P53 pathway is a major determinant in the radiosensitizing effect of Palbociclib: implication in cancer therapy

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Abstract

Targeting cell cycle has become one of the major challenges in cancer therapy, being Palbociclib, a CDK4/6 inhibitor, an excellent example. Recently, it has been reported that Palbociclib could be a novel radiosensitizer agent. In an attempt to clarify the molecular basis of this effect we have used cell lines from colorectal (HT29, HCT116) lung (A549, H1299) and breast cancer (MCF-7). Our results indicate that the presence of a p53 wild type is strictly required for Palbociclib to exert its radiosensitizing effect, independently of the inhibitory effect exerted on CDK4/6. In fact, abrogation of p53 in cells with functional p53 blocks the radiosensitizing effect of Palbociclib. Moreover, no radiosensitizing effect is observed in cells with non-functional p53, but restoration of p53 function promotes radiosensitivity associated to Palbociclib. Furthermore, the presence of Palbociclib blocks the transcriptional activity of p53 in an ATM-dependent-fashion after ionizing radiation exposure, as the blockage of p21/WAF1 expression demonstrates. These observations are a proof of concept for a more selective therapy, based on the combination of CDK4/6 inhibition and radiotherapy, which would only benefit to those patients with a functional p53 pathway.

1. Introduction

Cancer therapy has evolved from chemotherapy to novel approaches by targeting specific alterations present in tumours, being protein kinases inhibitors a novel weapon in this therapeutic approach[1]. In this sense, cyclin-dependent kinases (CDKs), prolin-targeted serine/threonine kinases, are key proteins in the control of cell cycle through their interaction with cyclins and the subsequent regulation of cell cycle progression, thus becoming an interesting field in cancer research[2]. Specifically, CDK4/6 control the G1 phase to the S phase transition of cell cycle trough the interaction with Cyclin D[3]. The main target of the CDK4/6-Cyclin D complex is the retinoblastoma gene product (pRB), in which phosphorylation blocks its tumour suppressor function by modulating the activity of the downstream transcription factor E2F[4,5], hence emerging as a very attractive target for cancer therapy[6]. Palbociclib is an specific inhibitor of CDK4/6 that has shown anti-tumour activity in preclinical models[7]. Indeed, its use has been investigated in several pathologies as myeloma[8], lymphoma[9], liposarcoma [10] lung cancer[11], and specially in breast cancer[12], were CDK4/6 inhibitors have become a novel approach to overcome resistance to other current therapies[13]. In addition, combination of Palbociclib with other therapeutic options is showing promising results, as in the case of other kinases or autophagy inhibitors[14–16].

In addition to surgery, one of the main therapeutic options in cancer is the use of ionizing radiation (IR) alone or in combination with chemotherapy. However, resistance to IR is still a main problem in cancer therapy and the search of compounds able to promote radiosensitivity is a major challenge[17]. In this regard, preliminary evidences have shown a potential role for Palbociclib in the modulation of the cellular response to IR by promoting radiosensitivity in different experimental models[18–20]. However,

few clues about the molecular mechanisms involved have been reported, except for the implication of the inhibition of MEK in K-ras mutant models[18], and the recent evidences found about the role of ATM[21]. Therefore, it is necessary to carry out studies to find the molecular determinants that control the radiosensitizing benefits associated to Palbociclib.

In an attempt to clarify this question, we have evaluated *in vitro* the effect of Palbociclib in different human cancer cells lines. Our data show that the tumour suppressor p53 is a critical determinant in the radiosensitizing effect associated to Palbociclib. Interestingly, the effect exerted by Palbociclib onto the p53 signalling pathway is due to the inhibition on ATM activity. In summary, our data support the idea that combination of Palbociclib and radiotherapy could be a promising approach to treat tumours, but only restricted to those patients with a functional p53 signalling pathway.

2. Materials and methods

2.1. Cell lines and plasmids

Lung cancer cell lines (A549 and H1299), colon cancer cell lines (HCT116 and HT-29) and 293T cells were purchased from ATCC (LCG Promochem). The breast cancer cell line MCF-7 was kindly provided by Dr. Alberto Ocaña (Translational Research Unit, Complejo Hospitalario Universitario de Albacete, Spain). GM00637 and GM09607 cell lines were obtained from Coriell Institute for Biomedical Research . Cells were maintained in 5% CO_2 and 37°C. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine plus antibiotics except for GM00637 an GM09607 cell lines that were grown in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, 1% glutamine plus antibiotics and 1% Non-essential Aminoacids (Sigma-Aldrich, {Citation}). All cell culture reagents were provided by Lonza .

Plasmids PLKO.1-puro-shRNAp53 (Sigma SHCLNG-NM_000546), PLKO.1-puro-shRNAp21 (Sigma SHCLNG-NM_000389) and PLKO.1-puro (Sigma SHC001) were obtained from Sigma-Aldrich. pcDNA3 p53 WT was a gift from David Meek (Addgene plasmid # 69003)[22]

2.2. Chemicals and antibodies

Antibodies against phospho-H2AX (Ser139), phospho-p53 (Ser15), phospho-Chk1 (Ser345), Chk1, phospho-pRB (Ser780), pRB, p21 Waf1/Cip1, CDK4 and CDK6 were purchased from Cell Signaling Technologies (Werfen). Antibodies against p53 and Tubulin were purchased from Santa Cruz Technology (Quimigen). Antibody anti-Vinculin was obtained from Sigma-Aldrich.

ATM inhibitor Ku-55933 (Calbiochem, Madrid Spain) and CDK4/6 inhibitors Palbociclib (Selleckchem) and Ribociclib (BioVision) were dissolved in DMSO and stored at -20ºC.

2.3. Transfections and infections

Lentiviral production and cell infection were performed as previously described [23,24]. Briefly, HEK293T cells were transfected overnight by using calcium phosphate with 9 μg of PLKO.1-shRNAp53 or PLKO.1-shRNAp21 or empty vector, plus 6 μg of PSPAX2, and 3 μg of the viral envelope protein, VSVG. Supernatants were collected 48 hours after transfection and added to the cells for 16 hours in the presence of 8 μg/ml polybrene. Forty-eight hours post-infection, cells expressing the shRNAs were selected with puromycin (Sigma-Aldrich) for 48 hours.

2.4. Western Blotting

Cells were collected in lysis buffer (100 mM HEPES, pH 7.5, 50 mM NaCl, 0,1% Triton X-100, 5 mM EDTA, 0,125 M EGTA). Protease and phosphatase inhibitors (Sigma-Aldrich) were added prior to lysis. Protein quantification was performed by using the BCA Protein Assay Kit (ThermoFisher) following the manufacturer's instructions. Indicated amounts of protein were loaded onto appropriate percentage SDS-PAGE, transferred to PVDF membranes with the semi-dry Pierce Power Blot (ThermoFisher) and blotted against different proteins using specific antibodies.

Antibody detection was achieved by enhanced chemiluminescence (Amersham) in a LAS-3000 system (FujiFilm). Results show a representative blot out of three with nearly identical results. Tubulin or Vinculin were used as a loading control.

2.5. Immunocytochemistry

Cells were grown onto SPL cell culture slides (Labclinic), fixed and permeabilized as previously described[25]. Then, samples were incubated overnight with an antibody against H2AX phosphorylated at Ser139 (1/500) and, after extensive wash, incubated for 60 minutes with an Alexafluor 488-conjugated anti-rabbit antibody (Invitrogen Molecular Probes). Subsequently, the samples were incubated for 30 seconds with DAPI (Sigma-Aldrich) and samples were mounted with Fluorosave (DAKO). Positive immuno-fluorescence was detected using a Zeiss LSM-710 confocal microscope. Images were acquired and processed using the Zen 2009 Light Edition program. Images show a representative field out of 5.

2.6. RNA isolation, reverse transcription and Real-time Quantitative PCR

Total RNA was obtained, and reverse transcription was performed as previously described[26] cDNA synthesis was performed with RevertAid First Strand cDNA synthesis Kit (Thermo Scientific) following manufacturer's protocol in a iCycler thermal cycler (Biorad). Real time PCR was performed with Fast SYBR Green Master kit (Thermo Scientific) in a 7500 Fast Real-Time PCR instrument (Applied Byosystems). The PCR conditions were performed as previously described [26]. Primers for all the target sequences were designed by using the NCBI BLAST software. PCR primers were purchased from Sigma-Aldrich. Primers sequences used are as follows: p21: forward 5'-ACTCTCAGGGTCGAAAACGG-3', reverse 5'-CTTCCTGTGGGCGGATTAGG-3'; for p53: forward 5'-TCCCCTGCCATTTTGGGTTT-3' - reverse 5'-GCAGGCCAACTTGTTCAGTG-3'; and for GAPDH: forward 5'-TCGTGGAAGGACTCATGACCA-3', reverse 5'-CAGTCTTCTGGGTGGCAGTGA-3'. Data shown are the average of, at least, three independent experiments performed in triplicate.

2.7. Irradiation and clonogenic assays

Cells were irradiated by the technical staff of the Radiotherapy Unit at the Universitary General Hospital of Albacete, in a Clinac Low Energy 600C linear electron accelerator from Varian (Palo Alto, California, USA) at a dose rate of 600 cGy/min in a radiation field of 40x40 cm. Clonogenic assays were performed as previously described[26]. Briefly, 80 cells/well were seeded in 6-well plates 48 hours prior to irradiation. Sixteen hours before irradiation cells were treated with 1 μM Palbociclib or Ribociclib. Culture medium was replaced 3 hours after IR and refreshed every 3 days until the end of the experiment. Finally, cells were washed with 1X PBS and incubated with a solution containing 0.5% glutaraldehyde and crystal violet (10 mg/ml) for 20 min at room temperature in mild rocking. The plates were photographed and the colonies were counted with the ImageJ plugin "Cell counter". The colonies with less than 5 mm diameter were discarded. Values were referred to untreated controls, set at 1. Data shown are the average of, at least, three independent experiments performed in duplicate cultures.

2.8. Flow cytometry

For cell cycle analysis, $2 \cdot 10^5$ cells were seeded in 10 cm plates, 24 hours later cells were treated for 16 hours with the indicated amounts of Palbociclib or Ribociclib and then trypsinized. Next, cells were washed with PBS, fixed with cold 70% ethanol in PBS at 4ºC and extensively washed in cold PBS. The cells were then incubated with 10 μg/ml propidium iodide (PI) and 20 μg/ml RNase for 20 min in darkness.

Samples were analysed in a MACSQuant Analyzer 10 (Miltenyi Biotec). Data were analysed by using FlowingSoftware (University of Turku).

2.9. Dose-response and cell proliferation measurements

For dose-response assays and cell proliferation measurements, 10^4 cells/well were seeded in 24-well plates and treated 24 hours later. Toxicity in dose-response assays was measured after 72 hours of treatment. Cell proliferation was analysed at 1, 2 and 3 days after plating by an MTT-based assay. Briefly, MTT at 0.5 mg/ml was added to the medium in each well and plates were returned to the incubator for 1 hour. The medium-MTT was then removed, 500 μl DMSO were added to each well, and the plate was kept in agitation, for 5 min in the dark to dissolve the MTT-formazan crystals. The absorbance of the samples was then recorded at 570 nm. Data shown are the average of three independent experiments performed in triplicated cultures.

2.10. Statistical analysis

Data are presented as mean ± standard deviation (S.D). Statistical significance was evaluated by Student's t test or ANOVA using GraphPad Prism software. The statistical significance of differences is indicated in figures by asterisks as follows: *$p < 0.05$, **$p < 0.01$ and ***$p<0.001$.

3. Results

3.1. Palbociclib blocks cell growth and promotes radiosensitivity in MCF-7 cells correlating with ATM signalling pathway inhibition.

To fully elucidate the effect of Palbociclib as a radiosensitizing agent, we chose the experimental breast cancer model of MCF-7, a cell line in which the effects of Palbociclib are well stablished[27]. As it is shown in figure 1, MCF-7 viability diminishes in presence of Palbociclib in a dose- (Fig. 1A) and time- (Sup. 1A) dependent fashion. This decrease correlates with a G0/G1 arrest (Fig. 1B and Sup. 1B) and a decrease in retinoblastoma (pRB) phosphorylation (Fig.1C). Next, we challenged the radiosensitizer potential of Palbociclib in our experimental model of MCF-7, showing a consistent effect (Fig 1D). Recent *in silico* and *in vivo* evidences[21,28] indicate that DNA damage signalling, mainly ATM kinase, could be a novel target of this CDK inhibitor. Therefore, phosphorylation of H2AX and CHK1, known to be mediated by ATM in response to IR at short time points [29,30], was evaluated (Fig 1E and F), supporting a correlation between the radiosensitizing effect of Palbociclib and the inhibition of ATM-dependent signalling. Furthermore, to evaluate the direct implication of ATM, we switched to an experimental model of fibroblasts derived from patients with functional ATM (GM00637) or annulated ATM (GM09607), and the results reinforce the idea that the presence of functional ATM is required for Palbociclib-associated radiosensitivity (Sup. 2).

3.2. Palbociclib radiosensitizing effect is not universal

To prove whether Palbociclib is able to promote radiosensitivity in a number of cell lines representative of different pathologies in which radiotherapy is currently used, we selected two cell lines from lung cancer (H1299 and A549) and two from colorectal

cancer (HCT116 and HT-29). Therefore, we challenged the radiosensitizing effect of Palbociclib in our experimental models of lung and colon cancer derived cell lines. As it is shown in Figure 2, pre-treatment for 16 hours with 1μM Palbociclib, promotes marked radiosensitivity in A549, HCT116 but not in HT-29 and H1299 cell lines.

3.3. Palbociclib is a universal CDK4/6 inhibitor.

In light of our previous findings, we decided to study if the different radiosensitizing effect observed could be mediated through a differential response to Palbociclib. Initially, we analysed the endogenous expression of CDK4/6 by western blotting and RT-qPCR in all the cell lines, observing similar expression levels of CDK4 among them, except for the high levels detected in MCF-7 (Fig. 3A and Sup. 3A). In the case of CDK6, HT-29 and H1299 cell lines displayed higher expression levels compared to the rest of cell lines at both RNA and protein levels (Fig. 3A and Sup. 3A). However, treatment with Palbociclib induced cell cycle arrest, loss of pRB phosphorylation and decrease of cell growth (Fig. 3B and C and Sup. 3B) in a similar fashion in all the cell lines tested regardless of the radiosensitizing effect observed.

3.4. Neither ATM nor CDK4/6 explain the lack of radiosensitizing effect associated to Palbociclib in HT-29 and H1299 cell lines.

Therefore, we assessed the activity of DNA damage signalling in these experimental models by evaluation of the H2AX and CHK1 phosphorylation. As it is shown, all the cell lines showed normal response after IR and a subsequent blockage of the pathway upon Palbociclib treatment (Fig. 4A and B), excluding a deficiency in the early DNA damage response, mainly dependent on ATM, as a mechanism to explain the lack of radiosensitizing effect.

To fully discard the role of CDK4/6, particularly considering the higher level of expression of CDK6 in H1299 and HT-29 cells compared to A549, HCT116 and MCF-7 cell lines, we took advantage of the availability of another well-established CDK4/6 inhibitor, namely Ribociclib[31]. As it is shown, Ribociclib was unable to promote radiosensitivity in two experimental models such as A549 and HT-29 (Fig. 5A) in spite of being able to affect cell cycle, pRB phosphorylation and cell growth (Fig. 5B, C and Sup. 4A) in a similar fashion to Palbociclib. Furthermore, Ribociclib was unable to affect H2AX and CHK1 phosphorylation (Fig. 5D and Sup. 4B). In fact, similar results were obtained in MCF-7, HCT116 and H1299 cell lines (data not shown).

Therefore, the lack of radiosensitizing effect associated to Palbociclib in cell lines as HT-29 or H1229, in which DNA damage response and CDK4/6 activity was clearly inhibited by the drug, suggests the existence of other determinants to induce this effect.

3.4. P53 is a key determinant of Palbociclib-associated radiosensitivity.

The p53 tumour suppressor protein is known to be a critical effector of ATM-mediated DNA damage response effects and the above results suggest an apparent correlation between the presence of wild type p53 and the radiosensitizing effect of Palbociclib. To fully prove this apparent correlation, we knocked down p53 in A549 cells by shRNA. After achieving an effective interference at the mRNA and protein levels (Sup. 5A), cells with reduced p53 expression showed a total loss of the radiosensitizing effect after Palbociclib exposure, but with no effect onto cell cycle distribution or pRB phosphorylation (Fig. 6A and B). In fact, similar results were obtained in MCF-7 (data not shown). In the case of HCT116, we switched to the isogenic experimental model of HCT116 $p53^{+/+}$ and $p53^{-/-}$ colorectal cancer cells[32] obtaining the same result as in the case of the A549 cell line (Sup. 6A and B). Next, we decided to challenge if restoration

of p53 function could promote the radiosensitizing effect of Palbociclib. To this end, we overexpressed p53 in a null context, as is the H1299 cell line (Sup. 5B). Interestingly, the presence of p53 wt renders radiosensitivity in the presence of Palbociclib without effect onto the cellular response to Palbociclib (Fig. 6C and D).

In light of our findings, we decided to evaluate whether ATM could be mediating the effects of Palbociclib onto p53 signalling axis by means of p53 Ser15 phosphorylation, a known target of ATM kinase activity implicated in DNA damage[33]. To this end, we took advantage of the high expression level of p53 in HT-29 and 293T cells, in which p53 is easily detected due to mutation or by the presence of viral proteins [34,35]. As it is shown in figure 7, p53 Ser15 phosphorylation was clearly increased in both cell lines after exposure to IR. However, pre-treatment with Palbociclib dramatically reduced Ser15 phosphorylation in a similar extent to Ku5993, a well-known inhibitor of ATM kinase activity (Fig.7A and B), suggesting that ATM exerts its effect in Palbociclib-associated radiosensitivity through the control onto the p53 signalling axis. Finally, we challenged how Palbociclib affects p53 activity. Among the several targets of p53, we chose the cell cycle inhibitor p21/WAF, known to be activated by IR in a p53-dependent fashion[36]. As it is shown in figure 7, pre-treatment with Palbociclib blocks p21/WAF induction at the protein and RNA levels in A549 cells after IR exposure (Fig. 7C and D) Furthermore, the interference of p21/WAF in A549 cells (Fig 7E) was able to block the radiosensitivity associated to Palbociclib (Fig. 7F). In fact, similar results were obtained in MCF-7 and HCT116 cell lines (Sup. 7 and 8).

In sum, this set of experiments unequivocally demonstrate the important role of p53 signalling axis in the radiosensitivity associated to Palbociclib.

4. Discussion.

Several conclusions can be obtained from this study:

The most obvious conclusion is that Palbociclib has a radiosensitizing effect independently of its activity as a CDK4/6 inhibitor. This observation was inferred from two key experiments. First, the similar effect observed in the different experimental models analysed, in which Palbociclib induces a comparable cell cycle arrest and a blockage of pRB phosphorylation, although only in some cases it is unable to promote radiosensitivity. Second, the use of Ribociclib, unable to promote radiosensitivity but being able to block cell cycle and pRB phosphorylation in the same extent than Palbociclib, definitively discarding the inhibitory effect on CDK4/6 as the mechanism of radiosensitivity. Our observations are in agreement with recent evidences[21] and opposite to another work proposing that CDK4 annulation promotes radiosensitivity[37]. However, several differences should be considered between this work and our report. For example, the use of cell lines versus cancer stem cells, the different origin of the cells used and the distinct CDK4 inhibitor used could account for the dissimilar result observed. Anyway, our data support the lack of implication of the inhibitory effect exerted onto CDK4 and CDK6 in the radiosensitivity associated to Palbociclib.

Second, the present report indicates that the ATM signalling pathway is a major determinant of radiosensitivity mediated by Palbociclib in agreement with recent works[21]. This is an interesting issue, reinforcing the critical role of ATM in the response to IR. However, it also shows the existence of “off target effects” for Palbociclib, unrelated to the CDK inhibitory function, which could affect other cellular responses. In this regard, and at least in terms of p53 Ser15 phosphorylation, Palbociclib inhibits ATM function in a similar fashion that the specific inhibitor Ku5995 [38]. Nonetheless, further studies are necessary to elucidate whether the effect of Palbociclib

is limited to ATM or other key molecules in the DNA damage response as ATR or DNAPK could also be affected by this CDK4/6 inhibitor[39].

In addition, our report adds a new player to this intriguing puzzle, the tumour suppressor p53, a key molecule in cancer[40]. Interestingly, our data support that the presence of a functional p53 is a critical requirement for the Palbociclib-associated radiosensitivity in a context of a functional ATM signalling pathway. This observation is extremely important considering that p53 is the most commonly mutated gene in cancer as well as one of the major targets of ATM after DNA damage[41]. P53 connection with the cellular response to IR has been known for more than 30 years and its lack of activity has been related to radioresistance [42,43]. Therefore, the lack of p53 function and the appearance of radiosensitivity instead of resistance in the presence of Palbociclib could be an apparent contradiction. This seeming discrepancy can be easily explained by the context of the presence or absence of a functional ATM signalling pathway. In fact, it is important to mention that other signalling pathways implicated in the cellular response to IR, vg. Akt[44], are modulated by ATM, which could account for the appearance of radiosensitivity or resistance. In addition, our data indicate that the effect of Palbociclib onto p53 activation renders a diminished transcriptional activity, as the expression levels of p21/WAF, a direct transcriptional target of p53[45], indicate. In keeping with this, we have been able to observe that the interference of p21/WAF abolishes the effect of Palbociclib in terms of radiosensitivity, suggesting that downstream targets of p53 could also have a critical role in Palbociclib-associated radiosensitivity.

From the clinical point of view, the use of p53 as a biomarker could be an ideal tool to classify patients for future combination of CDK4/6 inhibitors and radiotherapy. In this regard, p53 is a perfect candidate because its genetic analysis is much more affordable than ATM, a very big gene. Regarding p21/WAF, it seems to be an unlikely candidate

for future screening tests due to the low frequency of mutations detected on it [46,47]. However, this screening would only have full sense for CDK4/6 inhibitors able to promote radiosensitivity, such as Palbociclib and, more recently, Abemaciclib [48], but not for others, such as Ribociclib. Finally, it is important to mention that future studies are necessary to evaluate the effect of Palbociclib in non-transformed cells in order to identify possible side effects associated to the combination of Palbociclib plus radiotherapy. On this subject, Palbociclib has been shown to protect from side effects associated to radiotherapy in normal tissue [49,50] reinforcing the use of Palbociclib in combination with IR.

In sum, our data support unequivocally that Palbociclib is a novel radiosensitizer agent that strictly requires a functional ATM→p53→TARGETS signalling axis. These molecules, especially p53, could be ideal biomarkers allowing choosing the right patients for the combination of Palbociclib plus radiotherapy as a therapy for cancer.

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CONFLICT OF INTEREST

Authors declare that there are no competing financial interests in relation to the work described.

Figure Legends

Fig 1. **Palbociclib promotes radiosensitivity in MCF-7 cells correlating with ATM inhibition**.

A) MCF-7 cells were treated with Palbociclib for 48 hours at the indicated concentrations and viability was assessed by MTT. Bars mean standard deviation (S.D).

B) MCF-7 cells were exposed to Palbociclib 1 μM for 16 hours and cell cycle was evaluated by flow cytometry. Histogram shows a representative experiment out of 3, with nearly identical results. C) MCF-7 cells were exposed to Palbociclib at the indicated concentrations for 16 hours and total cell lysates were collected. Then, protein extracts (50 μg) were blotted against the indicated antibodies. Vinculin was used as a loading control. Image shows a representative blot out of 3 with nearly identical results. D) Clonogenic assays for MCF-7 cells exposed to the indicated doses of X rays. Cellular radiosensitivity was plotted using control cells (DMSO) versus pre-treated cells for 16 hours with 1 μM of Palbociclib. Bars mean standard deviation (S.D). E) MCF-7 cells were treated with Palbociclib (10 μM) for 16 hours prior to irradiation (10 Gy). Then, samples were collected at the indicated times, processed as in B and protein extracts (50 μg) were blotted against the indicated antibodies. Vinculin was used as a loading control. Image shows a representative blot out of 3 with nearly identical results. F) MCF-7 were plated onto cell culture slides 48 hours prior to irradiation (10 Gy). Sixteen hours before irradiation, cells were pre-treated or not with Palbociclib (10 μM). Thirty minutes after irradiation cells were fixed and processed for immunocytochemistry against phospho-H2AX (Ser139). Scale bars represent 25 μm. Images show a representative field out of 5 of at least 2 experiments.

Fig 2. **Palbociclib promotes radiosensitivity in HCT116 and in A549 but not in HT-29 and H1299 cell lines**.

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Fig 3. **Functionality of Palbociclib in cell lines derived from lung and colorectal cancer.**

A) Total cells lysates from the different cell lines were collected and protein extracts (X µg) were blotted against the indicated antibodies. Vinculin was used as a loading control. Image shows a representative blot out of 3 with nearly identical results. B) A549, H1299, HCT116 and HT-29 cells were exposed to 1 µM Palbociclib for 16 hours and cell cycle was evaluated by flow cytometry. Histograms show a representative experiment out of 3, with nearly identical results. C) A549, H1299, HCT116 and HT-29 cells were exposed to Palbociclib at the indicated concentrations for 16 hours and total cell lysates were collected. Then, 50 µg of protein extracts were blotted against the indicated antibodies. Vinculin was used as a loading control. Images show a representative blot out of 3 with nearly identical results.

Fig 4. **Palbociclib blocks ATM signalling independently of its radiosensitizing effect.**

A) A549, H1299, HCT116 and HT-29 cells were exposed or not to Palbociclib at 10 µM for 16 hours prior to irradiation (10 Gy). Then, total cell lysates were collected at the indicated times and protein extracts (50 µg) were blotted against the indicated antibodies. Vinculin was used as a loading control. Images show a representative blot out of 3 with nearly identical results. B) A549, H1299, HCT116 and HT-29 cells were plated onto cell culture slides 48 hours prior to irradiation (10 Gy). Sixteen hours before irradiation, cells were pre-treated or not with Palbociclib (10 µM). Thirty minutes after irradiation cells were fixed and processed for immunocytochemistry against phospho-H2AX (Ser139). Scale bars represent 25 µm. Images show a representative field out of 5 of at least 2 experiments.

Fig 5. **Ribociclib is not able to promote radiosensitivity but is functional in cell lines derived from lung and colorectal cancer.**

A) Clonogenic assays for A549 and HT-29 cells exposed to the indicated doses of X rays. Cellular radiosensitivity was plotted using control cells (DMSO) versus pre-treated cells for 16 hours with 1 μM of Ribociclib. Bars mean standard deviation (S.D). B) A549 and HT-29 cells were exposed to 1 μM Ribociclib for 16 hours and cell cycle was evaluated. Histograms show a representative experiment out of 3, with nearly identical results. C) A549 and HT-29 cells were exposed to Ribociclib at the indicated concentrations and total cell lysates were collected. Then, protein extracts (50 μg) were blotted against the indicated antibodies. Vinculin was used as a loading control. Images show a representative blot out of 3 with nearly identical results. D) A549 and HT-29 cells were plated onto cell culture slides 48 hours prior to irradiation (10 Gy). Sixteen hours before irradiation, cells were pre-treated or not with Palbociclib (10 μM). Thirty minutes after irradiation cells were fixed and processed for immunocytochemistry against phospho-H2AX (Ser139). Scale bars represent 25 μm. Images show a representative field out of 5 of at least 2 experiments.

Fig.6. **p53 functionality is required for Palbociclib-associated radiosensitivity but not for Palbociclib cell cycle effects.**

A) Upper panel: Clonogenic assays for A549 cells infected with empty vector pLKO-puro (E.V.). Cellular radiosensitivity was plotted using control cells (DMSO) versus pre-treated cells for 16 hours with 1 μM of Palbociclib. Bars mean standard deviation (S.D). Mid panel: A549 E.V. cells were exposed to 1 μM Palbociclib for 16 hours and then cell cycle was evaluated by flow cytometry. Histogram shows a representative experiment out of 3, with nearly identical results. Lower panel: A549 E.V. cells were

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Fig. 7. **Palbociclib blocks transcriptional activity of p53 and modulates Ser15 phosphorylation.**

A) HT-29 cells were exposed to Palbociclib or Ku-55933 for 16 hours at the indicated concentrations, prior to irradiation (10 Gy) and 3 hours after irradiation samples were collected. Then, 100 µg of protein extracts were blotted against the indicated antibodies. Vinculin was used as a loading control. Images show a representative blot out of 3 with nearly identical result. B) Same as in A for 293T cells. C) A549 cells were exposed to Palbociclib (10 µM) or vehicle (DMSO) for 16 hours prior to irradiation (10 Gy). Once irradiated, total cell lysates were collected at the indicated times. Then, 50 µg of protein extracts were blotted against the indicated antibodies. Tubulin was used as a loading control. Image shows a representative blot out of 3 with nearly identical results. D) A549 cells were treated as in C and RNA samples were collected 3 hours after irradiation. Next, p21 mRNA was evaluated by qRT-PCR referred to unirradiated cells. Bars mean standard deviation. E) Total cell lysates from A549 cells infected with Empty vector (E.V.) and A549 sh-p21 cells were collected and protein extracts were blotted against p21/WAF. Tubulin was used as a loading control. Image shows a representative blot out of 3 with nearly identical results. F) Clonogenic assays for A549 E.V. (left panel) and A549 sh-p21 cells (right panel) exposed to the indicated doses of X rays.

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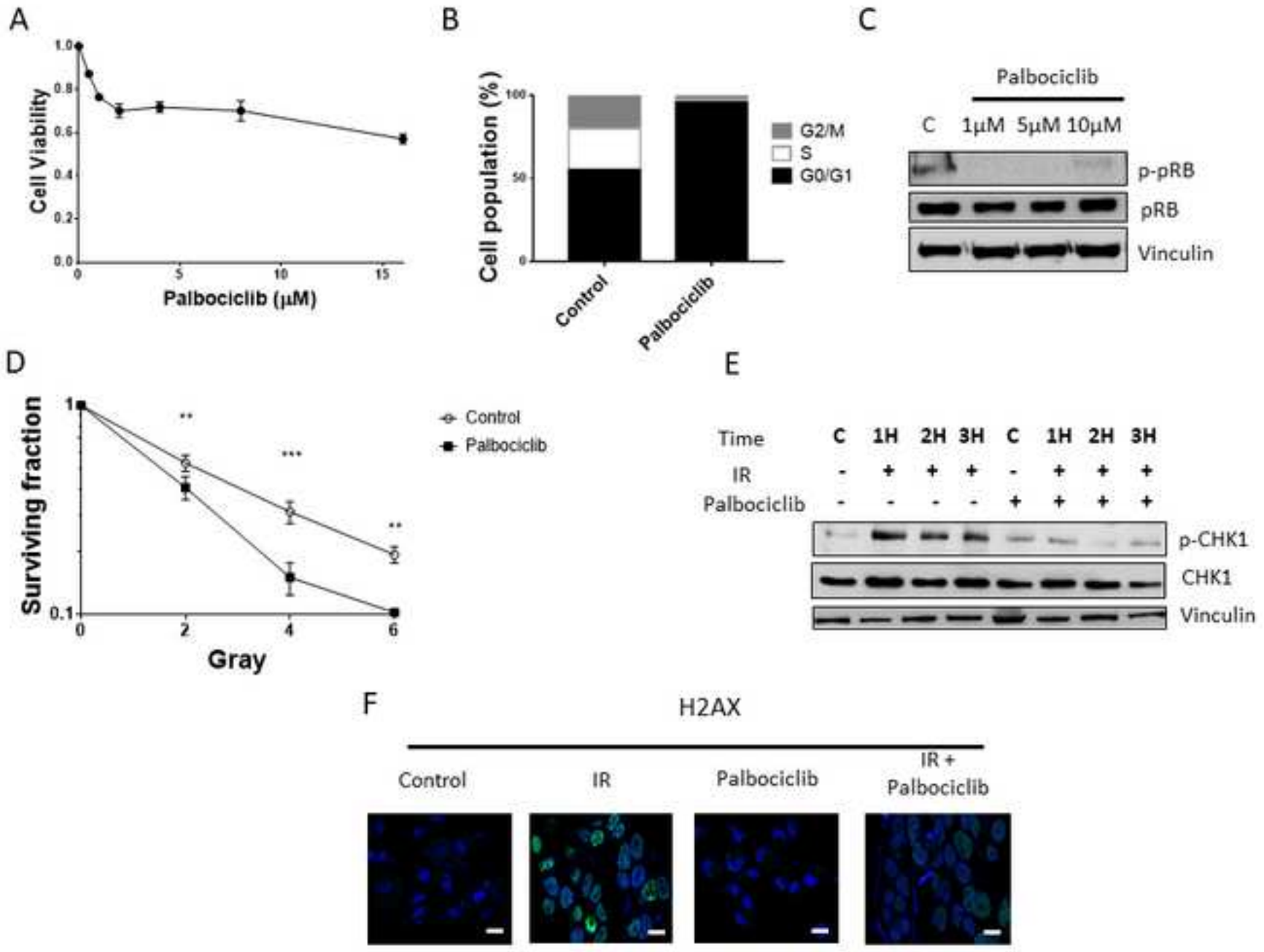

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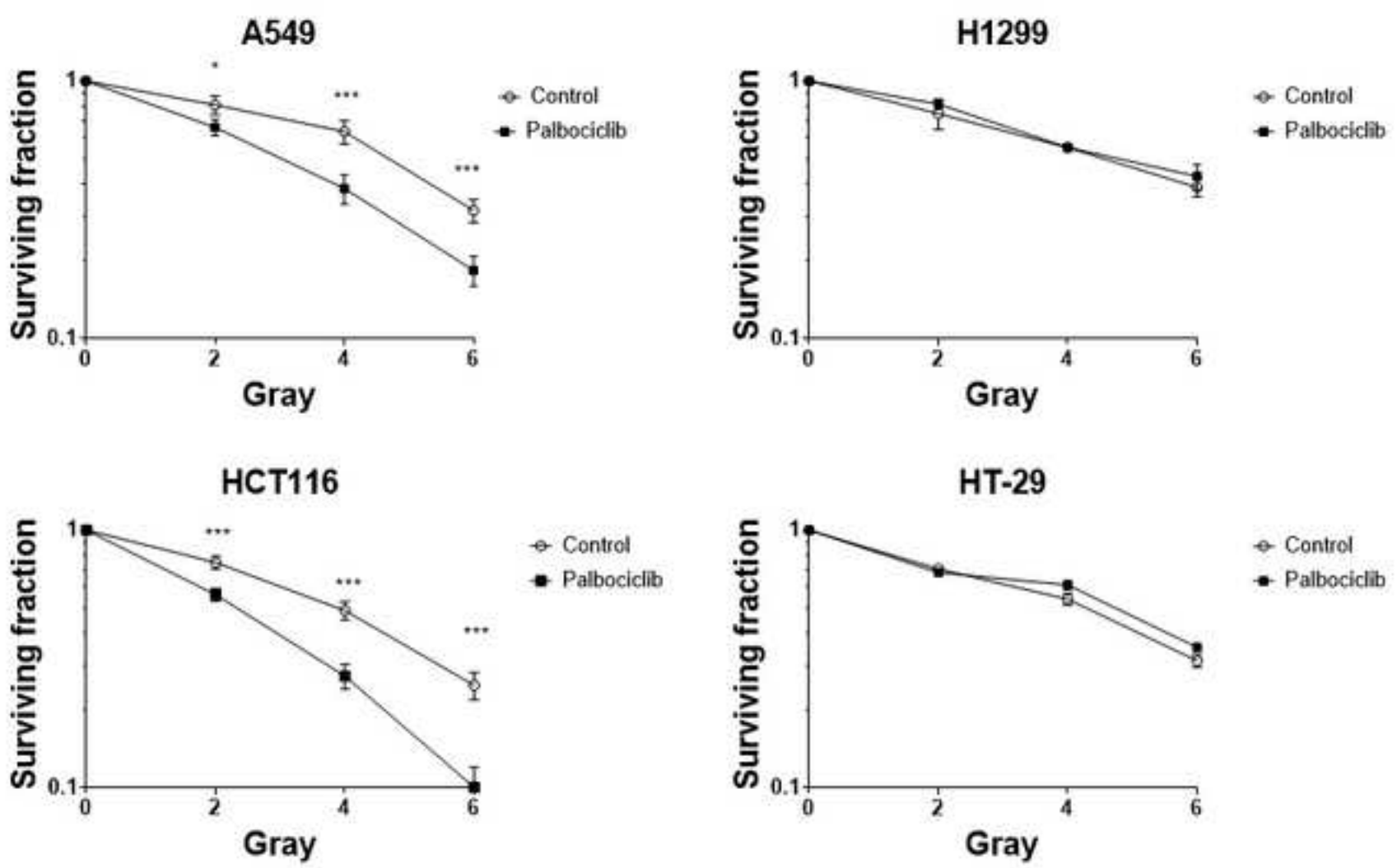
Fig 2



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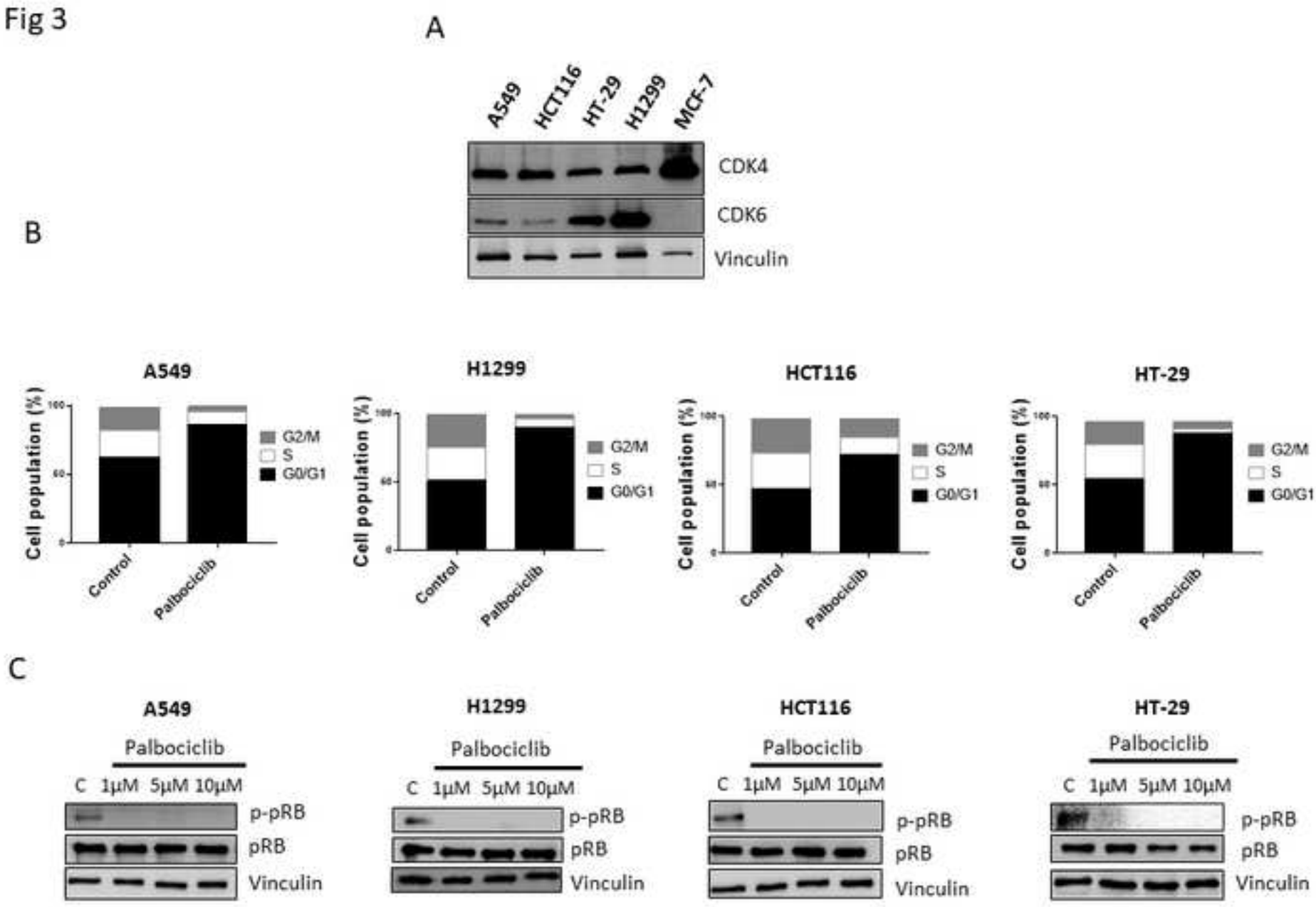

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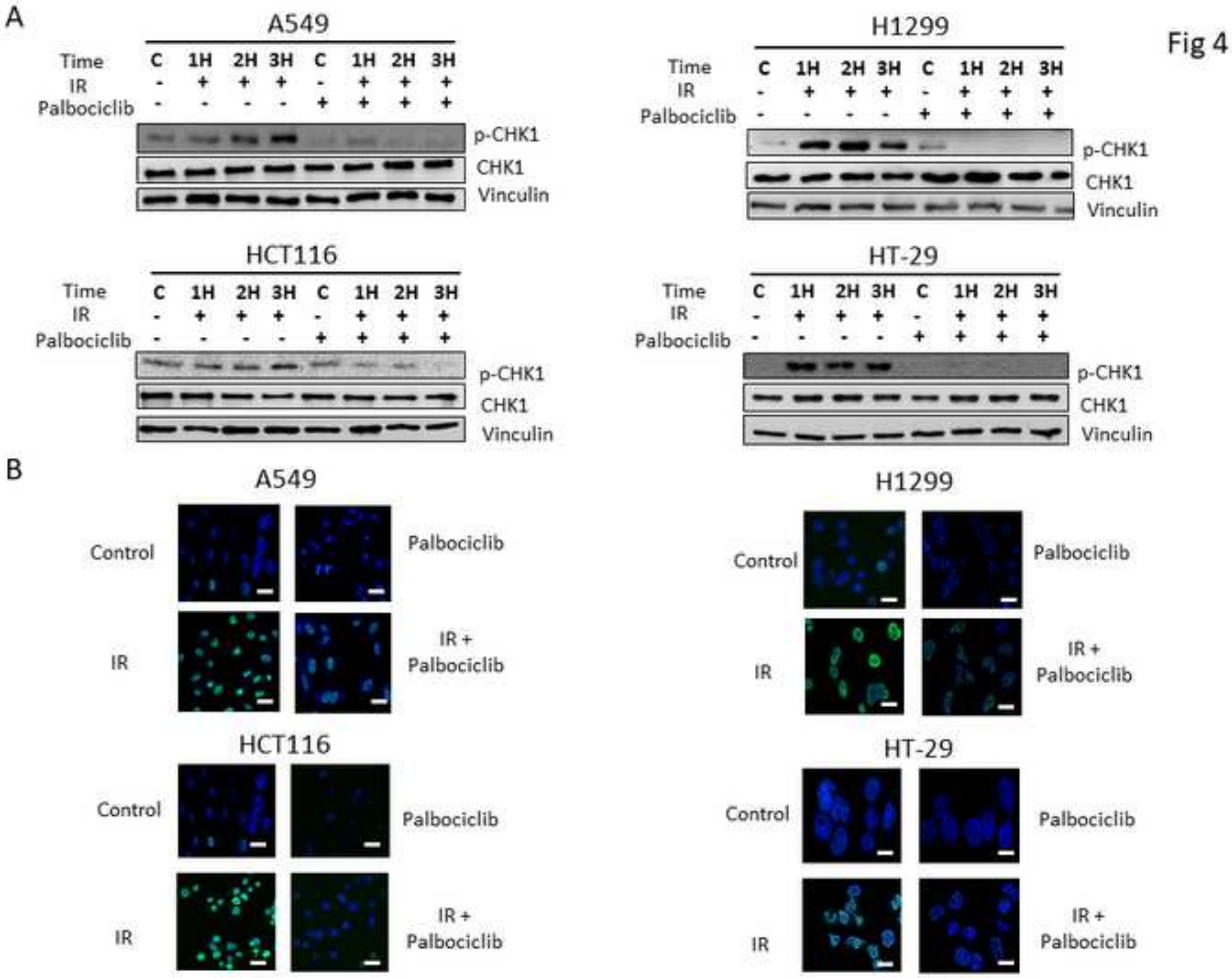

Fig 4

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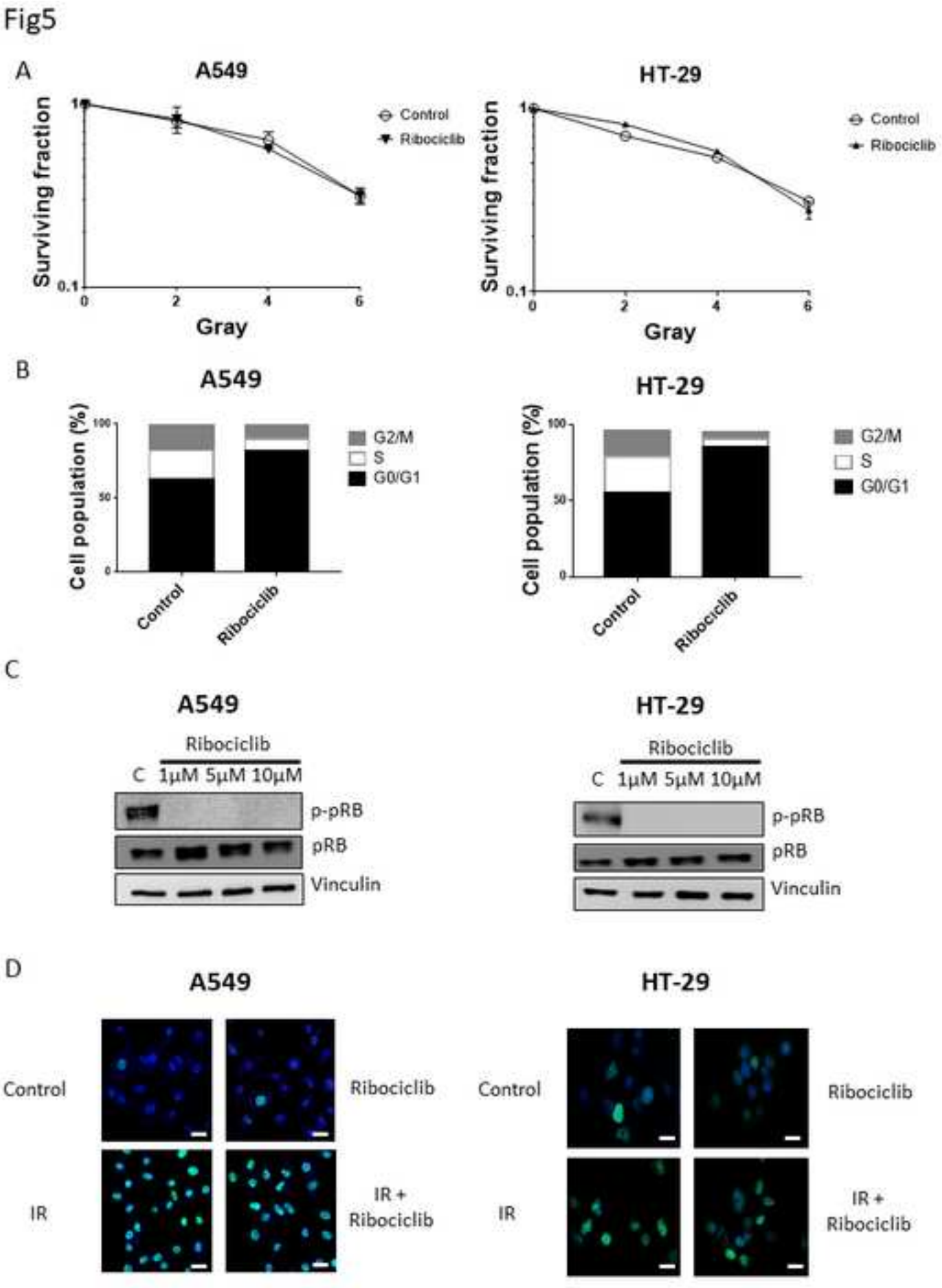

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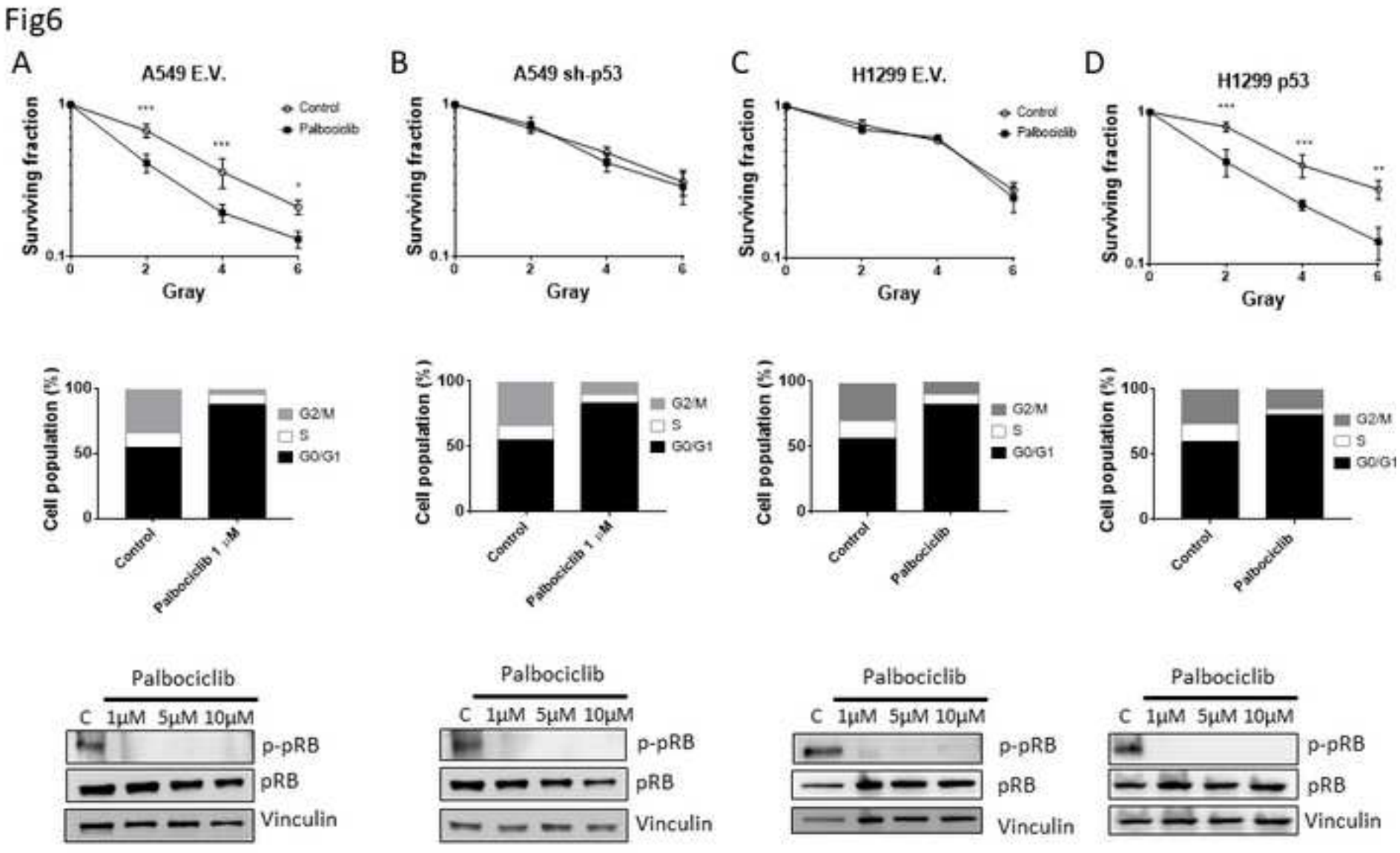

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