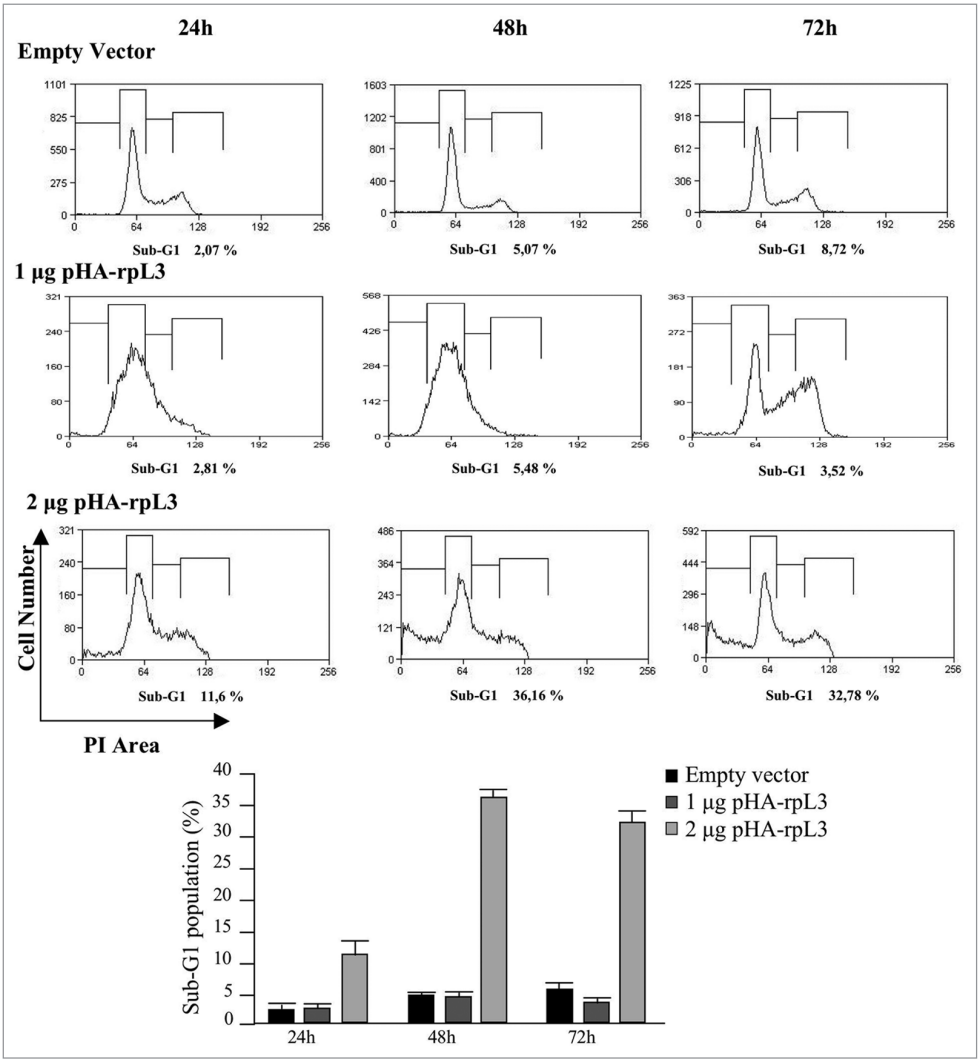

Figure 7. Effects of rpL3-mediated upregulation of p21 gene expression on the cell viability. Calu-6 cells transfected with 2 µg of pcDNAHAV3 (empty vector) or 1 µg and 2 µg of pHA-rpL3 were analyzed for DNA content by propidium iodide staining 24 h, 48 h and 72 h after transfection. On the bottom, a quantification of percentage of cells in sub-G_1 phase is shown.

apoptosis in Calu-6 cells by positively modulating the activity of p21 promoter in a p53-independent pathway. To our knowledge, the present study demonstrates for the first time a direct role of an r-protein, specifically rpL3, in the regulation of p21 transcription.

In an attempt to understand the mechanism of the rpL3-mediated upregulation of p21 expression, we focused our studies on the identification of protein partners of rpL3 protein involved in this activity. It is known that NPM is able to indirectly bind to p21 proximal promoter and activate its transcription.[22] Furthermore, our previous results demonstrated that NPM is able to interact directly with rpL3, and that this interaction is functional to the regulation of the rpL3 protein levels inside the cells.[24] Data reported in this paper clearly demonstrate that NPM represents a positive regulator of rpL3-mediated transactivation of p21 gene, although it is not a crucial player in this event (**Fig. 3**).

It is well-known that p21 proximal promoter contains six Sp1 binding sites, and that Sp1 is able to transcriptionally regulate the activity of different genes including those involved in the regulation of cell cycle.[8] Specifically, Sp factors have been proposed to be essential for transactivation of the p21 promoter by members of the p53 family proteins.[41] Here, we demonstrate that rpL3 associates in vivo with Sp1 on the p21 proximal promoter only in absence of MTM. Although treatment with MTM did not prevent the formation of a complex between rpL3 and Sp1 into the cells, it hindered its recruitment on p21 promoter (**Fig. 4**). Data from rpL3 and Sp1 overexpression in Calu-6 cells in absence of MTM indicated that expression of Sp1, in combination with rpL3, strongly increased rpL3-mediated p21 transactivation; these findings, together with the observation that rpL3, even in excess, was unable to transactivate p21 promoter in presence of MTM, strongly indicated that the binding of Sp1 to the proximal

p21 promoter region is essential for the rpL3-mediated transactivation of p21 promoter. Thus, we propose that Sp1 is a key component of rpL3-mediated p21 transactivation; in fact, Sp1 binding to GC boxes 3 and 4 is a prerequisite for rpL3 recruitment on p21 proximal promoter (**Fig. 5**).

In the light of these results, we propose a working model in which overexpression of rpL3, which may mimic situations of alteration in ribosome biogenesis, leads to a formation of a multiprotein complex containing at least rpL3, Sp1 and NPM on the proximal region of p21 promoter with consequent upregulation of p21 expression. This upregulation leads to a dual effect; arresting the cell cycle progression at G_1/S phase or promoting apoptosis in dependence of rpL3 and p21 protein levels into the cells (**Fig. 6**). To better understand the dynamics of these events, we performed a time-dependent analysis of the DNA content in Calu-6 cells transfected with different amounts of pHA-rpL3. Interestingly, we observed that 1 μg of pHA-rpL3 induced a reversible G_1/S arrest of the cell cycle. In fact, Calu-6 cells restored their capability to progress through the G_1/S transition at 72 h after transfection. Instead, transfection with 2 μg of pHA-rpL3 triggered mitochondrial apoptosis even at 24 h after transfection (**Figs. 7 and 8**). Moreover, we show that p21 is essential for rpL3-induced cell cycle arrest or mitochondrial apoptosis, since stable depletion of p21 in Calu-6 cells completely abolished both events (**Fig. 9**). Taken together, these data demonstrate that rpL3 plays an important role in the activation, through modulation of p21 levels, of two independent pathways; i.e., reversible arrest of cell cycle or mitochondrial apoptosis.

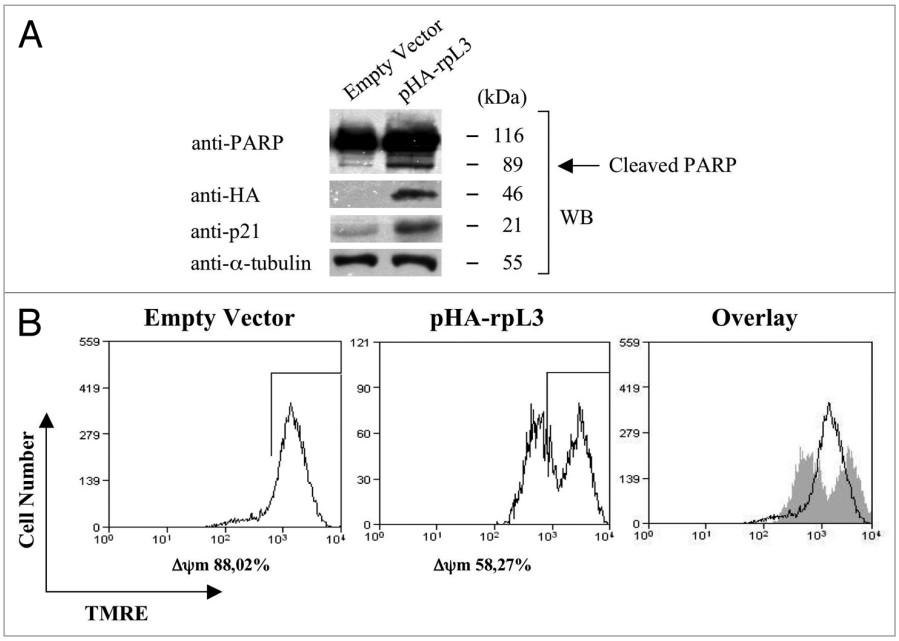

Figure 8. Characterization of rpL3-mediated apoptosis in Calu-6 cells. (**A**) Calu-6 cells transfected with 2 μg of pcDNAHAV3 (empty vector) or pHA-rpL3 were analyzed by WB assay with antibodies directed against PARP, the HA tag and p21, 48 h after transfection. Loading in the gel lanes was controlled by detection of α-tubulin protein. (**B**) The same samples were analyzed for mitochondrial membrane potential by tetramethyl-rhodamine ethyl ester (TMRE) staining, and fluorescence was measured by flow cytometry 48 h after transfection.

These data are in complete agreement with recent evidences demonstrating a much more complex scenario than had been expected regarding p21's role in the regulation of cell cycle. In particular, p21 has proved to exert also a proapoptotic function under certain conditions in specific systems beyond the well-known role in growth inhibition.[42] Notably, in most of these cases, the cellular systems used express a non-functional p53 isoform. The mechanism by which p21 may promote apoptosis is poorly understood, and different working models have been proposed. However, the common assumption is that the cellular activities of p21 are tightly regulated by multiple factors that seem to be specific for each function in different systems. For example, the interaction of p21 with PCNA is implicated in sodium butyrate-induced apoptosis,[43] other studies indicate that the modulation of proapoptotic or antiapoptotic genes is responsible for p21-induced apoptosis.[44,45]

Data reported in this paper indicate that rpL3 in a complex with Sp1 and NPM is able to positively transactivate p21 promoter. The p21 overexpression induced by this complex could trigger two distinct pathways: cell cycle arrest or mitochondrial apoptosis in a context devoid of p53. Although the molecular details underlining these mechanisms remain to be clarified, we speculate that the intracellular levels of p21 achieved upon modulation of the rpL3 amounts are determinant of cell fate. In the lights of these observations, our working model predicts that the intracellular levels of rpL3 could be crucial in regulating the hierarchy of interactions of p21 with multiple factors, leading to the assembly of specific protein complexes, which could trigger two distinct effects: cell cycle arrest or apoptosis.

At the present, the challenge is to identify the p21 molecular partners involved in the cellular response to rpL3 overexpression. This will help to elucidate the role of ribosomal proteins in the control of cell cycle and cell death through p21-dependent molecular pathways.

Materials and Methods

Cell cultures, transfections and drug treatment. Human cell line Calu-6[23] was cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids (Euroclone), 2 mM L-glutamine and penicillin-streptomycin 50 U/ml.

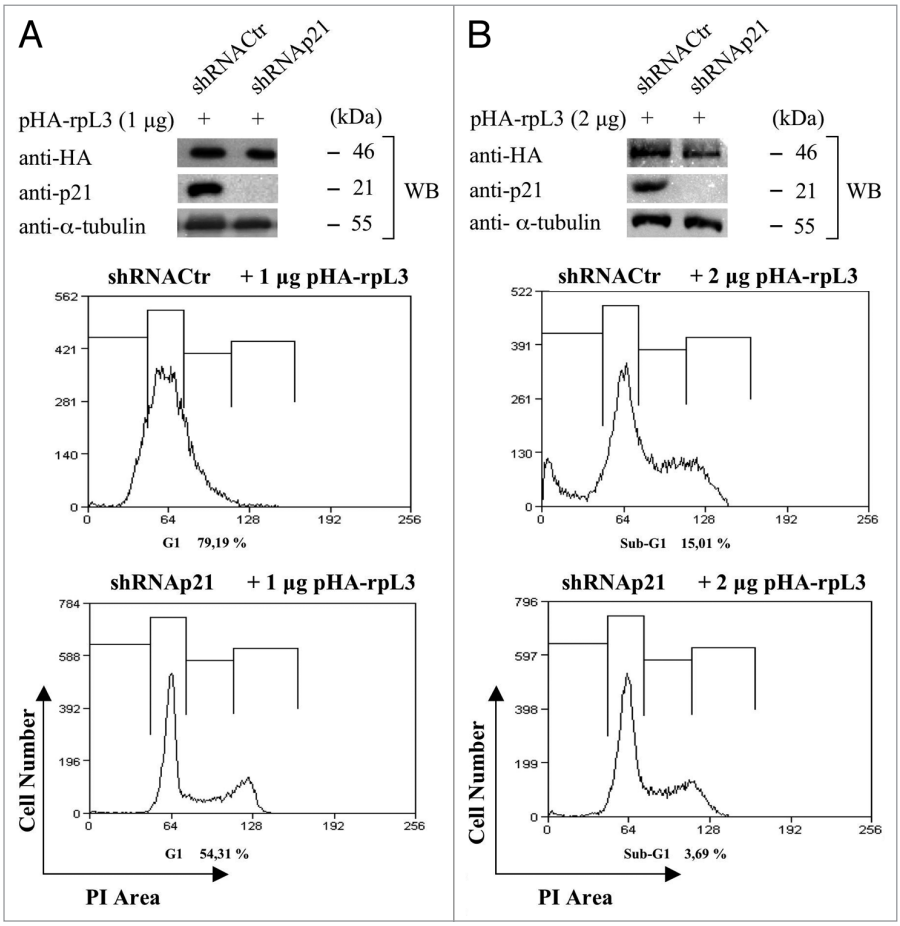

Figure 9. Role of p21 on rpL3-mediated cell cycle arrest and apoptosis. (**A**) Protein extracts from Calu-6 cells and p21ΔCalu-6 cells transiently transfected with 1 µg of pHA-rpL3 were analyzed by WB assay with antibodies directed against the HA tag and p21. Loading in the gel lanes was controlled by detection of α-tubulin protein. The same samples were analyzed for DNA content by propidium iodide staining, and fluorescence was measured by flow cytometry 24 h after transfection. (**B**) Protein extracts from Calu-6 cells and p21ΔCalu-6 transiently transfected with 2 µg of pHA-rpL3 were analyzed by WB assay with antibodies directed against the HA tag and p21. Loading in the gel lanes was controlled by detection of α-tubulin protein. The same samples were analyzed for DNA content by propidium iodide staining, and fluorescence was measured by flow cytometry 24 h after transfection.

p21ΔCalu-6 cell line, derived from Calu-6 cell line and stably silenced for p21, was grown in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids (Euroclone), 2 mM L-glutamine, penicillin-streptomycin 50 U/ml and 0.5 µg/ml puromycin (Sigma-Aldrich).

siRNA transfections were performed in Calu-6 cells (2.5×10^6 cells, 6 mm-well plate) at a concentration of 150 nM using Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions.

Plasmids were transfected in Calu-6 cells and p21ΔCalu-6 cells (2.5×10^6 cells, 60 mm-well plate) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

After DNA transfections, RNA and proteins were extracted by using the Trizol procedure (Invitrogen) for RT-PCR analysis and western blot, respectively.

Transfection efficiency was assessed by cotransfecting a GFP-expressing vector and normalizing RNA levels against GFP mRNA levels (data not shown).

Calu-6 cells (2.5×10^6 cells, 60 mm-well plate) were treated with 200 nM Mithramycin A (Sigma-Aldrich) for 16 h before transfections or chromatin immunoprecipitation.

Plasmids and production of recombinant proteins. The cDNAs of NPM and Sp1 were obtained by RT-PCR from Calu-6 cells using specific primers (**Table 1**) and cloned into a version of the eukaryotic expression vector pcDNA3.1/His C (Invitrogen) containing the FLAG epitope and into a version of the eukaryotic expression vector pcDNA3.1/HisMyc A (Invitrogen) containing the His epitope, respectively. The constructs were verified by DNA sequencing.

The plasmids encoding HA-rpL3, HA-NPM, rpL7a-His and GST-rpL3 were available.[24] The plasmids pWWP, pWP124, pWP101 and pWPdel-SmaI were kindly provided by Dr. Yoshihiro Sowa.[29]

The recombinant proteins GST-rpL3 and GST were produced as previously reported.[24]

RNA interference. The siRNAs targeting NPM were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology).

To stably silence p21, Calu-6 cells were seeded the day before transfection in a 60-mm plate, and 24 h later they were transfected with 2 µg of a p21 short hairpin RNA (shRNA) expressing pSM2c vector contained in the Expression Arrest™ human shRNA library (Open Biosystems). Transfected cells were selected with 1 µg/ml of puromycin (Sigma-Aldrich) for 7 d, and then the p21 depletion was evaluated by western blotting.

The sense strand of the p21 shRNA sequence is: 5'- TGC TGT TGA CAG TGA GCG ACC AGC CTC TGG CAT TAG AAT TTA GTG AAG CCA CAG ATG TAA ATT CTA ATG CCA GAG GCT GGG TGC CTA CTG CCT CGG A-3'.

A short hairpin non-silencing construct was used as control.

PCR. Reverse transcriptase-PCR analysis was performed as described previously.[24] The primers used to amplify p21 cDNA and β-actin, were reported in **Table 1**.

Real-time PCR was used to analyze DNA in ChIP experiments as described in this section (see sub-section "Chromatin immunoprecipitation").

Western blotting. Western blotting assay was performed as described previously,[24] using antibodies anti-rpL3 (Primm), anti-FLAG (Sigma-Aldrich), anti-Sp1 (Millipore), anti-p21, anti-NPM, anti-HA, anti-His, anti-PARP and anti-α-tubulin (Santa Cruz Biotechnology).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assay was performed as previously reported,[27] using anti-rpL3, anti-NPM, anti-Sp1 antibodies or normal mouse IgG. Aliquots of DNA-proteins immunocomplexes were resolved by 12% SDS-gel electrophoresis and transferred into nitrocellulose filters analyzed by western blotting. The chromatin was extracted from the remaining aliquots of DNA-proteins immunocomplexes, the protein-DNA crosslink was reversed, and the proteins were digested with proteinase K. DNA was then purified and analyzed by real-time PCR analysis with the primers reported in **Table 1** (p21 promoter -194/+88 and p21 3'-UTR +3455/+3771), using SYBR GreenEr qPCR SuperMix Universal (Invitrogen) following the manufacturer's instructions.

Dual luciferase assay. Luciferase assays were performed with the Dual-Glo Luciferase assay system (Promega) following manufacturer's instructions. Samples were read with Turner Luminometer and expressed as relative luciferase, i.e., R_T/R_C, where R_T and R_C are (Firefly luciferase)/(Renilla luciferase).

Flow cytometry. Cell cycle analysis, BrdU (5'-Bromo-2'-deoxyuridine) incorporation and tetramethylrhodamine ethyl ester (TMRE) staining were performed as previously reported.[46,47] Samples were analyzed by a CyAn ADP Flow Cytometer (DAKOCytomation) and quantified using Summit Software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The Authors wish to thank Prof. Angelo Lupo for mediating our contact with Dr. Yoshihiro Sowa, who donated the p21 promoter plasmids and Prof. Tommaso Russo for kindly providing the plasmid expressing p21 shRNA. This work was supported by Regione Campania, L5/2002 (grant number A.10057. RUSGRC05C, 2005).

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