

REPORT

## Regulatory role of rpL3 in cell response to nucleolar stress induced by Act D in tumor cells lacking functional p53

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### ABSTRACT

Many chemotherapeutic drugs cause nucleolar stress and p53-independent pathways mediating the nucleolar stress response are emerging. Here, we demonstrate that ribosomal stress induced by Actinomycin D (Act D) is associated to the up-regulation of ribosomal protein L3 (rpL3) and its accumulation as ribosome-free form in lung and colon cancer cell lines devoid of p53. Free rpL3 regulates p21 expression at transcriptional and post-translational levels through a molecular mechanism involving extracellular-signal-regulated kinases1/2 (ERK1/2) and mouse double minute-2 homolog (MDM2). Our data reveal that rpL3 participates to cell response acting as a critical regulator of apoptosis and cell migration. It is noteworthy that silencing of rpL3 abolishes the cytotoxic effects of Act D suggesting that the loss of rpL3 makes chemotherapy drugs ineffective while rpL3 overexpression associates to a strong increase of Act D-mediated inhibition of cell migration. Taking together our results show that the efficacy of Act D chemotherapy depends on rpL3 status revealing new specific targets involved in the molecular pathways activated by Act D in cancers lacking of p53. Hence, the development of treatments aimed at upregulating rpL3 may be beneficial for the treatment of these cancers.

### ARTICLE HISTORY

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p21; ribosomal protein; Actinomycin D; apoptosis; nucleolar stress; cancer; MDM2; ERK; p53


### Introduction

The main role of the nucleolus is the production of small and large ribosome subunits, but it appears to be involved in additional cellular functions that may not be directly related to ribosome subunit biogenesis.<sup>1,2</sup> The protein content of the nucleolus is dynamic and alters under stress condition.<sup>2,3</sup> In eukaryotes, ribosome biogenesis is a complex process that requires a number of coordinate events prior to nuclear export of the mature subunits to the cytoplasm.<sup>4</sup> In growing and proliferating tumor cells, the process of ribosome biogenesis is enhanced compared with normal somatic cells<sup>5</sup> suggesting that tumor cells are more sensitive to nucleolar stress than normal cells. Thus, chemotherapeutic agents that selectively target ribosome biogenesis could be less toxic to normal and differentiated cells. Moreover, unlike traditional anti-cancer drugs such as cisplatin and doxorubicin that are genotoxic, they did not cause DNA damage. Several anti-cancer agents that specifically inhibit RNA Pol I inducing nucleolar stress have been identified.<sup>6,7</sup> Among these, Actinomycin D (Act D), a widely used anti-cancer drug, is the best example of nucleolar stress-inducing chemical agents. Low doses of Act D (<10 nM) have been shown to exclusively inhibit RNA Polymerase I activity, leading to impaired ribosomal RNA (rRNA) transcription.<sup>8</sup> The mode of action and specific targets involved in the molecular pathways activated by Act D are less

defined. The identification of the cellular targets of a chemotherapeutic drug is an important challenge for the improvement of therapy regimens for cancer treatment. Perturbations in processes such as rRNA synthesis, rRNA modification as well as ribosomal protein (r-protein) imbalance produce alterations in ribosome biogenesis and resulting in nucleolar stress, causing an accumulation of ribosome-free form of r-proteins.<sup>9–11</sup> In response to this event some r-proteins translocate from the nucleolus to the nucleoplasm where bind to (mouse double minute-2 homolog) MDM2 thus promoting p53 stabilization and subsequent p53 mediated cell cycle arrest or apoptosis.<sup>12</sup> Recent findings revealed novel stress response pathways, which function independently of p53 and still lead to cell cycle arrest and/or apoptosis. Given that most cancers lack functional p53, it is of great importance to study these emerging molecular mechanisms. p53-independent pathways that require nucleolar proteins, such as Nucleophosmin (NPM), free r-proteins or MDM2 have also been identified.<sup>13</sup> It has been demonstrated that the expression of p21 protein, a cyclin-dependent kinase inhibitor (CKI), is negatively regulated by MDM2 protein independently of p53.<sup>14,15</sup> Recently, we demonstrated that ribosome-free rpL3 plays a crucial role in cell response to nucleolar stress induced by anticancer drugs known to interfere with DNA metabolism, but also able to act on RNA metabolism.

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Indeed, the rpL3 absence is dramatically associated to drug ineffectiveness.<sup>16</sup>

In this paper we investigated the regulatory mechanism of p21 expression mediated by rpL3 upon Act D-induced nuclear stress. We show that rpL3 is involved in drug-induced apoptosis and inhibition of cell proliferation and migration by controlling p21 expression both at transcriptional and post-translational levels independently from p53. We identify ERK 1/2 and MDM2 as new molecular targets of rpL3. Our findings extend the scenario of mechanisms involved in tumor cell response to drugs that specifically impact ribosome biogenesis for the treatment of cancers lacking active p53.

## Results

### **Act D increases the intracellular levels of ribosome-free rpL3**

It has been previously demonstrated that low doses of Act D (5 nM) exclusively inhibits RNA polymerase I-driven transcription<sup>17</sup> and induces ribosomal stress. In order to study the alteration of rpL3 and p21 expression in condition of ribosomal stress, Calu-6 and HCT 116 p53<sup>-/-</sup> cells were treated or not with 5 nM of Act D for 24 h. Then, cells were lysated and protein extracts were analyzed by western blotting with anti-rpL3, anti-p21, anti-rpL7a, anti-rpS19 and anti- $\alpha$ -tubulin. The results demonstrated that Act D treatment induced a specific increase of rpL3 levels that was associated to higher intracellular amounts of p21. No changes were observed in the expression levels of rpL7a and rpS19, 2 arbitrary ribosomal proteins of large and small subunits, respectively (Fig. 1A-B). To understand whether Act D-induced upregulation of rpL3 was also associated to its release from the ribosome, we treated Calu-6 cells with 5 nM of Act D or not. Then cells were collected, lysated (total fraction, TF) and fractionated to obtain the ribosome-associated fraction (RF) and the ribosome-free fraction (FF).<sup>16</sup> Proteins extracted from each fraction were analyzed by western blotting with anti-rpL3, anti-rpL7a, anti-rpS19, anti-rpL11 and anti- $\beta$ -actin. The results demonstrated that Act D treatment caused a significant increase of ribosome-free rpL3 level whereas it failed to increase the levels of the arbitrary rpL7a and rpS19, which remained largely associated to the ribosome (Fig. 1C).

### **rpL3 regulates p21 gene transcription upon Act D treatment**

Calu-6 and HCT 116 p53<sup>-/-</sup> cells, untreated or treated with 5 nM of Act D for 24 h, were collected and subjected to Chromatin immunoprecipitation experiments by using anti-rpL3 and anti-IgG as control. The presence of rpL3 in DNA-immunoprecipitated complexes was assayed by western blotting. qPCR assay on the samples was performed as previously reported.<sup>16</sup> Figure 2 shows that in untreated cells, rpL3 is able to bind p21 promoter, as we previously demonstrated.<sup>16</sup> After Act D treatment, the binding of rpL3 on p21 promoter was significantly increased compared to that observed in the control. Next, to test the role of rpL3 on p21 promoter activity in the response to drug exposure, we performed a reporter luciferase assay in condition of rpL3 silencing and drug treatment. To

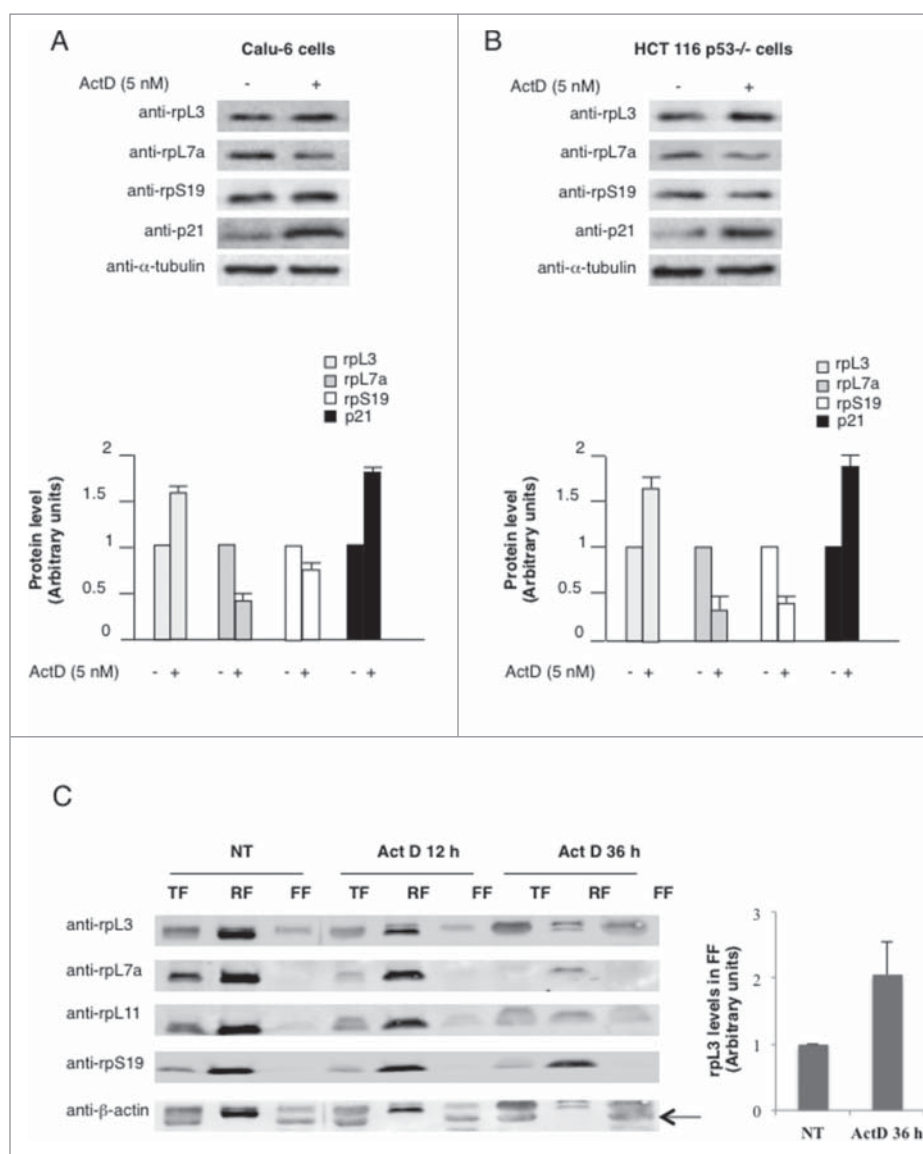
this aim, Calu-6 and HCT 116 p53<sup>-/-</sup> cells were transiently cotransfected with the full-length p21 promoter luciferase reporter plasmid and siRNA specific for rpL3 (rpL3-siRNA) or scrambled siRNA (Src-siRNA). Then, cells were treated with 5nM of Act D for 24 h or untreated and analyzed by western blotting with anti-rpL3 and anti-p21 (Fig. S1). As shown in Fig. 2C-D in untreated cells the rpL3 silencing did not cause any significant alteration on p21 promoter activity compared to untreated cells transfected with scrambled siRNA. The treatment with Act D increased p21 gene promoter activity. When rpL3 expression was switched off, the effect on p21 gene transcription of Act D was completely abolished since p21 promoter activity was similar to that observed in the control (Fig. 2C-D). These data indicated that rpL3 was necessary for regulating p21 promoter activity in the cell response to Act D.

### **rpL3 interacts with p21 in vivo and in vitro and positively regulates p21 half-life**

The translocation of rpL3 from the nucleolus to the nucleoplasm under ribosomal stress drove us to explore whether the subcellular localization change of rpL3 favored its interaction with p21 protein. To this aim, Calu-6 cells were collected and subjected to co-immunoprecipitation experiments. rpL3 was specifically immunoprecipitated from protein extracts. Then, the obtained immunocomplexes were analyzed by western blotting using anti-rpL3 and anti-p21. The presence of p21 in the rpL3-immunoprecipitate was consistent with a specific association between rpL3 and p21 *in vivo*. A control immunoprecipitate obtained with anti-IgG antibodies did not give any signal when probed with anti-rpL3 or anti-p21 (Fig. 3A).

To further analyze the interaction between rpL3 and p21, we performed GST pull-down assay by using purified recombinant proteins. His-tagged proteins rpL3 and NPM as control (His-rpL3 and His-NPM, respectively) were tested for their ability to bind to immobilized GST-tagged p21 (GST-p21). GST-p21, and GST as control, were immobilized using GSH-sepharose beads and incubated with purified His-rpL3 and His-NPM. Western blot analysis was performed on each pull-down sample. Fig. 3B shows that His-rpL3 was revealed in the pull-down preparation of GST-p21, but not in that of GST. These results provide evidence for a direct binding between rpL3 and p21 *in vitro*. No interaction was detected with GST-rpL7a used as a further control (data not shown).

p21 is a short-lived protein and post-translational regulations play a critical role in p21 expression.<sup>18</sup> On the basis of interaction between rpL3 and p21, we explored the possibility that rpL3 could regulate p21 amounts also at post-translational level. Therefore, in order to assess whether rpL3 affects the turnover of p21 we performed the analysis of p21 half-life in condition of alteration in rpL3 expression. To this aim, we generated a cell line stably silenced for rpL3 derived from Calu-6 cells namely rpL3 $\Delta$ Calu-6 cells. Fig. S2 shows the decrease of rpL3 levels in different obtained clones. We chose the cell clone expressing the lowest level of rpL3 for further experiments. Thus, Calu-6 and rpL3 $\Delta$ Calu-6 cells were treated with 5nM of Act D for 24 h and exposed to 10  $\mu$ g/ml of the inhibitor of protein synthesis Cycloheximide (CHX) for different times (0, 0.5, 1, 1.5 and 2 h). Protein extracts from the samples were analyzed by western



**Figure 1.** Expression profiles of rpl3 and p21 in Calu-6 and HCT 116 p53<sup>-/-</sup> cells upon Act D treatment. (A) Calu-6 and (B) HCT 116 p53<sup>-/-</sup> cells were treated with 5 nM of Act D for 24 h or untreated. Protein extracts from untreated or treated cells were analyzed by western blotting with indicated antibodies. Anti- $\alpha$ -tubulin was used as loading control. Quantification of protein levels is shown. (C) Ribosome distribution profile of rpl3 protein upon Act D treatment. Calu-6 cells, untreated and treated with 5 nM of Act D for 12 and 36 h were collected, lysated (total fraction, TF) and fractionated to obtain the ribosome-associated fraction (RF) and ribosome-free fractions (FF). The fractions were analyzed by western blotting with the indicated antibodies. The per-cell ratio of the amount of protein loaded onto a gel was TF:RF:FF/1:10:1. Quantification of rpl3 protein levels in FF (panel on the right) is shown. Results illustrated in Figures 1–6 are representative of 3 independently performed experiments.

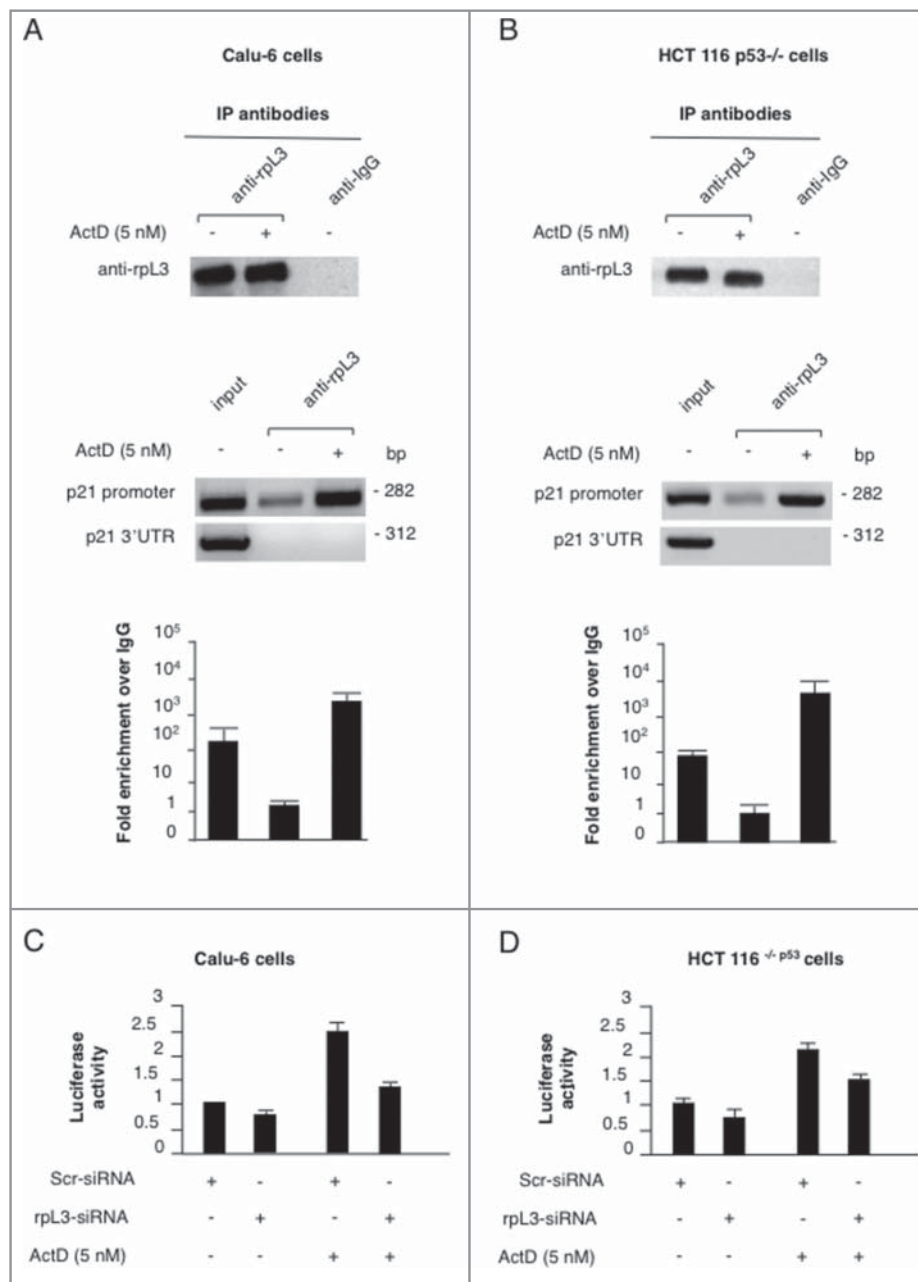
blotting using antibodies against p21. Fig. 3C showed that the half-life of p21 was greater in Calu-6 cells upon exposure to Act D (1 h vs 1.5 h). Of interest, in rpl3 $\Delta$ Calu-6 cells the silencing of rpl3 impaired the upregulation of p21. These data clearly demonstrate that rpl3 positively affect p21 stability and suggest that in condition of drug-induced nucleolar stress rpl3 becomes a positive regulator of p21 half-life.

### rpL3 regulation of p21 involves pERK and MDM2

As reported in the literature, p21 expression is mainly a result of ERK activation in a number of cell types.<sup>19,20</sup> Recent findings demonstrated that Raf/MEK/ERK activates Sp1-mediated p21 promoter activity in p53 null cells<sup>21</sup>. It has also been demonstrated that p21 protein expression is negatively regulated by MDM2 protein independently of p53.<sup>15</sup> These findings prompted us to analyze whether

ERK1/2 and MDM2 could play a role in rpl3-induced regulation of p21 expression. To this aim, we analyzed the effects of the alteration in the expression levels of rpl3 on the phosphorylated ERK and MDM2 protein levels. Calu-6 cells, untransfected or transiently transfected with pHA-rpl3 for 24 h, and rpl3 $\Delta$ Calu-6 cells were treated or not with 5 nM of Act D. Twenty-four h later, cells were lysated and protein extracts were analyzed by western blotting with anti-pERK, anti-p21 and anti-rpl3.

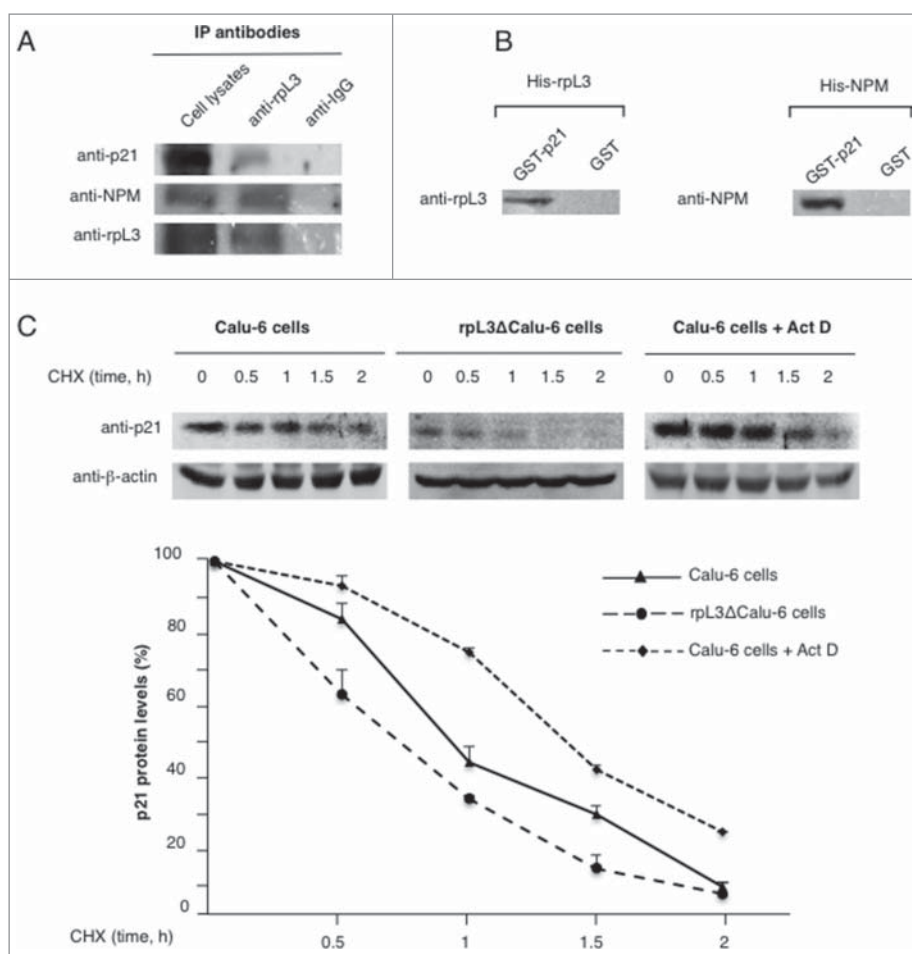
Fig. 4A shows that Act D treatment did not cause any changes in pERK levels. When rpl3 was overexpressed in untreated and Act D-treated cells a strong increase in the phosphorylation of ERK was detected. According to this, in untreated or treated cells the silencing of rpl3 inhibited the activation of ERK. These results clearly indicate that rpl3 positively affects the phosphorylation of ERK. As expected, the activation of pERK was associated to an increase of p21.



**Figure 2.** Analysis of the interaction between rpl3 and p21 gene promoter in response to ActD treatment. Protein samples of DNA-rpl3 or DNA-IgG immunocomplexes from (A) Calu-6 cells and (B) HCT 116 p53<sup>-/-</sup> untreated or treated with 5 nM Act D for 24 h were analyzed by western blotting assay with antibodies against rpl3. Note the absence of signal in DNA-IgG immunocomplex. The same DNA-immunoprecipitates were subjected to qPCR with primers specific for the proximal region of p21 gene promoter or control loci (p21 3'-UTR). (C) Calu-6 cells and (D) HCT 116 p53<sup>-/-</sup> cells were transiently cotransfected with the full-length p21 promoter luciferase reporter plasmid and siRNA specific for rpl3 (rpl3-siRNA) or scrambled siRNA (Scr-siRNA). Then, cells were treated with 5 nM of Act D for 24 h or untreated. Analysis of the relative luciferase activity, normalized against Renilla Luciferase (pRL) activity, of the samples is shown.

Moreover, by examining MDM2 levels, we observed that Act D treatment induces an increase of MDM2 intracellular amounts while the enforced expression of rpl3 impairs Act D-mediated MDM2 upregulation. In rpl3 depleted cells an increase in MDM2 levels was observed (Fig. 4A) and this effect was stronger upon Act D treatment. Of note, the upregulation of MDM2 was associated to a decrease in pERK and p21 protein levels. It is known that MDM2 is a negative regulator of p21. Since rpl3 overexpression correlates to MDM2 downregulation and p21 upregulation we hypothesized that rpl3 could regulate p21 stability by modulating the interaction between p21 and its negative regulator MDM2. An attractive possibility

was that rpl3 might stabilize p21 by abrogation of MDM2-p21 interaction. Thus, we looked for MDM2 in rpl3 immunoprecipitated complexes. The absence of signal for MDM2 in the immunoprecipitates demonstrated that rpl3 and MDM2 did not associate *in vivo* (Fig. S3). Furthermore, we verified whether rpl3 could regulate MDM2 expression acting as transcriptional factor. To this aim we analyzed MDM2 mRNA levels upon alteration in rpl3 expression levels and Act D treatment. No change in MDM2 mRNA amounts in all tested conditions was observed indicating that rpl3 is not involved in the regulation of MDM2 expression at transcriptional levels in normal condition or in condition of nucleolar stress (Fig. S4). These data



**Figure 3.** Analysis of the interaction between rpL3 and p21 proteins upon Act D treatment *in vivo*. (A) rpL3 was specifically immunoprecipitated from Calu-6 cells extracts with antibodies against the endogenous rpL3. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies versus the indicated proteins. Note the absence of signal in IgG immunocomplex. (B) Analysis of the interaction between rpL3 and p21 proteins *in vitro*. GST-p21 or GST (control), were immobilized on glutathione-sepharose beads and incubated with His-rpL3 or His-NPM. The eluted proteins were then analyzed by immunoblot with antibodies anti-rpL3 and anti-NPM. Note the absence of signal in control GST pull-down preparations. (C) Effect of rpL3 on p21 half-life. Calu-6, rpL3ΔCalu-6 and Calu-6 cells treated with 5 nM of Act D for 24 h were incubated with CHX for the indicated times. Western blot was performed using anti-p21 antibody and anti-β-actin as loading control.

suggest that a more complex mechanism of regulation remains to be clarified.

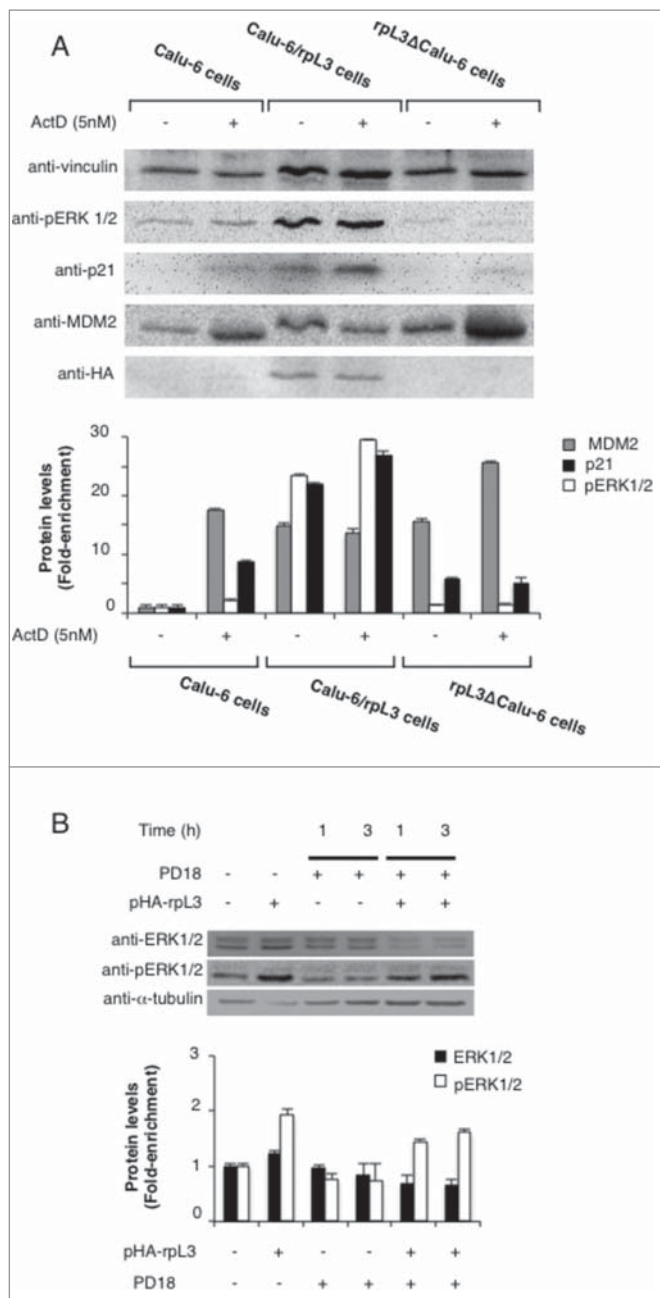
To better understand whether pERK was required for the rpL3-mediated induction of p21 expression, we treated cells with MEK1/2 inhibitor (PD18). To this aim, Calu-6 cells were transiently transfected with pHA-rpL3. Twenty-four h later, untransfected and transfected cells were treated with 10 μM of the inhibitor PD18 for 1 and 3 h. Then, cell were collected, lysated and protein extracts were analyzed by western blotting. As shown in Figure 4B, the addition of PD18 inhibited ERK phosphorylation. Of interest, the ectopic expression of rpL3 was able to overcome PD18 inhibition suggesting that rpL3 was crucial for ERK phosphorylation.

#### **rpL3 is involved in cell response to ribosomal stress induced by Act D**

To study the involvement of rpL3 on cell response to ribosomal stress induced by Act D, we firstly analyzed the influence of rpL3 on cell proliferation. To this aim, Calu-6 and rpL3ΔCalu-6 cells were treated with 5 nM of Act D for 24 h. In Calu-6 cells, the colony number was reduced upon exposure to Act D thus

confirming the ability of the drug to inhibit clonogenicity. It is noteworthy that in rpL3ΔCalu-6 cells the capacity of cells to produce colonies upon Act D treatment was comparable to the capacity of untreated rpL3ΔCalu-6 cells (Fig. 5A). These results suggest that the loss of rpL3 plays an important role in inhibition of cell proliferation upon exposure to Act D.

To study the effect of rpL3 on ActD-induced apoptosis, Calu-6 and rpL3ΔCalu-6 cells were treated with 5 nM of ActD or not. Twenty-four h later, modifications of mitochondrial inner membrane were estimated by tetramethylrhodamine (TMRE) staining and analyzed by flow cytometry. As expected, the percent of apoptosis increased after Act D treatment but, of note, rpL3 silencing caused a decrease of apoptotic cell number following Act D exposure (Fig. 5B). Having established the key role of rpL3 in cell response to Act D treatment, we wondered whether rpL3 overexpression could improve the cytotoxic effects of Act D. To this aim, we evaluated the *in vitro* cytotoxicity of Act D in combination with rpL3 overexpression. Calu-6 cells, untransfected and transiently transfected with pHA-rpL3, were treated with 5nM of Act D. Twenty-four h later, the cytotoxicity was evaluated by using MTT assay. Figure 5C shows that in Act D treated cells the cytotoxicity induced by rpL3 overexpression



**Figure 4.** Influence of rpL3 on MDM2, pERK1/2 and p21 proteins upon Act D treatment. (A) Calu-6 cells were transiently transfected with pHA-rpL3. Calu-6, Calu-6/rpL3 and rpL3ΔCalu-6 were treated with 5 nM Act D for 24 h or untreated. Protein extracts from the samples were analyzed by western blot with indicated antibodies. Anti-vinculin was used as loading control. (B) Effect of rpL3 on ERK expression upon ERK inhibition. Calu-6 cells were transiently transfected with pHA-rpL3. 24 h later, untransfected and transfected cells were treated with 10  $\mu$ M of the inhibitor PD18 for 1 and 3 h. Protein extracts from the samples were analyzed by western blot using anti-ERK1/2 and pERK1/2 antibodies. Anti- $\beta$ -tubulin was used as loading control.

was increased of about 20–25% as compared with cells treated with Act D alone suggesting that the ectopic expression of rpL3 allowed a more potent antiproliferative activity.

Furthermore, considering that rpL3 overexpression was associated to the upregulation of p21 and the role of p21 in preventing cell migration, we became interested to investigate the effect of rpL3 overexpression on cell motility. Calu-6 cell migration was determined using wound healing assay and quantitatively evaluated in terms of occupation rate of open

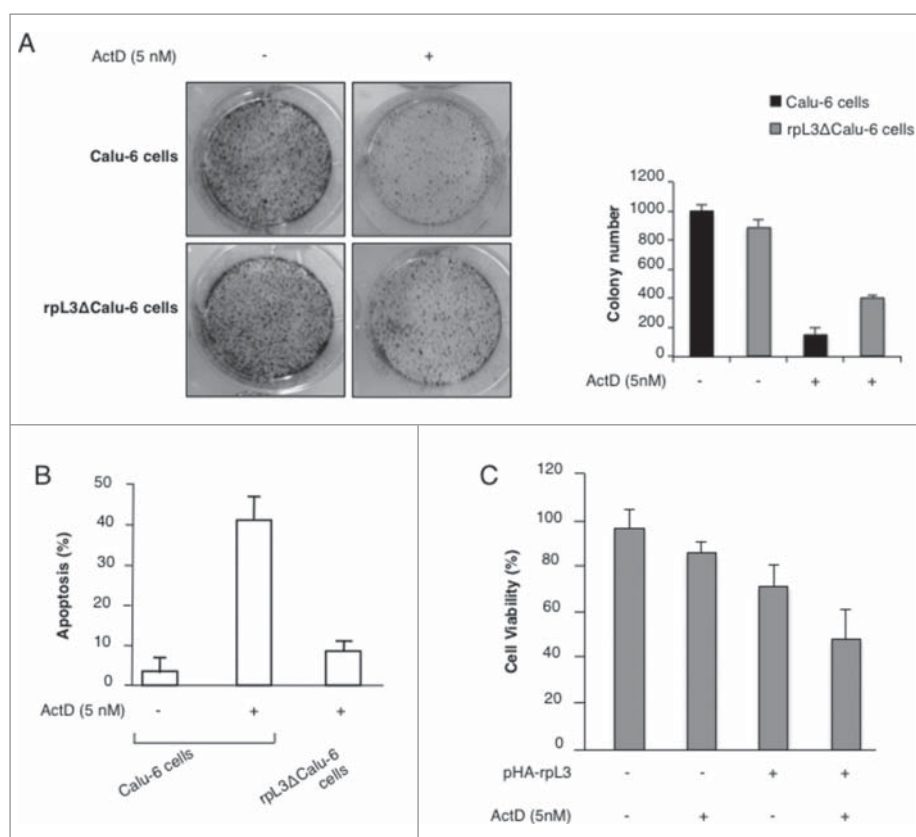
wound. As indicated in Fig. 6, the wound healing ability of Act D treated Calu-6 cells was reduced in time dependent manner compared to that observed in untreated cells. Likewise, the quantitative analysis showed that the open wound of Act D treated Calu-6 cells was increased of about 20% and 30% compared to untreated cells after 8 and 24 h, respectively. When rpL3 was overexpressed, the wound healing ability of Act D treated Calu-6 cells was markedly reduced, demonstrating that rpL3 overexpression was able to further decrease Act D-reduced cell motility. In particular, the quantification indicated that the open wound of Act D treated Calu-6/rpL3 cells and overexpressed of rpL3 was further increased of about 10% and 35% compared to that of Act D-treated cells after 8 and 24 h, respectively (Fig. 6).

## Discussion

Ribosome biogenesis is a complex process, highly regulated, and represents one of the major energy consuming processes of the cell.<sup>22</sup> The control of ribosome biogenesis is critical for cell cycle progression, cell growth and cell proliferation.<sup>23</sup> Perturbation in ribosome synthesis causes ribosomal stress with consequent release of nucleolar and ribosomal proteins that participate in a surveillance mechanism, regulating the cellular function of the critical tumor suppressor protein p53.<sup>1</sup> In human tumors, the disruption of p53 function represents the most frequent gene alteration and this occurs in more than 50% of cancers.<sup>24</sup> Recently, several p53-independent pathways that cause cell cycle arrest or apoptosis in response to alterations of ribosome biogenesis have been described.<sup>25,26</sup> Several findings have shown evidence that a variety of DNA damage agents including 5-FU and L-OHP impair ribosomal biogenesis.<sup>16,27</sup> We have recently demonstrated that human rpL3 acts as stress sensing molecule essential in the cell response to ribosomal stress caused by 5-FU and L-OHP in cancer cells lacking active p53.<sup>16</sup> In this scenario, our effort has been directed toward the study of p53 independent pathways activated upon Act D induced ribosomal stress in order to reveal new aspects of well studied chemotherapeutic drug and/or new therapeutic targets in the therapy of p53-negative cancers.

The antineoplastic antibiotic Actinomycin D represents one of the older clinically approved drug used to treat gestational trophoblastic cancer, testis cancer, Wilm's tumor, rhabdomyosarcoma and Ewing's sarcoma.<sup>28</sup> It is known that Act D, at a low cytostatic concentration, is specifically able to trigger ribosomal stress.<sup>29</sup> Although low doses of Act D have been studied in p53 cell system, it has been demonstrated that the selective inhibition of rRNA synthesis by Act D was able to cause a perturbation of cell cycle progression also in cells silenced of p53, but the molecular pathways by which this occurs has not been determined.<sup>9</sup>

Here we show that low doses of Act D (5 nM) induce the expression of rpL3 and its release from the ribosome in p53 mutated and p53 negative cells (Fig. 1). Data from the ChIP experiments and reporter luciferase assays reported here showed that upon Act D treatment free rpL3 binds p21 promoter and positively affects p21 expression at transcriptional level (Fig. 2). Analysis of immunoprecipitate of rpL3 in p53 null cells showed that rpL3 and p21 coimmunoprecipitate together indicating that



**Figure 5.** (A) Representative image of clonogenic analysis for cell proliferation in Calu-6 and rpl3 $\Delta$ Calu-6 cells after Act D treatment. Bar chart indicating clonogenic growth is shown. (B) Role of rpL3 on apoptosis upon Act D treatment. Calu-6 and rpl3 $\Delta$ Calu-6 were treated with 5 nM of Act D for 24 h or untreated. Then, cells were analyzed for mitochondrial membrane potential by TMRE staining and analyzed by flow cytometry. (C) Role of rpL3 on cell viability upon Act D treatment. Cell viability of untreated or Act D treated Calu-6 cells and Calu-6 cells transiently transfected with pHA-rpL3 was evaluated using the MTT assay. The cell viability was calculated vs untreated control cells at the respective time of incubation, set as 100%. Results are presented as percentage (mean  $\pm$  ds) (n = 3) of the control cells.

these proteins associate *in vivo*. In addition, a direct binding between these 2 proteins was observed *in vitro* by performing GST pull-down experiments (Fig. 3A and B). These results led us to hypothesize that the specific interaction between pL3 and p21 could affect p21 stability. We demonstrated that in cell stably depleted of rpL3, the half life of p21 was decreased, while ActD mediated rpL3 upregulation associates to a marked increase in p21 half life (Fig. 3C). These results indicate that rpL3 prolongs the half life of p21

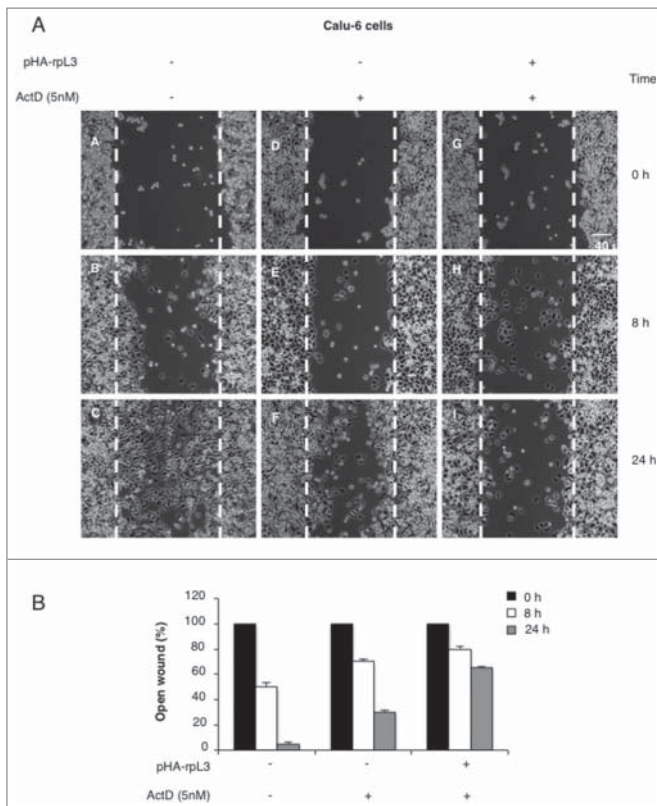
As expected, Act D treatment of Calu-6 cells caused an increase in the number of apoptotic cells. Of interest, the specific rpL3 impairment was able to markedly reduce the cytotoxic effects of the drug. In fact when rpL3 expression was switched off (rpl3 $\Delta$ Calu-6 cells) we observed a strong reduction in the percentage of apoptotic cells (10% vs 40%) following drug incubation and the cells became more resistant to Act D treatment (Fig. 5A and B). In addition, the ectopic expression of rpL3 strongly increased Act D mediated cytotoxic effects (Fig. 5C). Taken together, these results suggest that rpL3 exerts itself a cytotoxic activity that could be exploited to enhance the susceptibility of specific tumors lacking of p53 to the treatment with Act D. Furthermore, our studies demonstrate that rpL3-mediated upregulation of p21 contributes to the inhibition of cell migration (Fig. 6).

Next, we identified MDM2 and ERK 1/2 as a new molecular targets of rpL3 (Fig. 4). Specifically, we demonstrated that rpL3 was able to inhibit Act D mediated MDM2 upregulation. The

observed increased levels of MDM2 in cells treated with Act D and silenced of rpL3 correlated to decreased amounts of p21 protein. This result together with previous data indicating that p21 protein expression is negatively regulated by MDM2 protein independently of p53<sup>14,15</sup> led us to hypothesize that rpL3 is crucial for the regulation of p21 protein levels after Act D treatment by regulating MDM2-mediated stability of p21.

It is known that MEK/ERK pathways control the growth and survival of a large spectrum of human cancers<sup>30</sup> and affect p21 expression.<sup>31</sup> MEK1/2 is immediately upstream of ERK1/2 and has been reported to activate ERK1/2. The use of specific MEK inhibitor allowed us to selectively inhibit ERK1/2 activation thereby showing that ERK pathway represents the key mechanism of rpL3-mediated cell response (Fig. 4B). The proapoptotic function of the ERK pathway is well documented for apoptosis induced by DNA-damaging agents.<sup>32</sup> ERK activity has been particularly implicated in cisplatin-mediated apoptosis.<sup>33</sup> Mechanisms of ERK-mediated apoptosis include the upregulation of p53 associated to an increase of its stability by inhibiting the association with MDM2.<sup>34</sup> However, some studies have shown that ERK is also able to induce apoptosis independently of p53.<sup>35</sup>

All together our results led us to propose a working model predicting that Act D-induced ribosomal stress causes the upregulation of rpL3 expression and its dissociation from the ribosome. The increased ribosome-free form of rpL3 (free rpL3) translocates from nucleolus to nucleus where takes part to the transactivation of p21 promoter (Fig. 7).<sup>25,36</sup> We have



**Figure 6.** Role of rpL3 on cell migration upon Act D treatment. Calu-6 cells untransfected or transiently transfected with pHa-rpL3 were treated with 5 nM of Act D for 24 h. Wound widths were measured at 0, 8 and 24 h on 3 fields per well and averaged. Data is expressed as the fold-decrease of area respect to controls (A,D,G) set as 100%. Bars represent the mean of triplicate experiments; error bars represent the standard deviation.

previously reported that rpL3-mediated p21 upregulation requires the specific interaction between rpL3 and Sp1.<sup>36</sup> It has been shown that ERK1/2 is involved in p21 dependent G1 cell cycle arrest<sup>37</sup> and that ERK1/2 can phosphorylate Sp1 on Threonines 453 and 739 *in vitro* and *in vivo*.<sup>21</sup> These Sp1 phosphorylations were also detected when perifosine induced p21<sup>CIP1</sup> transcription via MEK/ERK.<sup>21</sup> Here, we show that the ectopic expression of rpL3 is able to induce the phosphorylation of ERK, thereby activating ERK, essential in mediating rpL3-induced p21 expression. Based on these observations, we

can speculate that the MEKs/ERKs pathway activated by free rpL3 could induce, in turn, the phosphorylation of Sp1 and promotes Sp1/rpL3 binding to the p21 promoter. These findings indicate that when overexpressed upon Act D induced nucleolar stress rpL3 becomes an inducer of p21 via ERK activation (Fig. 7). Furthermore, the finding that the increased ribosome-free rpL3 was associated to MDM2 downregulation and p21 upregulation led us to propose that rpL3 could modulate p21 stability by controlling the interaction between p21 and its negative regulator MDM2. rpL3 effects associate to cell cycle arrest, apoptosis and inhibition of cell proliferation and migration (Fig. 7).

In conclusion our findings provide new insight for the development of novel therapeutical strategy to improve the efficacy of clinically approved drugs that specifically impact ribosome biogenesis in the treatment of cancers lacking active p53.

## Materials and methods

### Cell cultures, transfections and drug treatment

Human Calu-6 and HCT 116 p53<sup>-/-</sup> cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin-streptomycin 50 U/ml and 0.1 mM non-essential amino acids (Euroclone).

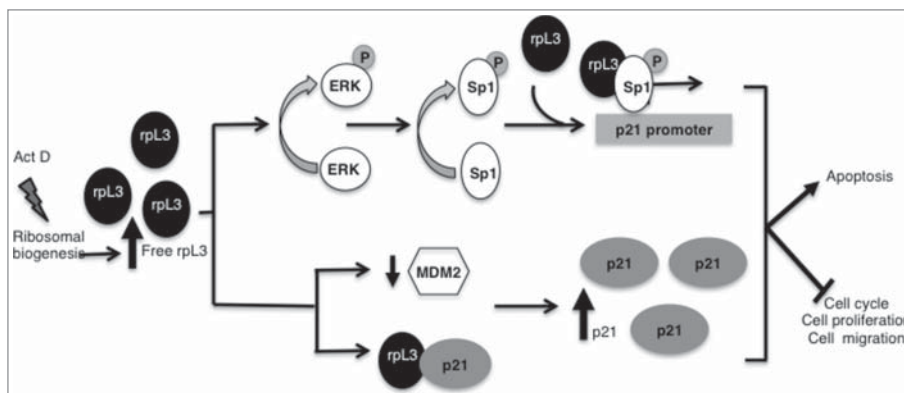
rpL3ΔCalu-6 cell line was obtained from Calu-6 cells. Cells were transfected with 2 μg of different shRNA rpL3 plasmids (Sigma-Aldrich) by using Lipofectamin 2000 (Life Technologies) according to the manufacturer's instructions. Stable clones were selected in medium containing 1 mg/ml of Puromycin (Sigma-Aldrich) and assayed for the detection of rpL3 expression level by western blotting.

Transfection of DNA, siRNA and shRNA targeting rpL3 (Sigma-Aldrich) was performed in cells using Lipofectamin 2000 as previously described.<sup>38</sup>

Drug treatments were performed by adding to cells 5 nM of Actinomycin D (Act D, Sigma-Aldrich) for 24 h.

### Ribosome isolation

Ribosome isolation was performed as previously reported.<sup>39</sup>



**Figure 7.** Schematic representation of proposed model. Act D induced nucleolar stress caused an induction of rpL3 total intracellular levels and the accumulation of rpL3 as ribosome-free form. Free rpL3 becomes a regulator of p21 expression. At transcriptional level, rpL3 induces the phosphorylation of ERK that, in turn, could promote the phosphorylation of Sp1. Then, rpL3 could recruit phosphorylated Sp1 and induce the transactivation of p21 promoter. At post-translational level, rpL3 binds and stabilizes p21 protein and downregulates MDM2. rpL3 effects associate to cell cycle arrest, apoptosis and inhibition of cell proliferation and migration.



### Plasmids and production of recombinant proteins

Plasmids encoding pHA-rpL3 and pGL3 control plasmid were already available. The recombinant proteins GST-rpL3, GST-p21, His-rpL3 and His-NPM were produced as previously reported.<sup>39</sup>

### Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed as previously reported.<sup>16</sup>

### Dual luciferase assay

Luciferase assays were performed as previously reported.<sup>16</sup>

### GST pull-down and Immunoprecipitation assay

GST pull-down assay was performed as previously reported.<sup>40</sup>

### p21 half-life analysis

Cells were treated with 5 nM Actinomycin D for 24 h or untreated. Then, cells were treated with Cycloheximide (CHX) 0,1  $\mu\text{g}/\mu\text{l}$  for different times, and subsequently harvested and lysed using RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0,5% Na-deoxycolate, 150 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1X EDTA-free Roche protease inhibitor cocktail). Protein extracts from samples were analyzed by western blotting.

### Western blotting

Western blotting analysis was performed as previously reported.<sup>41</sup> The membranes were challenged with anti-rpL3 and anti-rpL7a (Primm), anti-p21 (BD Pharmingen), anti-rpS19, anti-p70S6k, anti-anti-vinculin, anti- $\beta$ -actin and anti-HA-Tag (Santa Cruz Biotechnology), anti pERK 1/2 and anti-ERK 1/2 (Cell Signaling Technologies). Anti-MDM2 antibodies were kindly provided by Fabiola Moretti (CNR-Santa Lucia). Proteins were visualized with enhanced chemiluminescence detection reagent according to the manufacturer's instructions (Millipore).

### Mitochondrial membrane potential measurement

To quantify changes in mitochondrial membrane potential, cells were labeled with 50 nM of the mitochondrial membrane potential-sensitive fluorescent dye tetramethylrhodamine ethyl ester (TMRE, Invitrogen) for 30 min at 37 °C and analyzed by flow cytometry.

### 3-(4,5-Dimethylthiazole-2-yl)-2,5-biphenyltetrazolium bromide (MTT) assay

MTT assay was performed as previously described.<sup>42</sup>

### Clonogenic assay

For clonogenic assay, cells ( $4 \times 10^3$  in 6-well multidishes) were plated in triplicate and treated with 5nM of Act D for 24 h or

not. After 5 to 7 days, colonies were stained with 1% methylene blue in 50% ethanol.

### Wound healing assay

Cell motility was assessed using a wound healing assay. Calu-6 cells ( $1 \times 10^6$  per well) were seeded into 35 mm tissue culture plates and transiently transfected with pHa-rpL3. After 48 h, Calu-6 cells were treated with Act D 5 nM for 24 h or not. The confluent monolayer cells were then carefully wounded using a sterilized pipette tip. Monolayer cells were photographed at 0, 8 and 24 h with an objective 10X. Quantitative analysis of wound assay was performed by measuring the gap area. The gap area was defined by using ImageJ Software (National Institute of Health, USA). Data is expressed as the fold-decrease of area respect to controls set as 100%. Bars represent the mean of triplicate experiments; error bars represent the standard deviation.

### Abbreviations

Act D	Actinomycin D
CHX	Cycloheximide
CKI	cyclin-dependent kinase inhibitor
ERK	extracellular-signal-regulated kinase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GST	glutathione S-transferase
HA	hemagglutinin
His	histidine
L-OHP	Oxaliplatin
MEK	Mitogen-activated protein/Extracellular signal-regulated Kinase
MDM2	Mouse double minute-2 homolog
NPM	nucleophosmin
r-proteins	ribosomal proteins

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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