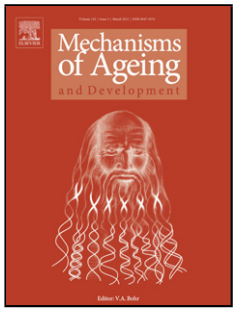

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Rapamycin prevents strong phosphorylation of p53 on serine 46 and attenuates activation of the p53 pathway in A549 lung cancer cells exposed to actinomycin D

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Abstract

The activation of the p53 pathway by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a molecule that mimics metabolic stress, is attenuated by rapamycin, an inhibitor of mTOR kinase, immunosuppressant, and cancer drug. Rapamycin also extends lifespan in experimental animals. Because AICAR is a relatively weak activator of p53, we investigated whether stimulation of p53 by the strong activator actinomycin D is also sensitive to the inhibitory effect of rapamycin. In A549 lung cancer cells, activation of p53 by actinomycin D was associated with phosphorylation of p53 on Ser46. Rapamycin inhibited the accumulation of phospho-Ser46 p53, attenuated upregulation of some p53 target genes, and altered cell-cycle progression. Moreover, in cells exposed to actinomycin D, rapamycin attenuated the accumulation of PML, a protein that in some conditions stimulates Ser46 phosphorylation. However, Ser46 phosphorylation was not diminished in PML-knockdown cells, suggesting that in our system PML does not play a major role in stimulating p53 phosphorylation on Ser46. Knockdown of p53 diminished the upregulation of PML by stress-inducing agents, consistent with the idea that *PML* is a p53-regulated gene. Our data suggest that the attenuation of p53 phosphorylation on Ser46 may play a significant role in the biological activity of anti-aging rapamycin.

Key words: p53; phosphorylation; actinomycin D; rapamycin; survivin

1. Introduction

p53 is a major tumor-suppressor protein that functions within an extensive signaling network (Kruse and Gu, 2009). Depending on cell type and the nature of the stress-inducing agent, p53 can induce transient cell-cycle arrest, cellular senescence, or apoptosis. p53 exerts many of its functions by acting as transcription regulator, e.g., it activates the *CDKN1A* gene, which encodes the p21 protein, a strong cell-cycle inhibitor. p53 also upregulates expression of the *MDM2* gene, which encodes a negative regulator of p53 itself. According to one current model, p53 can be activated at various levels. When mildly activated, p53 regulates genes with very sensitive p53-response elements. When the activation is very strong, p53 modulates less sensitive gene regulatory elements such as those that control genes encoding proapoptotic proteins. The "strength" of p53 activation depends on the degree, nature, and location of the protein's posttranslational modifications. For example, phosphorylation of serine 46 is associated with very strong activation of p53 and upregulation of proapoptotic genes (reviewed by Kruse and Gu, 2009; Gu and Zhu, 2012).

The AMP mimetic AICAR and low glucose concentration can both activate the p53 pathway, indicating that p53 participates in the cellular response to metabolic stress (Imamura et al., 2001; Jones et al., 2005; Okoshi et al., 2008). The activation of p53 by AICAR is sensitive to rapamycin, a specific inhibitor of mTOR kinase (Zajkowicz and Rusin 2011). By contrast, the activation of p53 by genotoxic doses of resveratrol is not sensitive to mTOR inhibition. Resveratrol and AICAR activate p53 to different degrees (Zajkowicz and Rusin 2011). These previous observations suggest that p53 might be sensitive to rapamycin treatment only at a low level of activation. To test this hypothesis, we examined the influence of rapamycin on the activation of the p53 pathway by very strong activators, such as actinomycin D and camptothecin, which stimulate p53 by different mechanisms. Actinomycin D is a DNA intercalator. At low concentrations, it inhibits transcription by RNA polymerase I,

resulting in nucleolar stress and the release of ribosomal proteins from nucleoli into the nucleoplasm. Some of the released proteins interact with MDM2, preventing it from inducing degradation of p53; ultimately, this results in p53 accumulation (reviewed by Deisenroth and Zhang, 2010). On the other hand, camptothecin is an inhibitor of topoisomerase I that blocks DNA transcription and replication; treatment of cells with this compound induces very strong activation of the p53 pathway (Liu et al., 2000; Jaks et al., 2001).

Studying the mechanism by which rapamycin modulates the activity of p53 will improve our understanding of the biological activity of this drug. For many years, rapamycin has been used as an immunosuppressant in kidney transplant recipients. Recently, it has been approved for treatment of renal cell carcinoma, mantle cell lymphoma, pancreatic cancer, and tuberous sclerosis (reviewed by Johnson et al., 2013). In addition, rapamycin extends lifespan in experimental animals including mice (Harrison et al., 2009; Neff et al., 2013 and references therein), although the mechanism of this extension is a matter of dispute (Blagosklonny, 2013). The mechanistic target of rapamycin (mTOR) is firmly established as a central, evolutionarily conserved regulator of longevity. Many genetic (e.g. S6 kinase 1 deletion in mice), dietary (e.g. caloric restriction) and pharmacological (e.g. rapamycin) interventions that extend lifespan of experimental animals target the mTOR signaling pathway (reviewed by Johnson et al., 2013). Thus, analyzing the molecular outcome of inhibition of mTOR kinase may help to understand the mechanism of its lifespan-regulating activity. The role of p53 in modulation of aging is not clear-cut. Based on the available experimental evidence, some authors hypothesize that mild or moderate levels of p53 activation would allow repair and survival of cells, whereas excessive p53 activation would eliminate cells. Thus, p53 can switch from a pro-survival to a pro-aging protein (reviewed by Sahin and DePinho, 2012). The mTOR and p53 pathways intersect (Feng and Levine, 2010) and the molecular details of this intersection may be relevant to the field of aging research.

The results of this study show that rapamycin attenuates the upregulation of some p53 target genes in cells treated with actinomycin D. This attenuation is associated with decreased phosphorylation of p53 on serine 46 and alterations in cell-cycle progression. Thus, our data suggest that mTOR kinase participates, by an unknown mechanism, in phosphorylation of p53 on a key serine residue.

2. Materials and methods

2.1. Cell culture and reagents

U-2 OS (human osteosarcoma, from American Type Culture Collection [ATCC]), A549 (human lung adenocarcinoma, ATCC), NCI-H292 (human mucoepidermoid pulmonary carcinoma, ATCC) and HCT116 (human colon adenocarcinoma, ATCC) cells were grown at 37°C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MI, USA) containing 1 g/1000 ml glucose, supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin–streptomycin (Sigma-Aldrich).

The following stock solutions of chemicals were used: AICAR (100 mM in H_2O; Cayman Chemical, Ann Arbor, MI, USA), actinomycin D (10 μM in DMSO; Sigma-Aldrich), camptothecin (4 mM in DMSO; Calbiochem-Merck, Darmstadt, Germany), MK-2206 dihydrochloride (10 mM in DMSO; Selleck Chemicals, Houston, TX, USA), rapamycin (40 μM in DMSO; Calbiochem-Merck, Darmstadt, Germany), and resveratrol (100 mM in DMSO; Sigma-Aldrich). Stock solutions were diluted in culture medium to obtain the required final concentrations. Control cells were mock-treated with medium containing DMSO (up to 11.8 μl in 10 ml).

Cell-cycle profiles were obtained by FACS analysis (FACSCanto flow cytometer, Becton Dickinson, Franklin Lakes, NJ) following trypsinization of attached cells, ethanol fixation, RNase treatment, and propidium iodide (PI) staining.

2.2. Suppression of PML and p53 expression by lentivirus-delivered shRNA

Lentiviral particles were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and the transduction was performed according to the manufacturer's protocol. A549 cells were treated with transduction-ready lentiviral particles containing three constructs encoding 19–

25-nucleotide shRNA sequences targeting *TP53* or *PML* mRNA. Control cells were transduced with lentiviral particles containing scrambled shRNA sequences that do not induce the specific degradation of any known mRNA. Positively transduced cells were selected using puromycin. Clonal selection was not performed, because most cells were puromycin-resistant after transduction. One day before treatment, the cells were trypsinized, seeded into culture dishes, and incubated in puromycin-free medium. The efficiency of silencing of PML or p53 was monitored by immunoblotting, as described below.

2.3. Western blotting

Cells growing on culture plates were harvested by trypsinization. For preparation of whole-cell lysates, PBS-washed cell pellets were frozen on dry ice and stored at −70°C. Subsequently, the frozen cell pellets were suspended in IP buffer (50 mM Tris-HCl, pH 8.0; 120 mM NaCl; 0.5% NP-40) supplemented with protease inhibitors (PMSF, pepstatin A, aprotinin, and leupeptin) and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). After incubation on ice for 20 minutes, lysates were cleared by centrifugation (14,000 rpm, 4°C, 20 minutes). Two volumes of cleared lysate were mixed with one volume of solution containing 150 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 0.01% bromophenol blue, and 7.5% β-mercaptoethanol. Lysates were then denatured (95°C, 5 minutes), chilled on ice, and stored at −70°C.

Nuclear extracts were prepared as described previously (Rusin et al., 2009). Cells were trypsinized, washed with PBS, and centrifuged. The resultant cell pellets were treated with ice-cold EC buffer (20 mM Tris, pH 7.6; 10 mM KCl; 2 mM $MgCl_2$; 1 mM DTT; 0.5 mM EGTA; 0.5% NP40; 2.5% glycerol) supplemented with the protease and phosphatase inhibitors mentioned above. After incubation on ice for 10 minutes, the samples were centrifuged at 310 × *g* at 4°C for 10 minutes. The supernatants containing the cytoplasmic

fractions were removed, and the pellets enriched in cell nuclei were frozen and stored at −70°C. The pellets were lysed by treatment on ice for 20 minutes with RIPA buffer (0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) supplemented with protease and phosphatase inhibitors. After centrifugation and denaturation as described above, the nuclear extracts were stored at −70°C.

Subsequently, 15–50-µg aliquots of whole-cell lysates or nuclear extracts were separated by 8% or 12% SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked for 1 hour at room temperature in blocking solution (5% skim milk solution in PBS with 0.1% Tween-20) and incubated with the indicated primary antibody. The following antibodies were from Cell Signaling Technology (Danvers, MA, USA): anti–phospho-Ser1981 ATM (D6H9), anti-ATM (D2E2), anti–acetyl-Lys382 p53, anti–phospho-Ser9 p53, anti–phospho-Ser15 p53 (rabbit polyclonal antibody), anti–phospho-Ser20 p53, anti–phospho-Ser37 p53, anti–phospho-Ser46 p53, anti–phospho-p70 S6 kinase (Thr389) (108D2), anti-survivin (71G4B7), anti-AKT (pan; C67E7), anti–phospho-Thr308 AKT (C31E5E), anti–phospho-Ser473 AKT (D9E). Anti-p53 (DO-1), anti-$p21^{WAF1}$ (F-5), anti-MDM2 (HDM2-323), and anti-PML (PG-M3) antibodies were from Santa Cruz Biotechnology. HSC70 (loading control) was detected using the B-6 antibody (Santa Cruz Biotechnology). All incubations with primary antibodies were performed overnight at 4°C in blocking solution. The secondary antibodies were HRP-conjugated and detected by chemiluminescence.

2.4. PCR arrays

A549 cells were either mock-treated or exposed to 5 nM actinomycin D, actinomycin D, and rapamycin (15 nM), or rapamycin alone for 48 hours. The cells were trypsinized and washed with PBS, and the cell pellets were frozen at −70°C. RNA samples from two independent

experiments were isolated from frozen pellets using the RNeasy Mini kit (Qiagen, Hilden, Germany) with DNase digestion, according to the manufacturer's protocol. The RNA integrity number (RIN) was determined using the RNA Nano Chip (Agilent Technologies); all samples were in the range 9.3–9.7. Subsequently, cDNA was synthesized from 3 μg of total RNA using the RT-First Strand kit (Qiagen, Hilden, Germany). Gene expression at the mRNA level was analyzed using the RT-Profiler PCR array for p53 signaling pathway (cat no. PAHS027D-12 as of March 2012, SABiosciences-Qiagen, Hilden, Germany) on a BioRad CFX96 real-time cycler. The web-based software provided by the manufacturer was used to perform quantitation, using the $\Delta\Delta C_T$ method, of the fold change in mRNA expression. Expression of the housekeeping gene *GAPDH* was used as a reference.

2.5. Immunofluorescence staining

Cells on glass slides (Lab-Tek II, NUNC, Roskilde, Denmark) were washed with PBS, fixed for 2 minutes at room temperature with 3.7% formalin in PBS, washed again, and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. After washing, the cells were incubated for 30 minutes in blocking solution (5% BSA and 0.15% glycine in PBS) at room temperature. Primary antibody (rabbit anti-PML polyclonal antibody; Chemicon International, Merck Millipore, Darmstadt, Germany) was diluted 1:500 in the blocking solution. After incubation (2 hours at room temperature) and washing, the primary antibody was detected with FITC-conjugated anti-rabbit IgG (1:300 dilution; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. The stained cells were embedded in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized on an AXIO Imager.M2 fluorescence microscope (Zeiss, Oberkochen, Germany).

3. Results

3. 1. Camptothecin and actinomycin D are more potent p53 activators than resveratrol or AICAR.

In a previous study, we found that resveratrol is a more potent activator of p53 than AICAR: resveratrol induced stronger phosphorylation of p53 on Ser15 and Ser37, and caused greater upregulation of p21 after extended treatment (Zajkowicz and Rusin 2011). At the outset of the current study, we compared the degree of p53 pathway activation in A549 and U-2 OS cells treated with resveratrol, AICAR, actinomycin D, or camptothecin. The concentrations of AICAR (1 mM) and resveratrol (50 μM) were as previously described (Zajkowicz and Rusin 2011). Actinomycin D was used at a low concentration (5 nM) that induces nucleolar stress (Deisenroth and Zhang, 2010), and camptothecin was used at the lowest concentration (1 μM) capable of activating p53 in most cell lines (Jaks et al., 2001). Cells were treated for either 24 or 48 hours. Under these experimental conditions, actinomycin D and camptothecin were more potent activators of the p53 pathway than resveratrol or AICAR (Fig. 1). This elevated activation manifested as greater upregulation of p53 and/or p21.

In A549 cells, strong activation of the p53 pathway by camptothecin or actinomycin D was associated with high levels of phosphorylation of p53 on Ser46. Phosphorylation of Ser15 was similar in both cell lines. These observations indicate that the signaling system responsible for p53 phosphorylation on Ser46 is attenuated in U-2 OS cells, relative to A549 cells. This finding was unexpected, because other authors have detected Ser46 phosphorylation in U-2 OS cells following treatment with actinomycin D (Smeenk et al., 2011). Overexposure of the blot shown in Fig. 1 revealed phosphorylation of Ser46 in actinomycin D-treated U-2 OS cells (data not shown). Moreover, an independent time-course experiment demonstrated detectable but relatively weak phosphorylation of Ser46 p53 in U-2 OS cells treated with actinomycin D (Fig. S1). Various kinases are responsible for p53

phosphorylation on Ser46 (Bulavin et al., 1999; D'Orazi et al., 2002; Yoshida et al., 2006; Taira et al., 2007). For instance, this residue is phosphorylated by ATM in the early phase after exposure to ionizing radiation (Kodema et al., 2010). However, we noticed that the level of ATM activation, as reflected by phosphorylation on Ser1981, was not correlated with phosphorylation of Ser46 (Fig. 1). Thus, the high level of ATM activation observed in U-2 OS cells is not sufficient for strong phosphorylation of p53 on Ser46.

3.2. Rapamycin attenuates activation of the p53 pathway in cells treated with actinomycin D.

Next, we investigated whether rapamycin can modulate the activation of the p53 pathway by actinomycin D. As a positive control we used AICAR, which activates p53 in a rapamycin-sensitive manner (Zajkowicz and Rusin 2011). We also examined the phosphorylation status of p70S6K on Thr389, an indicator of mTOR activity that can be used as a marker of the inhibition of mTOR by rapamycin (Shackelford and Shaw, 2009). Rapamycin attenuated AICAR-induced p53 activation in both A549 and U-2 OS (Fig. 2). Thus, the influence of rapamycin on AICAR-induced p53 activation is not limited to A549 cells. Moreover, in A549 cells, rapamycin also inhibited activation of p53 by actinomycin D. This inhibition manifested as attenuated upregulation of p21 and loss of strong phosphorylation of p53 on Ser46 (Fig. 2). The accumulation of p53 with phosphorylated Ser15 or acetylated Lys382 was also diminished by co-treatment with rapamycin. Thus, rapamycin is able to significantly modulate the activation of p53 pathway even by a strong inducer. To explain this modulation we hypothesized that the kinase responsible for p53 phosphorylation on Ser46 is sensitive to treatment with rapamycin. In principle, rapamycin might inhibit this kinase either directly or indirectly, or activate a mechanism that prevents phosphorylation of Ser46. One well-known inhibitor of p53 pathway is AKT kinase, a major pro-survival protein that inactivates many pro-apoptotic proteins by phosphorylating them (reviewed by Duronio, 2008). Rapamycin, by

blocking a p70S6 kinase-dependent negative-feedback loop, promotes strong activation of AKT (Shi et al., 2005; Harrington et al., 2004).

We hypothesized that AKT may promote growth in part by inhibiting a kinase responsible for p53 phosphorylation on Ser46. We initially tested this hypothesis by treating A549 cells with various p53 activators (camptothecin, actinomycin D, resveratrol, and AICAR) and co-treating the cells with rapamycin for 48 hours (Fig. 3A). By Western blot, we examined the activation status of the p53 pathway and the phosphorylation status of AKT (Thr308, Ser473), which reflects its activation by upstream kinases (Duronio, 2008). Consistent with the findings described above, p53 was strongly activated by camptothecin and actinomycin D, and moderately activated by resveratrol and AICAR (Fig. 3A). p53 activation by actinomycin D and AICAR was inhibited by rapamycin. As expected, p53 activation was associated with decreased phosphorylation of AKT. This observation is consistent with previous reports that p53 lowers AKT activity by various mechanisms (reviewed Feng and Levine, 2010). Rapamycin alone increased the phosphorylation level of AKT, consistent with results reported by others (Shi et al., 2005). Rapamycin also increased AKT phosphorylation in cells treated with p53 activators. Thus, rapamycin promotes AKT phosphorylation in untreated cells, as well as in cells with activated p53.

3.3. The AKT inhibitor MK-2206 attenuates activation of the p53 pathway.

To determine whether AKT activation by rapamycin is responsible for attenuation of the p53 pathway, we used a specific inhibitor of AKT, MK-2206, which has been used in phase I clinical trials in patients with solid tumors (Yap et al., 2011). In these experiments, the p53 pathway was activated by actinomycin D in A549 cells, and the cells were also treated with rapamycin, or rapamycin and MK-2206, to prevent rapamycin-induced hyperactivation of AKT (Fig. 3B). MK-2206 prevented AKT activation, but did not prevent inhibition of the p53

pathway by rapamycin. Unexpectedly, inhibition of AKT by MK-2206 in the absence of rapamycin also attenuated activation of the p53 pathway by actinomycin D. Thus, paradoxically, in some circumstances AKT may be required for strong activation of the p53 pathway. This result indicates that the relationship between the p53 and AKT pathways may be more complicated than it is currently considered to be (Feng and Levine, 2010).

3.4. PCR-microarray analysis reveals genes regulated at the transcriptional level by actinomycin D and rapamycin treatment.

To determine how rapamycin modulates expression of genes considered to be p53 targets, we performed semi-quantitative RT-PCR analysis using PCR-microarray technology. Our microarrays contained genes encoding 84 p53-related proteins, including some that act upstream of the p53 pathway (e.g., ATM and ATR kinases) and others regulated by p53 at the transcriptional level (e.g., *CDKN1A*, which encodes p21). We identified 12 transcripts that were upregulated at least 3-fold (Fig. 4), and 15 transcripts downregulated at least 3-fold, by actinomycin D (Fig. 5). The transcript encoding p21 (*CDKN1A*) exhibited the highest level of upregulation. Furthermore, consistent with the Western-blot results, rapamycin attenuated actinomycin D-induced upregulation of p21 (*CDKN1A*) mRNA. Rapamycin also significantly reduced the upregulation of another p53 target, *RPRM*; this gene encodes reprimo protein, a cell-cycle regulator that arrests cells in G2 phase (Taylor and Stark, 2001). *BIRC5*, the gene most strongly repressed by actinomycin D, encodes survivin, which belongs to the inhibitor of apoptosis (IAP) family (Kelly et al., 2011). Survivin gene repression was slightly weaker in the presence of rapamycin. Other genes downregulated by actinomycin D encode cell-cycle regulators: BRCA1, BRCA2, cyclins B2 and E2, cyclin-dependent kinase 1, and transcription factor E2F1. In general, the microarray data show that the influence of rapamycin on p53-dependent gene expression is gene-specific.

3.5. Rapamycin prevents high levels of accumulation of various posttranslationally modified forms of p53.

Rapamycin-induced attenuation of p53 phosphorylation on Ser46 was associated with slightly reduced accumulation of p53 with phosphorylated Ser15 or acetylated Lys382 (Fig. 2). To determine whether rapamycin can modulate the accumulation of p53 molecules modified at other residues, we performed a dose-response experiment. Cells were exposed for 48 hours to various concentrations of actinomycin D, either alone or in combination with rapamycin (Fig. 6 A). At 0.5 nM, actinomycin D induced an increase in the overall p53 level and accumulation of p53 phosphorylated at Ser9, Ser15, Ser20, and Ser37. The p53 targets p21 and MDM2 were also upregulated. All of these events were prevented by rapamycin. Thus, rapamycin inhibits accumulation of p53 protein as well as various posttranslationally modified forms of p53. However, at 5 nM actinomycin D, the reduction in Ser46 phosphorylation induced by rapamycin was lower than the reduction in the total amount p53 (Fig. 6A). Densitometric quantitation of bands from three independent experiments confirmed this conclusion (Fig. 6B). This observation indicates that the reduction in the level of phospho-Ser46 p53 is not simply caused by a decrease in the total amount of p53.

Consistent with the microarray data (Fig. 5), actinomycin D at 5 nM prevented expression of survivin (encoded by *BIRC5*; Fig. 6). At this concentration of actinomycin D, rapamycin did not influence the expression of this protein. However, rapamycin was able to inhibit repression of survivin when p53 was moderately activated by 1 nM actinomycin D (Fig. 6). The experiment was performed twice with similar results. Thus, in A549 cells, rapamycin not only inhibits upregulation of p53-dependent genes, but can also inhibit repression of genes downregulated by p53.

3.6. Rapamycin attenuates accumulation of PML induced by actinomycin D.

The phosphorylation of p53 on Ser46 in cells with damaged DNA is mediated by HIPK2 and other kinases (Bulavin et al., 1999; D'Orazi et al., 2002; Yoshida et al., 2006; Taira et al., 2007). Under some conditions, Ser46 phosphorylation by HIPK2 is strictly dependent on the presence of PML protein, which forms nuclear bodies that are involved, among other things, in promoting activation of p53 (Möller et al., 2003). Hence, we hypothesized that rapamycin prevents strong p53 phosphorylation on Ser46 by blocking expression of PML. Immunocytochemical staining revealed that actinomycin D strongly upregulated PML (Fig. 7A). Consistent with the hypothesis described above, this upregulation was attenuated by rapamycin co-treatment. To further test the hypothesis, we investigated whether PML participates in Ser46 phosphorylation in response to actinomycin D. To this end, we knocked down the expression of PML in A549 cells using lentivirus-delivered shRNAs; reference cells were transduced with control lentivirus. Subsequently, these cell populations were mock-treated or exposed to actinomycin D, actinomycin D, and rapamycin, or rapamycin alone (Fig. 7B). Unexpectedly, we found that downregulation of PML did not attenuate Ser46 phosphorylation induced by actinomycin D; rather, the level of phosho-Ser46 p53 was slightly elevated in these cells. This experiment confirmed the immunocytochemical data showing that rapamycin attenuates PML upregulation induced by actinomycin D. These results indicate that PML does not promote phosphorylation of Ser46 in A549 cells exposed to actinomycin D, leading us to conclude that rapamycin does not influence phosphorylation of this residue by inhibiting PML expression. However, it remains unclear how rapamycin attenuates the upregulation of PML. One possible explanation might be that PML gene is a direct transcriptional target of p53, and that p53 activation results in increased expression of PML (de Stanchina et al., 2004). Therefore, it is conceivable that, in cells exposed to

actinomycin D, we observed p53-dependent stimulation of PML expression and the attenuation of this process by rapamycin.

To determine whether PML expression is regulated in a p53-dependent manner in A549 cells, we monitored the effect of p53 knockdown on the expression of PML. The accumulation of p53-regulated proteins (p21 and MDM2) served as a control. In these experiments, we used cells transduced with lentiviruses expressing either shRNAs directed against p53 mRNA or control shRNAs. Both p53-knockdown and control cells were mock-treated, exposed to 1 μM camptothecin (CPT) for 24 hours, or exposed to actinomycin D (5 nM), resveratrol (50 μM), or AICAR (1 mM) for 48 hours. Western blots (Fig. 7C) revealed that expression of PML was significantly upregulated by camptothecin, actinomycin D, and AICAR, and that this upregulation was dramatically reduced by knockdown of p53. This finding demonstrates that upregulation of PML by these compounds is p53-dependent. Based on the observations presented in Fig. 7, we conclude that in A549 cells, rapamycin attenuates the upregulation of PML by inhibiting activation of p53, a transcriptional stimulator of the gene encoding PML (de Stanchina et al., 2004).

3.7. The pattern of cell-cycle inhibition induced by actinomycin D is modulated by rapamycin.

To study the biological consequences of p53 modulation by rapamycin, we examined the cell cycle of A549 cells treated for 48 hours with actinomycin D or co-treated with actinomycin D and rapamycin (Fig. 8A). Actinomycin D alone strongly inhibited progression through the cell cycle at either G1 (79%) or G2 phase (20%); cells with S-phase DNA content were virtually absent (Fig. 8A and B). Rapamycin co-treatment significantly reduced the percentage of cells in G2 phase (to 6.4%) and increased the percentage of cells in G1 phase (to 93%). Thus, the rapamycin-induced attenuation of p53 activation in cells treated with actinomycin D is

associated with a statistically significant decrease in the proportion of cells in G2 phase. Rapamycin alone did not induce major changes in the pattern of cell-cycle progression

Next, we examined the influence of actinomycin D on cell growth by treating cells with 1 nM actinomycin D for 48 hours. This concentration was selected because it was the lowest examined value that induced significant upregulation of p53 target genes (coding for MDM2 and p21 proteins, Fig. 6). Growth of the cell population was reduced by actinomycin D to 31% of the level in untreated control cells (Fig. 8C). Rapamycin alone also reduced cell growth to 63% of the level in control cells. Co-treatment of cells with actinomycin D and rapamycin had neither an additive nor a synergistic effect. Rapamycin slightly increased (from 31% to 40%) the growth of cells treated with actinomycin D; this increase was moderate, but statistically significant ($p=0.024$ by paired t-test). This result suggests that attenuated upregulation of p21 (Fig. 6), which slows cell-cycle progression, may be responsible for the more rapid proliferation of cells treated with actinomycin D and rapamycin, relative to cells treated with actinomycin D alone.

3.8. The activation of p53 by actinomycin D is modulated neither by rapamycin nor by AKT inhibitor in human colon carcinoma cell line HCT116.

We demonstrated that rapamycin and MK-2206 significantly attenuated activation of the p53 pathway induced by actinomycin D in the A549 cell line. To determine whether rapamycin and MK-2206 have similar activities in other cell lines with wild-type p53 and strong p53 phosphorylation on Ser46, we performed the experiment in HCT116 cells. Rapamycin did not inhibit p53 activation by any of the p53 inducers employed in this study (Fig. 9A). Moreover, in contrast to the situation in A549 cells, the phosphorylation of Ser46 was not attenuated by MK-2206, an AKT kinase inhibitor (Fig. 9B). Thus, the ability of rapamycin to inhibit the

activation of p53 pathway may be cell line-specific, a further illustration of the enormous complexity of the regulation of the p53-dependent signaling system.

3.9. The p53 phosphorylation and survivin expression are modulated by rapamycin in actinomycin D-treated NCI-H292 cells.

In order to expand the panel of cells examined for rapamycin's influence on p53 phosphorylation, we have selected two additional cell lines derived from mesothelioma (NCI-H28) and mucoepidermoid lung carcinoma (NCI-H292). Both lines have functional p53 (Nogi et al., 2012, Ge et al., 2012). After treatment of these cells with 5 nM actinomycin D for 48 hours, we found that Ser46 phosphorylation was very strong in NCI-H292 cells and very weak in NCI-H28 cells (data not shown). Hence, for further experiments we have selected NCI-H292 cells. The DNA sequencing showed that they have wild-type *TP53* gene (Zhou et al., 2010). We treated them with increasing concentrations of actinomycin D, either alone or in combination with rapamycin (Fig. 10) analogously to the experiment performed on A549 cells (Fig. 6). Even at the lowest concentration (0.5 nM), actinomycin D induced an increase in the overall p53 level and accumulation of p53 phosphorylated at Ser15, and Ser46. The p53 target p21 was upregulated, while the p53-repressed survivin (Mirza et al., 2002) was downregulated by actinomycin D. The phosphorylation of p53 at both examined sites was attenuated by rapamycin. It was associated less efficient repression of survivin. The influence of rapamycin on p21 expression was barely detectable (Fig. 10). Interestingly, rapamycin interfered with phosphorylation of p53 without noticeably changing the overall p53 level. We conclude that the ability of rapamycin to modulate the phosphorylation of p53 and the expression of p53-regulated genes is not limited to A549 cell line.

4. Discussion

The results of this study show that in A549 and NCI-H292 cells, rapamycin strongly inhibits actinomycin D-induced accumulation of p53 phosphorylated at Ser46. At the concentration used in our experiments, actinomycin D preferentially inhibits biosynthesis of rRNA, resulting in nucleolar stress (reviewed by Deisenroth and Zhang, 2010). According to a current model, nucleolar stress activates the p53 pathway through the inhibition of MDM2 activity by ribosomal proteins (e.g., RPL23) released from the stressed nucleoli; inhibition of MDM2 in turn leads to p53 accumulation and upregulation of p53-dependent genes (reviewed by Deisenroth and Zhang, 2010). We found that that activation of p53 by actinomycin D is also accompanied by phosphorylation of p53 at residues (Ser9, Ser15, Ser20, Ser37, and Ser46) that are phosphorylated when p53 is activated by various stress agents (Boehme and Blattner, 2009). Phosphorylation of Ser46 in cells treated with actinomycin D has been reported by others (Smeenk et al., 2011); those authors used U-2 OS cells as an experimental model. We also demonstrated that Ser 46 can be phosphorylated in response to actinomycin D in U-2 OS cells, but this phosphorylation was significantly weaker than in A549 cells (Fig. S1). Apparently, cells differ in the activity of the protein(s) responsible for modification of this residue.

Our previous experiments revealed that the activation of the p53 pathway by resveratrol in U-2 OS cells is weaker than in A549 cells (Rusin et al., 2009; Zajkowicz et al., 2013). The results reported here show that U-2 OS cells are characterized by reduced ability to phosphorylate Ser46 of p53 in response to camptothecin or actinomycin D treatment (Fig. 1, Fig. 2, Fig. S1). Future studies should focus on identifying the molecular mechanism of this apparent attenuation. Previous work toward this goal led us to the detection of a mutation in the *PPM1D* gene in U-2 OS cells (Zajkowicz et al., 2013). This mutation was independently reported by others, along with the discovery that the mutant PPM1D protein from U-2 OS

cells is enzymatically active and more stable than the wild-type protein (Kleiblova et al., 2013). This and similar mutations in *PPM1D* could have important clinical implications (Ruark et al., 2013, Kleiblova et al., 2013).

In A549 cells, the mechanism responsible for p53 Ser46 phosphorylation is sensitive to rapamycin and MK-2206 (AKT kinase inhibitor, Hirai et al., 2010). Rapamycin blocks the activity of mTOR kinase towards some of its substrates (Zoncu et al., 2011). MK-2206 also inhibits mTOR by direct inhibition of AKT kinase, which stimulates mTOR (Zoncu et al., 2011; Ma et al., 2013). The mechanistic details underlying this observation remain to be elucidated. mTOR activity stimulates the translation of a subset of mRNA molecules, ultimately promoting cellular growth (Zoncu et al., 2011). We speculate that mTOR may activate a kinase that phosphorylates p53 on Ser46. Alternatively, translation of the Ser46 kinase may be mTOR-dependent. Our results in this study, together with our previously published data (Zajkowicz and Rusin, 2011), clearly demonstrate that rapamycin is able to inhibit p53 pathway activation induced by various stress factors. Our observations in HCT116 and NCI-H292 cells (Fig. 9 and 10) indicate that the inhibitory effect of rapamycin on p53 activation may be cell line-specific, suggesting that rapamycin influences the activation of p53 in a targeted manner. Other authors also demonstrated that mTOR promotes p53 activation by stimulating its translation; consequently, rapamycin inhibits p53 accumulation in cells with constitutively active mTOR (Lee et al., 2007). Our study extends these findings by demonstrating that rapamycin prevents p53 activation by inhibiting at least one posttranslational modification of p53, the phosphorylation of Ser46. Besides Ser46, rapamycin also clearly attenuated the phosphorylation of p53 on Ser9 and Ser37 (Fig. 6). The attenuation of Ser46 phosphorylation may not be the sole mechanism responsible for inhibition of p53 pathway observed in cells co-treated with rapamycin. We focused on Ser46 because it is phosphorylated in strongly activated p53, which induces expression of pro-

apoptotic genes (reviewed by Gu and Zhu, 2012). Moreover, in our experiments, the influence of rapamycin on phosphorylation of p53 was best visible for this residue. Interestingly, in two cell lines (A549 and NCI-H292) treated with actinomycin D, rapamycin modulated the expression of the gene (*BIRC5*) coding for survivin, an antiapoptotic protein. The reduced phosphorylation of Ser46 was associated with higher expression of survivin. This is another observation supporting the hypothesis that strong phosphorylation of Ser46 may promote apoptosis, although in our experiment, the appearance of apoptotic cells, defined as the population of cells with sub-G1 DNA content, was not detected (Fig. 8). Apparently, some crucial pro-apoptotic signals were missing.

We observed that rapamycin co-treatment lowers the proportion of cells arrested at G2/M phase by actinomycin D, and slightly accelerates the growth of cells treated with actinomycin D. This apparent loss of G2/M arrest may be associated with the repression by rapamycin of *RPRM* gene (Fig. 4), which codes for reprimo - the protein acting as an inducer of cell cycle arrest at G2 (Taylor and Stark, 2001). It is plausible that modulation of the p53 pathway plays a role in the biological activity of rapamycin, which not only serves as an mTOR inhibitor in laboratory research, but is also used as an immunosuppressant and cancer drug (reviewed by Johnson et al., 2013).

Recent studies have shown that rapamycin extends lifespan in experimental animals, including mammals (Harrison et al., 2009; Neff et al., 2013, reviewed by Johnson et al., 2013). It remains unclear whether this lifespan extension is associated with modulation of the p53 pathway; however, several published observations may provide clues regarding a plausible mechanism. As mentioned earlier, in some experiments, strong phosphorylation of p53 Ser46 is associated with the upregulation of proapoptotic genes (Oda et al., 2000). In our study, the downregulation of survivin, an antiapoptotic protein regulated by p53 (Kelly et al., 2011), was inhibited by rapamycin in two cell lines (Fig. 6A, Fig. 10). Thus, rapamycin has

the potential to inhibit p53-dependent apoptosis, e.g., by promoting expression of pro-survival proteins under some stress conditions. A variant of p53 with proline at codon 72 is a weaker inducer of apoptosis than a variant with arginine at this position (Dumont et al., 2003). This difference in apoptotic potential is relevant to epidemiological observations showing that individuals homozygous for 72Pro allele exhibit significantly increased lifespan relative to 72Arg/Pro heterozygotes and 72Arg homozygotes (Ørsted et al., 2007). The molecular mechanism underlying the pro-survival effect of the 72Pro allele is not known, but it is biologically plausible that decreased apoptosis of somatic cells, stem cells specifically, might decrease the rate of organ failure (Campisi, 2005). Further studies should explore the mechanisms of inhibition of the p53 pathway and lifespan extension by rapamycin.

Acknowledgements

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Appendix A.

Supplementary information

Supplementary figure legend

Fig. S1. Actinomycin D induces stronger p53 Ser46 phosphorylation in A549 cells than in U-2 OS cells.

The expression of p53 and phospho-Ser46 p53 in whole-cell lysates of cells treated with 5 nM actinomycin D in a time-course experiment. HSC70 is a loading control.

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Figure legends

Figure 1. Camptothecin and actinomycin D are more potent p53 activators than resveratrol or AICAR.

Expression of ATM, p53, and p21, and these proteins phosphorylated on the indicated residues, in whole-cell lysates from A549 or U-2 OS cells that were untreated (untr) or treated with 1 μM camptothecin (CPT), 5 nM actinomycin D (ActD), 50 μM resveratrol (Res), or 1 mM AICAR for the indicated number of hours. HSC70 is a loading control.

Figure 2. Rapamycin attenuates activation of the p53 pathway in A549 cells treated with actinomycin D.

Expression levels in whole-cell lysates of total p53, p53 phosphorylated on Ser15 or Ser46, and p53 acetylated on Lys382. Expression of p21 and MDM2 is also shown. The level of p70S6K protein with phosphorylated Thr389 was used as a marker of mTOR activity. A549 and U-2 OS cells were treated for 48 hours with AICAR (1 mM), actinomycin D (5 nM), rapamycin (15 nM), or the indicated combination of drugs.

Figure 3. Inhibition of AKT kinase attenuates activation of p53 pathway in A549 cells.

A. Immunodetection of indicated proteins in A549 cells. AKT-Thr308 antibody recognizes AKT protein with phosphorylated threonine 308, whereas AKT-Ser473 recognizes AKT with phosphorylated serine 473. Cells were treated for 48 hours with camptothecin (1 μM, CPT), actinomycin D (5 nM, ActD), resveratrol (50 μM, Res), or AICAR (1 mM); co-treated with these substances and rapamycin (15 nM, Rap); or treated with rapamycin alone. **B.** Immunoblot for indicated proteins expressed in A549 cells either mock-treated or treated for 48 hours with the indicated combinations of actinomycin D (5 nM), rapamycin (15 nM), and MK-2206 (3 μM). Both panels show data obtained from whole-cell lysates.

Figure 4. p53-related genes strongly upregulated at the mRNA level by actinomycin D in A549 cells.

The relative expression of mRNAs encoded by p53-related genes upregulated at least 3-fold by treatment with actinomycin D. Expression levels of 84 genes were determined using PCR arrays. The graph shows means and standard deviation from two independent experiments. The control cells were mock-treated, and the experimental cells were treated for 48 hours with actinomycin D (5 nM), actinomycin D and rapamycin (15 nM), or rapamycin alone. The expression of mRNA of each gene in control cells was defined as 1.

Figure 5. p53-related genes strongly repressed by actinomycin D in A549 cells.

The relative mRNA expression of p53 pathway genes that were downregulated at least 3-fold by treatment with actinomycin D. For additional details, see the legend of Fig. 4.

Figure 6. Rapamycin prevents high levels of accumulation of various posttranslationally modified forms of p53.

A. Levels of p53, its modified forms, and p53-related proteins, determined by Western blot in whole-cell lysates of cells treated for 48 hours with increasing concentrations of actinomycin D, with or without 15 nM rapamycin. **B.** Quantitative Western-blot analysis of total p53 and phospho-Ser46 p53 in cells treated as indicated (5 nM actinomycin D, 15 nM rapamycin) for 48 hours. The bands from three independent experiments were quantified using the Gene Tools software (Syngene, Cambridge, UK). The *t*-test was used to calculate statistical significance of differences.

Figure 7. Accumulation of PML is p53-dependent and is inhibited by rapamycin in cells treated with actinomycin D.

A. Immunocytochemical staining of PML protein (upper panel) in mock-treated A549 cells (U) and cells treated for 48 hours with actinomycin D (ActD, 5 nM), rapamycin (Rap, 15 nM), or both substances. Lower panel shows cell nuclei stained with DAPI. **B.** Expression of PML and p53 in nuclear extracts of A549 cells transduced with control lentiviruses (-) or with lentiviruses expressing shRNA molecules directed against PML mRNA (+). The cells were treated as described above for 48 hours. The size of the detected PML protein can be estimated from the position of molecular weight markers (kDa) shown on the left. **C.** Expression of the indicated proteins in nuclear extracts of A549 cells transduced with control lentiviruses (-) or lentiviruses expressing shRNA molecules directed against p53 mRNA (+). The cells were treated for 24 hours with 1 μM camptothecin (CPT), or for 48 hours with actinomycin D (ActD, 5 nM), resveratrol (Res, 50 μM), or 1 mM AICAR.

Figure 8. The pattern of cell-cycle inhibition induced by actinomycin D is modulated by rapamycin.

A. The cell-cycle distribution of untreated A549 cells (Con) or cells exposed for 48 hours to 5 nM actinomycin D (ActD), 15 nM rapamycin (Rap), or both (ActD+Rap). The horizontal axis shows DNA content. The values for G1 and G2/M phases are marked. The vertical axis shows the cell count. **B.** The percentage of cells in G1 or G2/M phases of the cell cycle in a population of cells treated (as in **A**) with actinomycin D or both actinomycin D and rapamycin. The graphs show the means and standard deviations from three independent experiments. The paired t-test was used to determine statistical significance. **C.** Growth-inhibitory effect of actinomycin D and rapamycin in A549 cells in a short-term assay. Equal

numbers of cells were seeded onto culture dishes at the start of the experiment. The next day, control cells were mock-treated, while experimental cells were treated with actinomycin D (ActD, 1 nM), rapamycin (Rap, 15 nM), or a combination of both substances. After 48 hours, the attached cells were washed, trypsinized, and counted. The graph was drawn from means and standard deviations from four independent experiments performed in triplicate. The number of cells on control dishes after 48-hour culture was defined as 100%. The paired t-test was used to calculate the statistical significance.

Figure 9. The activation of p53 by actinomycin D in HCT116 cells is not modulated by rapamycin or MK-2206.

A. Immunoblot of HCT116 cells, showing expression of the indicated proteins in untreated cells (U) and cells treated for 48 hours with camptothecin (1 μM, CPT), actinomycin D (5 nM, ActD), resveratrol (50 μM, Res), AICAR (1 mM); co-treated with these substances and rapamycin (15 nM, Rap); or treated with rapamycin alone. **B.** Immunoblot for the indicated proteins expressed in untreated HCT116 cells, and in cells treated for 48 hours with the indicated combinations of actinomycin D (5 nM), rapamycin (15 nM), and MK-2206 (3 μM). Both panels show data obtained from whole-cell lysates.

Figure 10. Rapamycin modulates the p53 phosphorylation in NCI-H292 cell line.

Levels of p53, its phosphorylated forms, and p53-related proteins, determined by Western blot in whole-cell lysates of NCI-H292 cells treated for 48 hours with increasing concentrations of actinomycin D, with or without 15 nM rapamycin.

Highlights

Actinomycin D strongly activates the p53 pathway in the A549 lung cancer cell line.
Actinomycin D promotes phosphorylation of p53 on Ser46.
Rapamycin inhibits phosphorylation of Ser46 in cells exposed to actinomycin D.
Rapamycin modulates the expression of selected genes regulated by p53.
Rapamycin significantly alters the division cycle of cells exposed to actinomycin D.

Figure 1

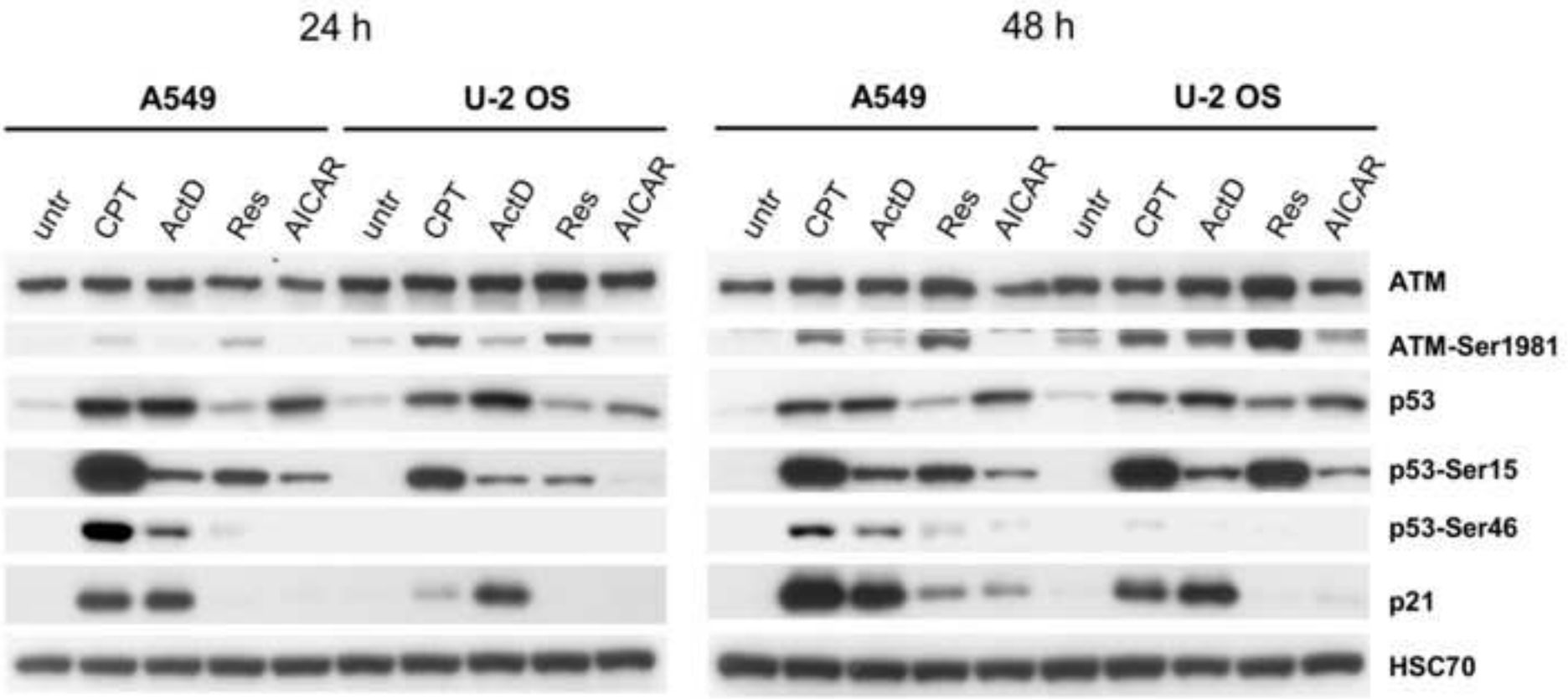

24 h
48 h
A549
U-2 OS
A549
U-2 OS
untr
CPT
ActD
Res
AICAR
ATM
ATM-Ser1981
p53
p53-Ser15
p53-Ser46
p21
HSC70

Figure 2

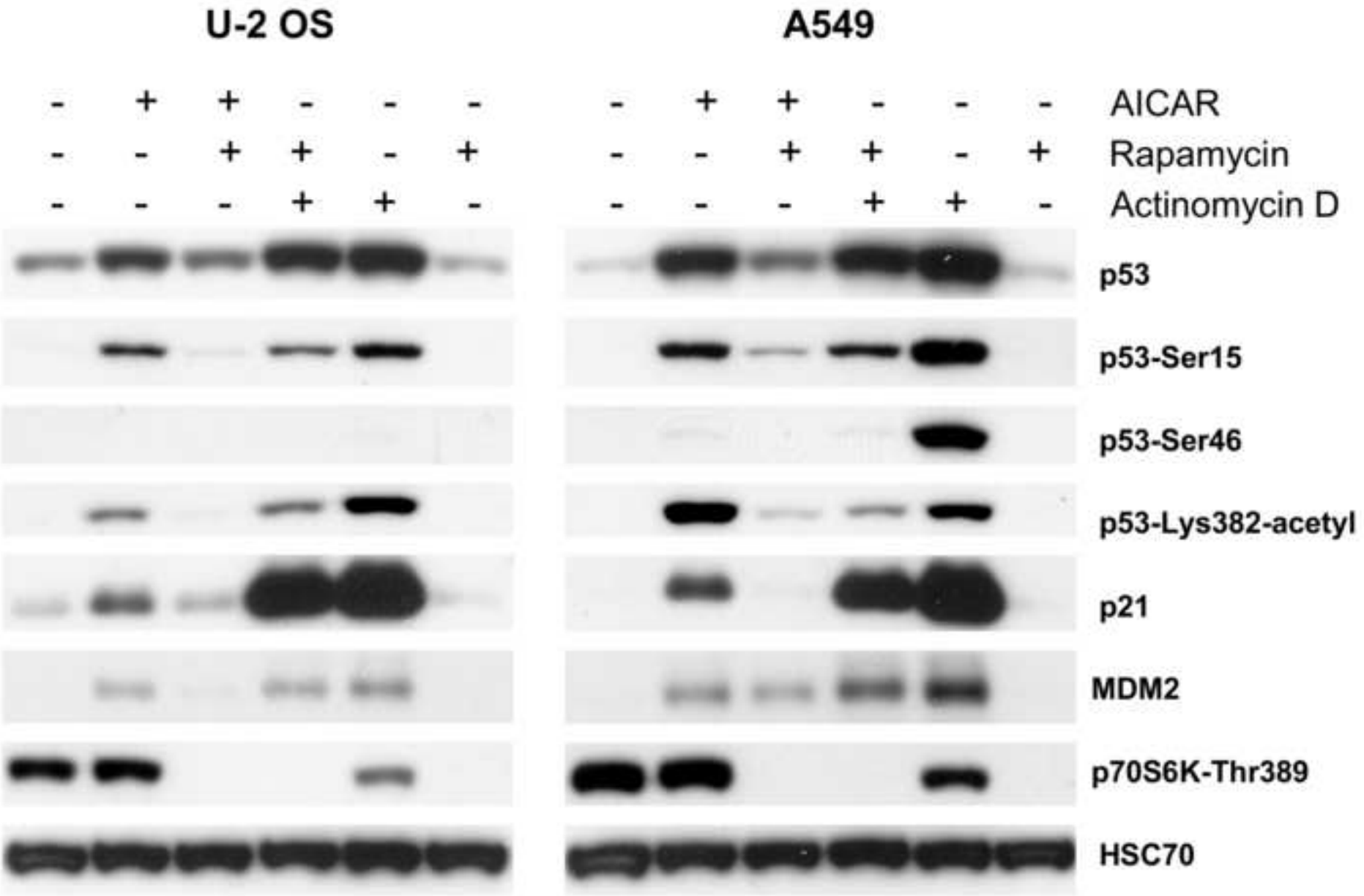

U-2 OS
A549
AICAR
Rapamycin
Actinomycin D
p53
p53-Ser15
p53-Ser46
p53-Lys382-acetyl
p21
MDM2
p70S6K-Thr389
HSC70

Figure 3

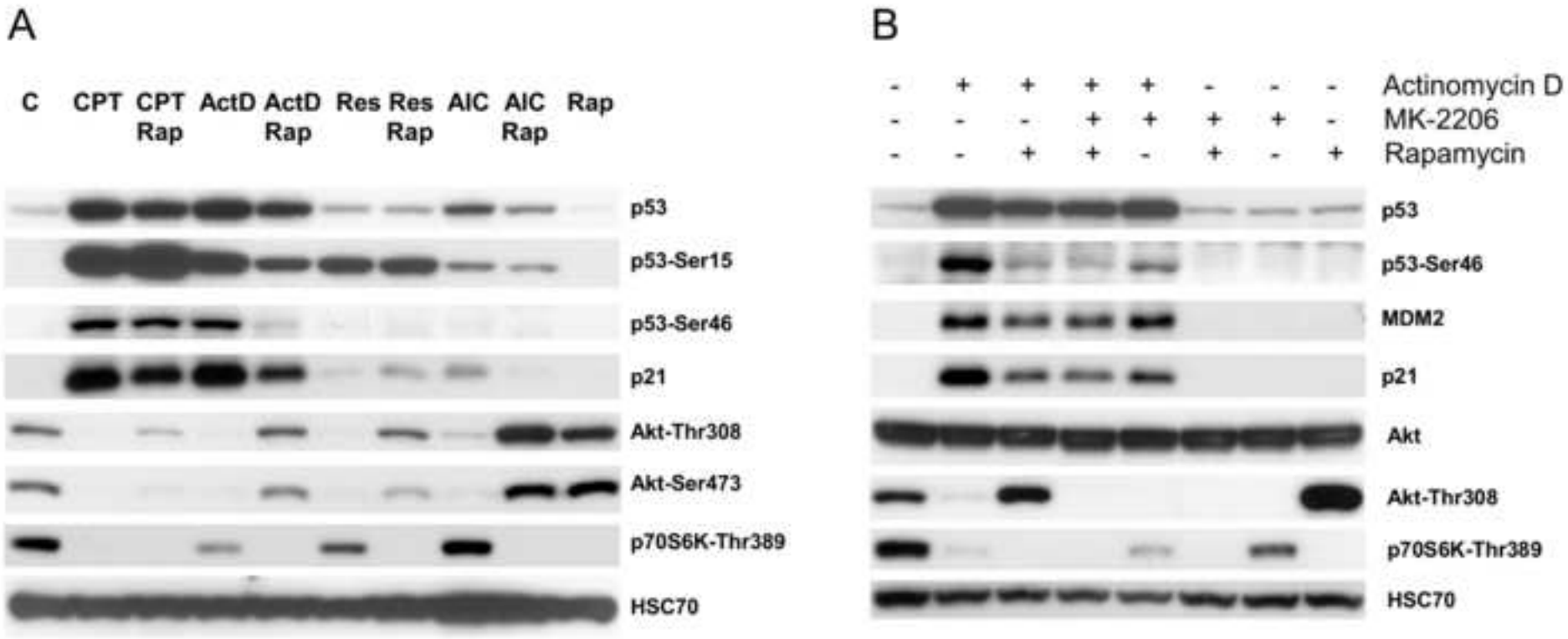

A
C
CPT
CPT Rap
ActD
ActD Rap
Res
Res Rap
AIC
AIC Rap
Rap
p53
p53-Ser15
p53-Ser46
p21
Akt-Thr308
Akt-Ser473
p70S6K-Thr389
HSC70
B
Actinomycin D
MK-2206
Rapamycin
p53
p53-Ser46
MDM2
p21
Akt
Akt-Thr308
p70S6K-Thr389
HSC70

Figure 4

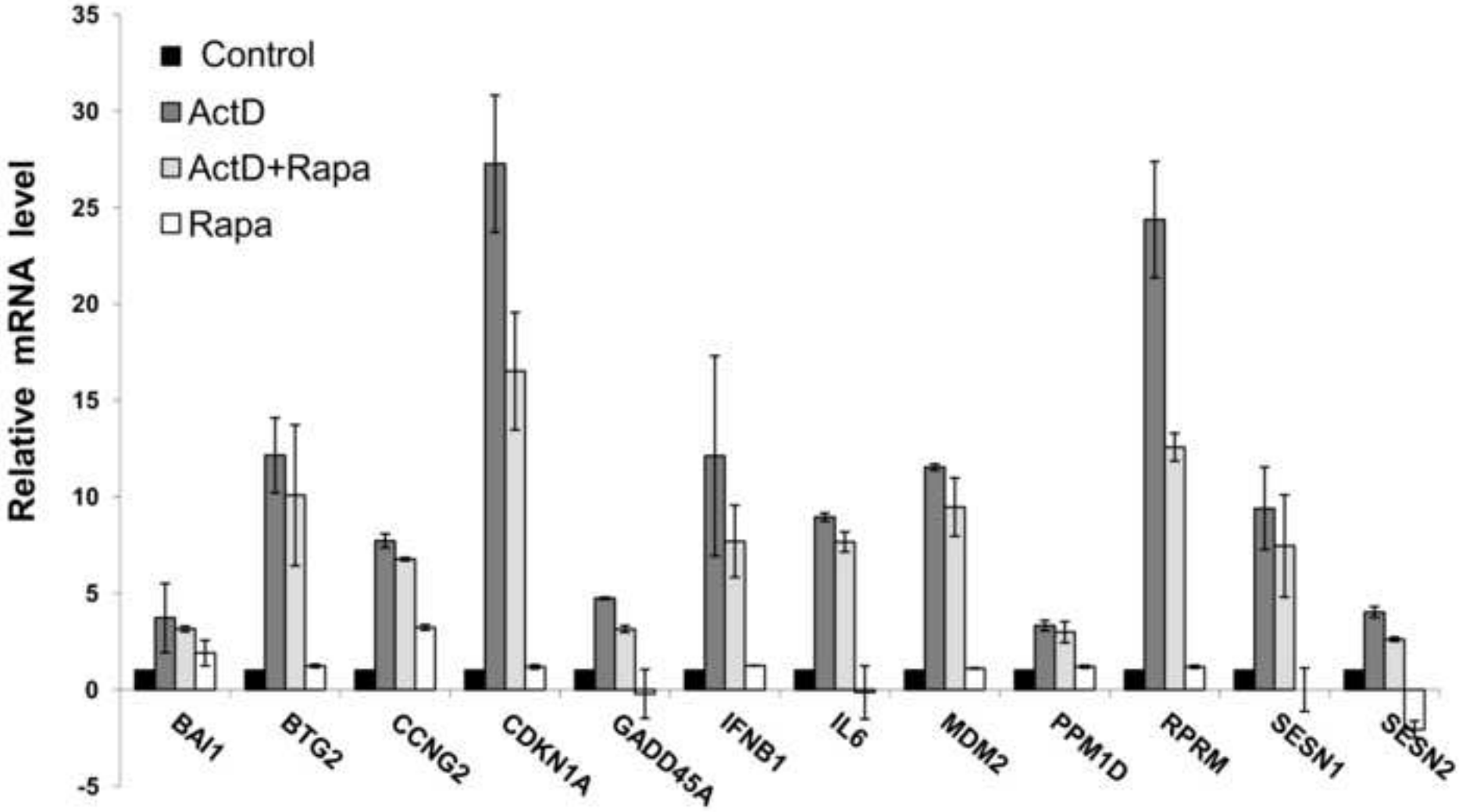
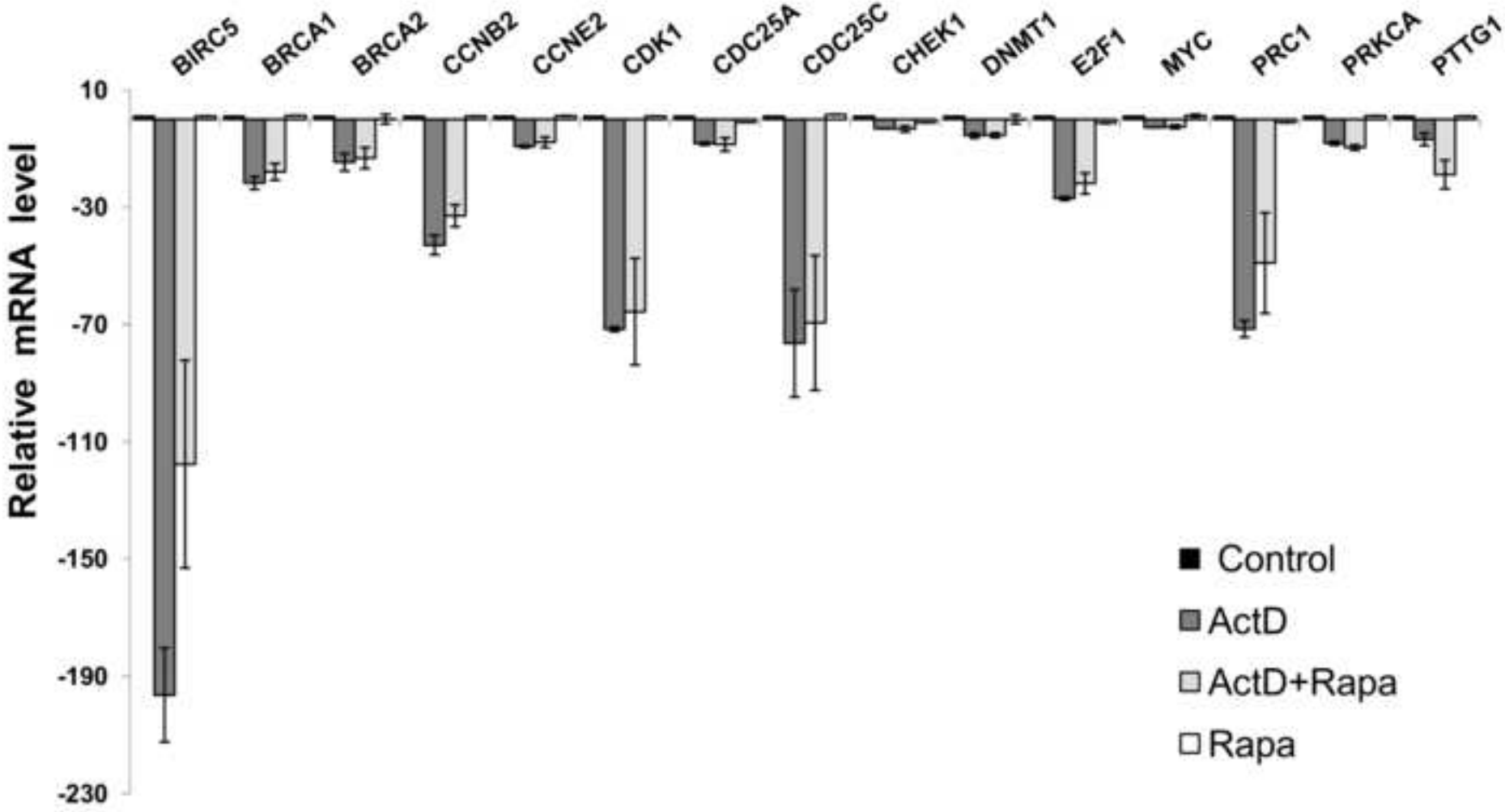

Relative mRNA level
35
30
25
20
15
10
5
0
-5
Control
ActD
ActD+Rapa
Rapa
BAI1
BTG2
CCNG2
CDKN1A
GADD45A
IFNB1
IL6
MDM2
PPM1D
RPRM
SESN1
SESN2

Figure 5


BIRC5
BRCA1
BRCA2
CCNB2
CCNE2
CDK1
CDC25A
CDC25C
CHEK1
DNMT1
E2F1
MYC
PRC1
PRKCA
PTTG1
10
-30
-70
-110
-150
-190
-230
Relative mRNA level
Control
ActD
ActD+Rapa
Rapa

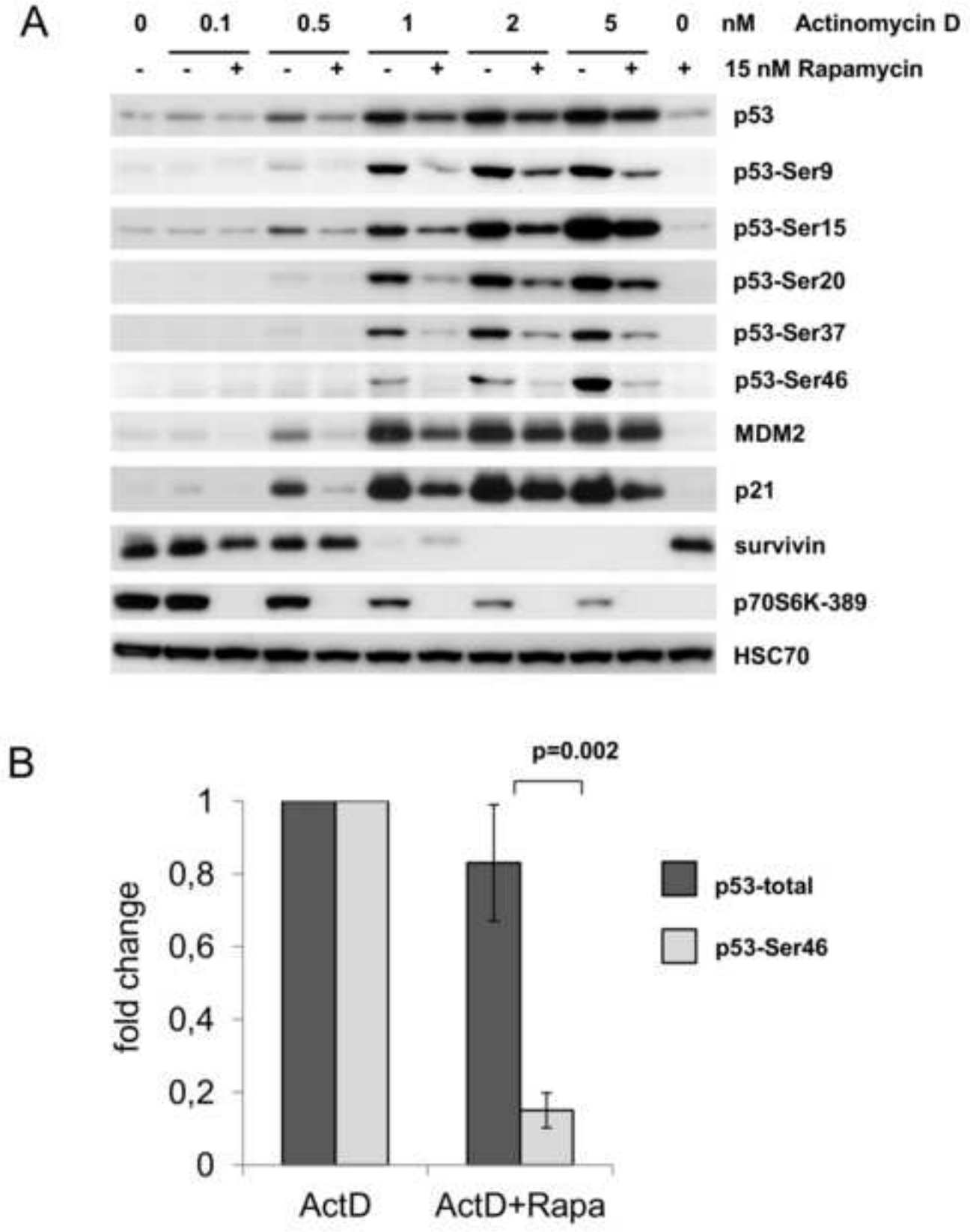


Figure 7

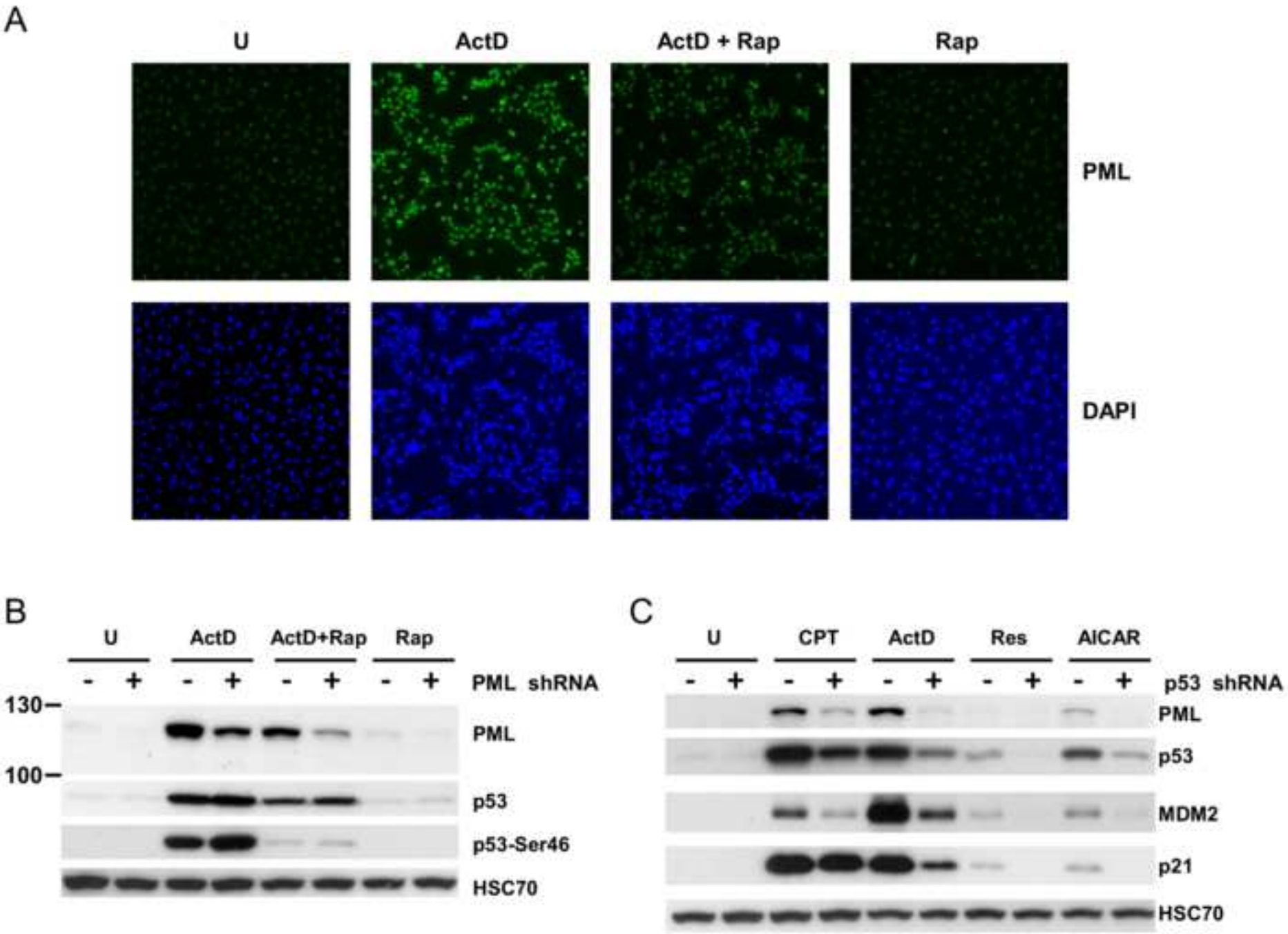

A
U
ActD
ActD + Rap
Rap
PML
DAPI
B
U
ActD
ActD+Rap
Rap
- + - + - + - +
PML shRNA
130
100
PML
p53
p53-Ser46
HSC70
C
U
CPT
ActD
Res
AICAR
- + - + - + - + - +
p53 shRNA
PML
p53
MDM2
p21
HSC70

Figure 8

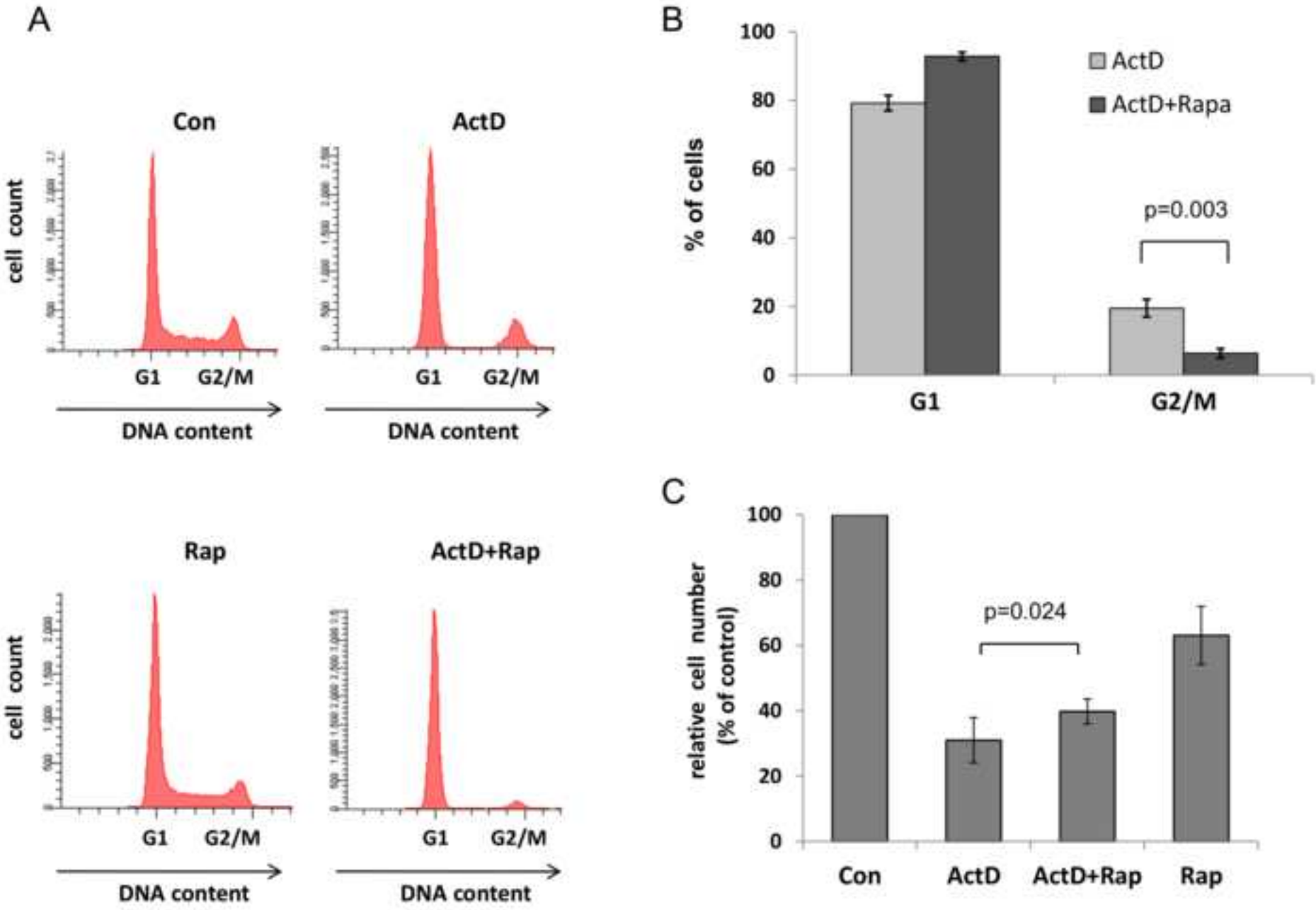

A
Con
ActD
cell count
G1
G2/M
DNA content
Rap
ActD+Rap
B
% of cells
100
80
60
40
20
0
ActD
ActD+Rapa
p=0.003
G1
G2/M
C
relative cell number (% of control)
p=0.024
Con
ActD
ActD+Rap
Rap

Figure 9

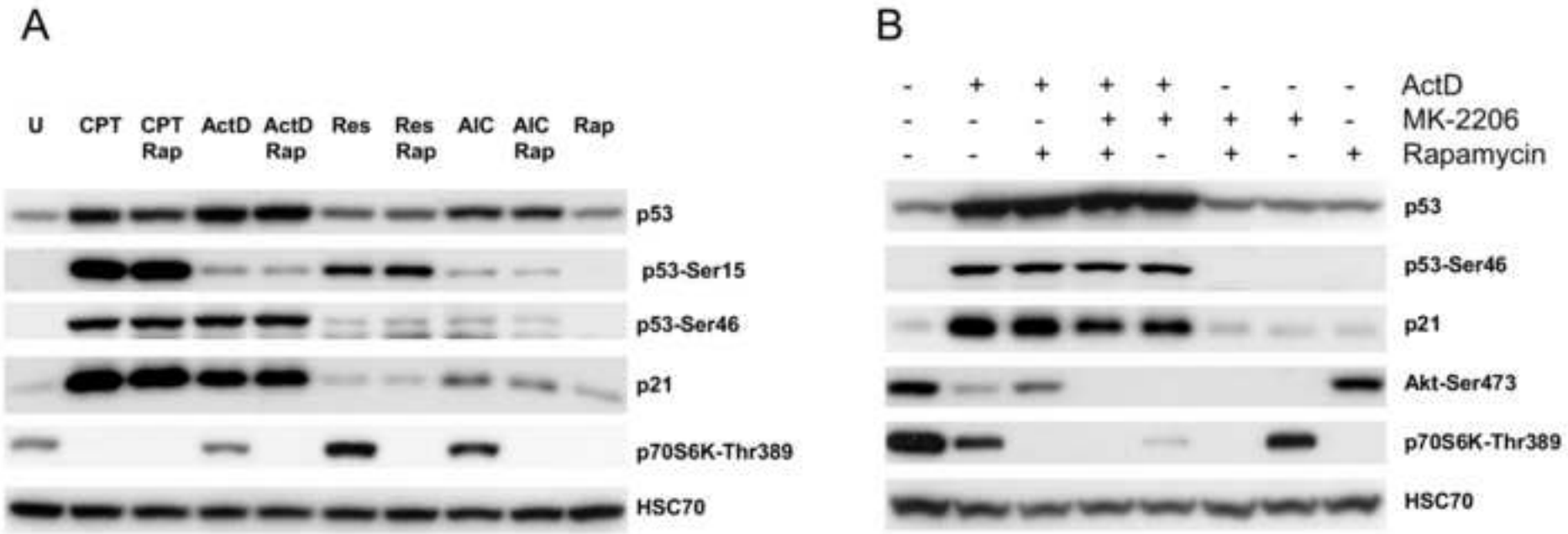
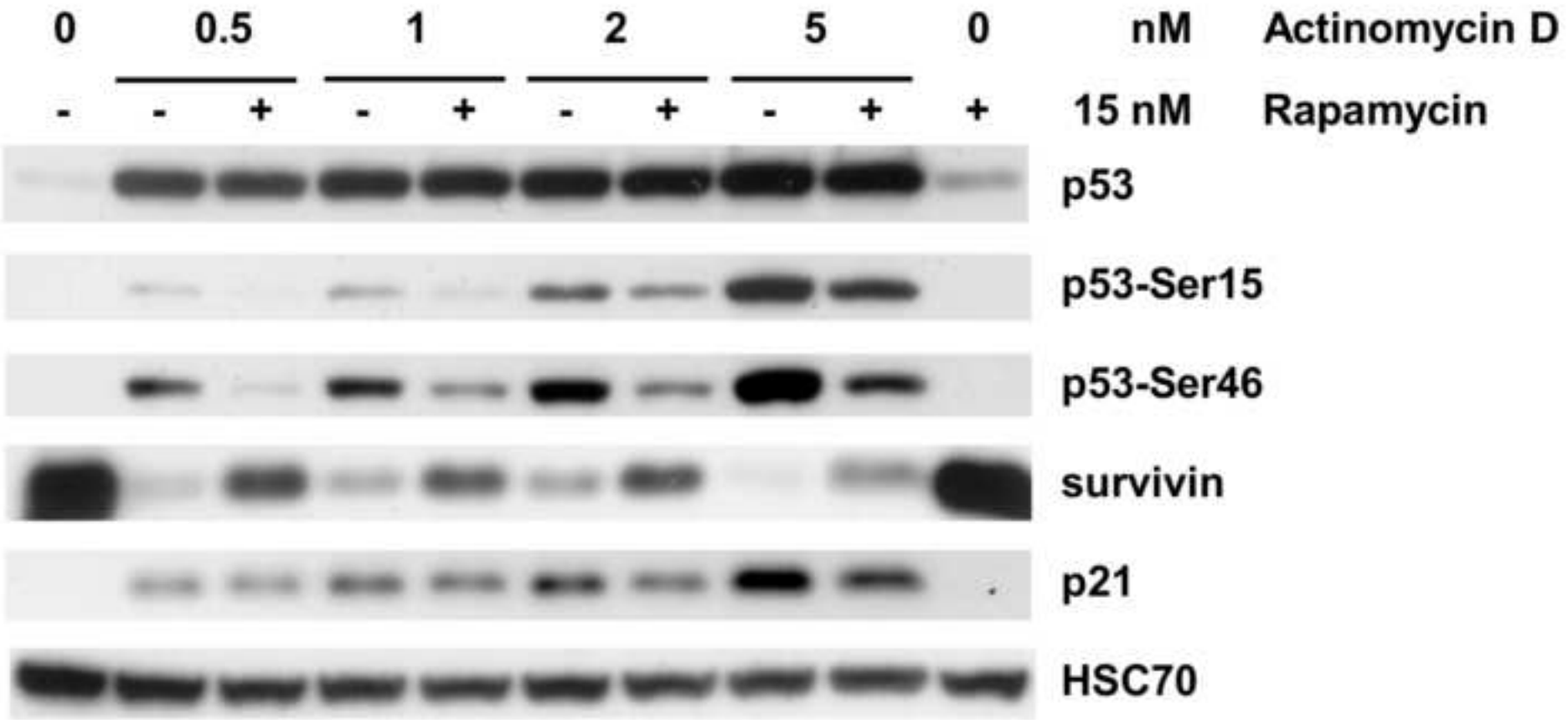

A
U
CPT
CPT Rap
ActD
ActD Rap
Res
Res Rap
AIC
AIC Rap
Rap
p53
p53-Ser15
p53-Ser46
p21
p70S6K-Thr389
HSC70
B
ActD
MK-2206
Rapamycin
p53
p53-Ser46
p21
Akt-Ser473
p70S6K-Thr389
HSC70

Figure 10


0
0.5
1
2
5
0
nM Actinomycin D
-
-
+
-
+
-
+
-
+
+
15 nM Rapamycin
p53
p53-Ser15
p53-Ser46
survivin
p21
HSC70