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**Drugs down-regulating E2F-1 expression
hinders cell proliferation
through a p53-independent mechanism**

Presentata da: Daniela Pollutri

Coordinatore Dottorato

Chiar.mo Prof.

Sandro Grilli

Relatore

Chiar.mo Prof.

Massimo Derenzini

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1. Introduction

1. E2F-1 transcription factor

E2F-1 is a transcriptional factor that plays a pivotal role in regulating cell-cycle progression by modulating the timely expression of target genes whose products are required for S phase entry, as Cyclin E, and for S phase-events, including dihydrofolate reductase (DHFR), thymidine kinase (TK), and DNA polymerase α (Pol α). These genes are coordinately regulated during the cell growth cycle, being induced in late G1 and S phases, at a time when active E2F-1 accumulates. (Müller H *et al.* 2001; Polanger S, Ginsberg D. 2009; Kowalik TF *et al.* 1995).

For this reason, E2F-1 transcription activity regulation appears to be the most important mechanism controlling cell cycle progression at G1/S check-point level and its activity must be tightly regulated in a cell cycle phases-dependent manner to allow gene expression to be closely coupled with cell-cycle position (Marti A *et al.* 1999).

1.1 E2Fs family

E2F-1 belongs to a family of transcriptional factor composed of 8 proteins (E2F-1 to E2F-8) encoded by distinct genes, overall referred to as E2Fs. These factors are central regulators of cell cycle progression regulating the timely expression of genes involved in mitosis, chromosome segregation, mitotic spindle checkpoints, DNA repair, chromatin assembly/condensation, apoptosis, differentiation and development. (Kel AE *et al.* 2001; Mundle SD *et al.* 2003; Reluca V *et al.*

1997, Wunderlich M and Berberich SJ. 2002). Based on structure and function studies, E2F family members have been divided into two main subclasses: activator and repressor E2Fs. E2F-1, E2F-2, and E2F-3 are potent trans-activators that bind and activate target genes promoters, while E2F-4 and E2F-5 are members of the repressor subclass. While activator E2Fs expression is timely regulate in response to growth stimulation, and their activities peak during G1/S, repressor E2Fs are constitutively and during G0 associates in complexes that repress transcription of target genes. During G0, both the subclasses associate with a class of proteins, “pocket proteins” resulting in their repression or activation respectively. Upon re-entry into the cell cycle, pocket proteins inactivation results in release of activators E2Fs and disruption of repressor complexes. E2F-6, E2F-7, and E2F-8 also contribute to gene silencing, but in a manner that is independent of pocket proteins. (Timmers C *et al.* 2007). Overall, E2Fs play a critical role in determining the extent and precise timing of target gene expression during cell cycle entry via an interesting interplay between repressor and activators members. Whereas repression and de-repression appear to be the principal mechanism of setting the overall pattern of expression, transcriptional activation would serve to accentuate and define the precise timing of E2F target expression at the G1-S boundary (Timmers C *et al.* 2007). With the exception of E2F7/8, these transcription factors function as heterodimers with members of the DP family (DP1 and DP2),

with the DNA-binding specificity being determined by the E2F subunit (Bell and Ryan, 2004).

1.2 E2F-1 regulation

Since its crucial role in timely control the cell cycle progression, E2F-1 is itself tightly controlled by different mechanisms. Retinoblastoma protein binding to E2F-1 regulates its trans-activation activity while E2F-1 protein levels are regulated both at transcriptional level and by the proteasome-dependent degradation pathway. Moreover, there's evidence that human Mouse Double Minute 2 protein (MDM2) binding increases E2F-1 stability (Zhang Z *et al.* 2005).

Activity regulation: pRb-inhibitory binding

The Retinoblastoma protein (pRb), encoded by *RB1* gene, belongs to a family of nuclear proteins collectively called pocket proteins that share a common structural unit (the pocket) which is critical for protein-protein functional interaction (Martelli F *et al.* 1999). All three pocket proteins bind and regulate one or more E2F –DP heterodimers. Among them, pRb has largely characterized as tumor suppressor protein for its ability to control proliferation by modulating E2F-1 activity.

During the cell cycle, pRb undergoes a switch active/inactive by phosphorylation. In quiescent cells, hypo-phosphorylated (active) pRb binds and inhibits E2F-1, preventing its trans-activation, and as a result,

inhibiting entry in S phase. When the cell is prompt to proliferate, Cyclin D increase and bind Cyclin dependent kinases 4 and 6 (Cdk4 and Cdk6), to form active complex that progressively phosphorylate pRb, in a stepwise manner that subsequently involves also Cyclin E-Cdk2 complex. As only the hypo-phosphorylated form of pRb binds and inhibits E2F-1, pRb phosphorylation by Cdk4 and Cdk6 complexes, finally results in release of free, transcriptional active, E2F-1, allowing to cell-cycle S-phase entry. (Udayakumar T *et al.* 2010; Vandel L, Kouzarides T. 1999, Mundle SD *et al.* 2003). (Fig.a).

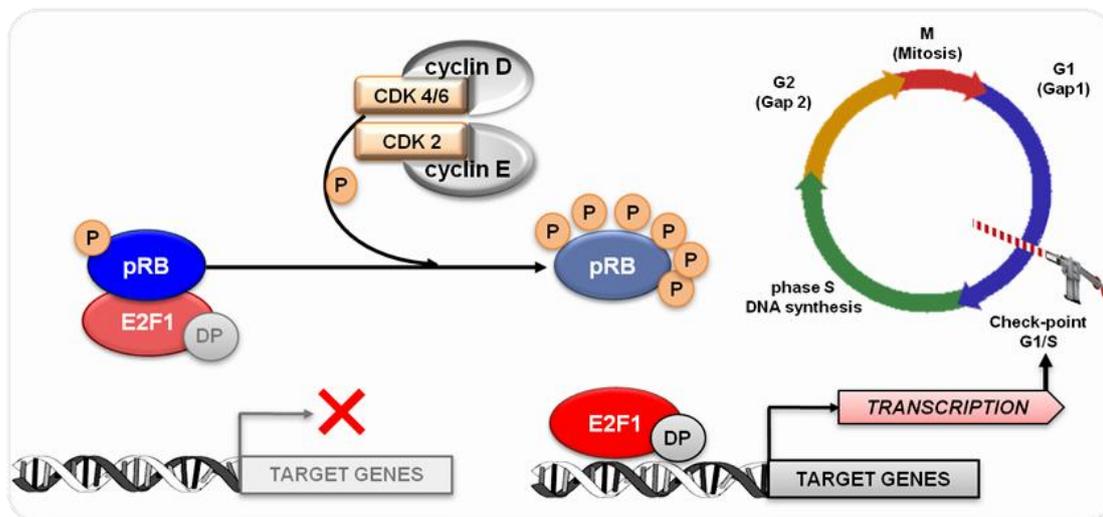


Fig.a Inhibition of E2F-1 transactivation by direct binding of hypo-phosphorylated Retinoblastoma protein. The transcriptional ability of E2F is cell cycle-regulated mainly through association with the retinoblastoma tumor suppressor proteins (pRb). During the cell cycle, pRb undergoes a switch active/inactive by phosphorylation. In quiescent cells, hypo-phosphorylated (active) pRb binds and inhibits E2F-1, preventing its transactivation. Upon mitogenic stimuli, Cyclin dependent kinases 4 and 6 (Cdk4 and Cdk6), each one complexed with Cyclin D, progressively phosphorylate pRb. As only the hypo-phosphorylated form of pRb binds and inhibits E2F-1, pRb phosphorylation by Cdk4 and Cdk6, results in release of free, transcriptional active, E2F-1, allowing expression of genes required for progression into the S phase.

Indeed, E2F-1 transactivation activity peaks at G1/S and early S and decreases in late S. The importance of the pRb/E2F-1 pathway for normal control of cell proliferation is highlighted by evidence that this regulation is inactivated in most types of tumor cells (Weinberg, 1995; Dyer and Bremner, 2005; Tsantoulis and Gorgoulis, 2005; Burkhart and Sage, 2008).

Transcriptional regulation

Another level of regulation of E2F-1 function is reflected by its cell cycle-dependent synthesis. There is evidence that the expression of E2F-1 protein, similarly to other cell cycle regulatory proteins, is closely related to the cell cycle phases. In G0 phase, E2F-1 gene expression is barely detectable. Upon mitogen stimuli, as cells approach late G1, early increasing regulator proteins appear to stimulate E2F-1 transcription leading to a progressive accumulation that reach its highest value in S phase (Marti *et al.*, 1999, Mundle *et al.* 2003, Jonson DG *et al.* 2014). Conversely, transcription of the E2F-1 gene decreases in late S and is kept at lower level till next cycle began.

Among positive regulators, cMyc transcriptional factor, D-type Cyclins and Cyclin E are reported to modulate E2F-1 expression by activating its promoter. Interestingly, E2F-1 itself is described to activate its transcription by binding E2F sites within its own promoter (Coller HA *et al.* 2007; O'Donnell KA, 2005; Jonson DG *et al.* 2014).

On the other hand, E2Fs of repressor subclass bind E2F sites and keeps target genes transcriptionally repressed in G0 and early G1. At this regard, an E2F DNA-binding complex containing the p130 protein and one or more repressor E2Fs, have been described to inhibit E2F-1 transcription by repressing its promoter, and recent experiments suggests that pRb does not simply negatively regulates E2F-1 activity but, rather, can repress its expression (Jonson DG *et al.* 2014, Weintraub M *et al.* 2002).

Ubiquitin-mediated degradation

The correct progression through the phases of cell cycle requires a strictly temporal control of key regulators so they can in turn, regulate the gene expression properly.

In mammalian cells, ubiquitin-mediated degradation is one among most important mechanisms to regulate protein amount, and it is particularly used to modulate cell cycle-related transcriptional regulators, by rapidly reducing their level when their function is not longer required. The process of the ubiquitin-mediate degradation starts with the addition of multiple molecules of ubiquitin to target proteins, to produce a poly-ubiquitin chain tag which is recognized by proteasome machinery as signal to destroy the target protein. The poly-ubiquitination reaction is carried out in three main steps, activation, conjugation and ligation, and requires the coordinate action of three enzymes, each one with a specific

function: an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2 and an ubiquitin-ligase enzyme E3. The high specificity of ubiquitin-mediated degradation is mediated by E3 enzyme that has a key role in substrate recognition.

Like other key regulators of the cell-cycle, E2F-1 levels are tightly regulated by this mechanism. Following its accumulation in the late G1 phase of the cell cycle, E2F-1 is rapidly degraded in S/G2 phase and is kept at low levels until the next G1/S phase transition. This event is linked to a specific interaction of E2F-1 with the p45Skp2 (Skp2) component of ubiquitin-ligase complex SCF^{Skp2}, which catalyzes E2F-1 ubiquitination, thus marking it for degradation by the 26S proteasome. Disruption of the interaction between E2F-1 and Skp2 results in a reduction in E2F-1 ubiquitination leading to stabilization and accumulation of transcriptionally active E2F-1 protein (Marti A *et al.* 1999; Harper JW and Elledge SJ, 1999).

MDM2 stabilizing binding

Even if E2F-1 is characterized by high instability, there are evidences that Mouse Double Minute 2 protein (MDM2) is able to prolong the half-life of E2F-1 by inhibiting its proteasome-dependent degradation. In fact, in a study of 2005, Zhang Z. *et al.* have observed that E2F-1 accumulates upon MDM2 over-expression, while MDM2 inhibition is associated with a decrease of E2F-1 protein level. In this study, it was then

demonstrated that MDM2 directly binds E2F-1 preventing its interaction with E3 ligase SCF^{Skp2} by competition for the same binding site. As a result, the attachment of ubiquitin to E2F-1 is inhibited (Zhang Z *et al.* 2005). Since the presence of a poly-ubiquitin chain is essential for the specific degradation of a protein by the proteasome, MDM2 binding to the E2F-1 result in the inhibition of its degradation (Zhang Z *et al.* 2005). The mechanism described above is schematized in figure b.

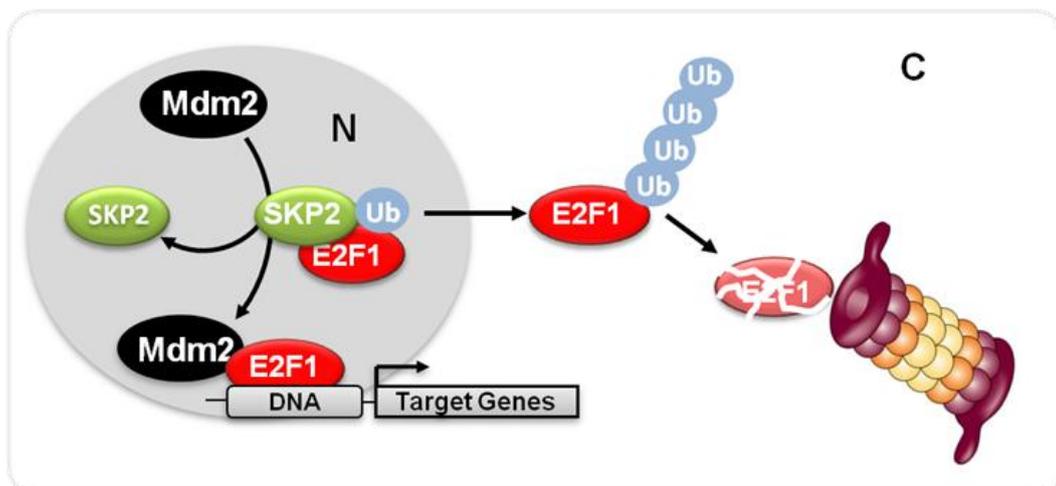


Fig.b Schematic representation of proteasome-dependent degradation of E2F-1 and stabilizing function of MDM2. SCF^{skp2} protein-ligase (SKP2) interacts with E2F-1 and catalyzes the addition of ubiquitin molecules (Ub) to form a poly-ubiquitin chain, which is the signal for degradation by 26S proteasome. On the contrary, Mouse Double Minute 2 protein (MDM2) is able to prolong the half-life of the E2F-1. In fact MDM2 binding to the E2F-1 protein displaces SCF^{SKP2}, protecting the factor from proteasome-dependent degradation.

1.3 E2F-1 and cancer

E2F-1 has long been considered an oncoprotein because it has the ability to promote quiescent cells to enter S phase and proliferate (Zhang *et al.* 2005).

E2F-1 was originally isolated as the cellular mediator of the transforming activity of the product of Adenovirus early region 1A (E1A) oncogene. Studies about E2F-1 activation by E1A protein led to elucidate the mechanism by which E2F-1 is regulated in normal cells. It was found that E1A protein caused pRb dissociation from E2F-1 committing cells in late G1 phase to initiate cell cycle progression. This early observation that E2F-1 was deregulated by a transforming virus was the first indication that E2F may be associated with cancer. It was subsequently found that other viral oncoproteins as Simian virus 40 tumor antigen, and E7 proteins from 'high-risk' human papilloma viruses, share with adenovirus E1A protein the capacity to disrupt the interaction between transcription factor E2F-1 and pRb (Chellappan SP *et al.* 1991; Bell and Ryan 2004; Chellappan S *et al.* 1992; Zhang Z *et al.* 2005).

Even if E2F-1 itself is rarely found to be mutated, it is now considered that deregulation of E2F-1 is an event in most, if not all, cancers. Given the clearly important role played by the pRb/E2F-1 pathway in controlling cell growth, it is not surprising that, as well as viral infection, a wide spectrum of mutations have been reported to disrupt normal function of this pathway. Along with mutation in *RB1* gene it-self, that

are actually frequent, deregulated expression of D-type Cyclins, Cyclin kinase dependent 4 (Cdk4) and p16INK4a Cyclin kinase inhibitor are more commonly reported to deregulate the pathway (Nevins JR. 1992). Zhan Z. *et al.* study about the ability of MDM2 to bind and stabilize E2F-1, suggests that in cells with MDM2 over-expression, the E2F-1 protein may be stabilized and kept at a higher level, which may contribute to deregulated proliferation and carcinogenesis (Zhang Z *et al.* 2005).

Studies using in vitro models, transgenic models with E2F-1 over-expression and clinical data from patient with different kind of tumor have depicted E2F-1 to have a role in oncogenesis, tumor progression and therapy resistance (Zhang Z *et al.* 2005).

Overall this data underlines the need to further understand pathways involved in E2F-1 function and develop new therapies targeting E2F-1.

2. Aims of the study

When a cell is stimulated to proliferate, a series of events occur ultimately resulting in release of free active E2F-1, leading to the expression of genes that are necessary for entering and progression through S phase. Therefore, E2F-1 plays a central role for a cell to execute the decision to re-enter cell cycle and proliferate, both in homeostatic and pathologic conditions. (Golias CH *et al.* 2004, Massagué J, 2004)

During recent studies conducted in my laboratory, it was observed that experimental ribosomal RNA (rRNA) synthesis inhibition hindered cell cycle progression in cells with inactive-p53, by down-regulating E2F-1. It was showed that the down-regulation of E2F-1 was due to its increased degradation as a consequence of ribosomal proteins interaction with MDM2, which inactivated the E2F-1 stabilizing function of MDM2. While in p53-proficient cells, the down-regulation of E2F-1 was only of little help to p53 in the control of proliferation, in cells with inactive p53, it appeared to have a key role in hindering cell proliferation after the inhibition of rRNA synthesis (Donati G. *et al.* 2011). This is, again, in keeping with the notion that this protein plays a critical role in controlling proliferation, but above all, bring at light a mechanism to control cell proliferation independent from p53 activity upon Ribosomal biogenesis inhibition. The evidence that the loss of p53 function very frequently characterizes human cancers, which are therefore less

sensitive to current cytostatic and cytotoxic drug treatment, underlines the importance of E2F-1 down-regulation and suggests that E2F-1 expression may represent a target for therapy of cancers with inactivated or deleted p53.

In this study was experimented a drug combination in order to obtain a strong down-regulation of E2F-1 by acting on two different mechanisms independent from p53. For this purpose I tested the anti-proliferative activity of Nutlin-3a and PD0332991 on a series of cell lines lacking or with inactivated p53.

Nutlin-3a is a selective inhibitor of Mdm2, largely studied for its ability to inhibit MDM2-p53 interaction leading to p53 stabilization, and inhibits growth in most tumor cells with wild-type p53 (Shen H *et al.* 2011). Worth of note, it has been recently shown that MDM2 direct binding to E2F1 prolongs the half-life of E2F1 protecting him from ubiquitination (Zhang Z *et al.*, 2005). Since it has been reported that p53 and E2F-1 bind to the same region of MDM2 through a well conserved sequence (Marti K *et al.* 2001), I supposed that Nutlin-3a could inhibits also MDM2 binding to E2F-1 by interfering with its stabilizing activity and thus leading to E2F-1 degradation with the same mechanism described by Donati G. (2011).

In preliminary experiments I found that Nutlin-3a treatment reduced cell proliferation rate in TP53^{-/-} and p53-mutated cancer cells as a consequence of E2F-1 levels down-regulation, due to its increased

degradation. Considering that a sufficient amount of E2F-1 protein was left to promote cell cycle progression after Nutlin-3a treatment, I expected that the inhibition of pRb phosphorylation should greatly increase the Nutlin-induced cytostatic effect by allowing the residual E2F-1 amount to be completely linked to hypo-phosphorylated pRb. This provided the rationale for testing inhibitors of Cdk4 and Cdk6, in combination with Nutlin-3a to obtain a stronger down-regulation of E2F-1. For this purpose I used Palbociclib (PD 0332991), an oral and selective inhibitor of Cdk4 and Cdk6, with ability to block pRb phosphorylation in the low nanomolar range, and is in a newly started Phase III clinical trial (Rocca A *et al.* 2014; Web ref. 1).

As expected, when combined, these drugs strongly hinder cell proliferation in p53^{-/-} and p53-mutated cells by blocking them in G1 phase of cell cycle.

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3. Material and Methods

3.1 Cell lines, growth conditions and drugs

The human colon cancer cell line HCT-116, human osteosarcoma Saos-2 and human osteosarcoma U2OS cell lines were obtained from the American Type Culture Collection (ATCC). HCT-116 p53DD cells were obtained in my laboratory by viral transduction of a retroviral vector expressing a double dominant-negative, truncated form of murine p53 (Derenzini M *et al.*, 2009). HCT116 p53^{-/-} cell line was kindly provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich). FBS was inactivated by heating to 56° C for 30 min. In addition HCT-166 p53DD were maintained in presence 0.7 µg/ml Puromycin (Sigma-Aldrich) selection antibiotic. HCT-116 p53^{-/-} were maintained in presence of 400µg/ml Geneticin (Sigma-Aldrich) and 100µg/ml Hygromycin B (Sigma-Aldrich) selection antibiotic.

All cell lines were cultured in monolayer at 37°C in humidified atmosphere containing 5% CO₂. When necessary, cells were frozen at -80° C in 90% FBS and 10% dimethyl sulfoxide (DMSO, Carlo Erba) and stored in liquid nitrogen.

For each experiment cells were seeded 24h before treatment.

10 mM working solution of Nutlin-3a (Sigma-Aldrich), PD0332991 (Pfizer) and MG132 (Sigma-Aldrich) were obtained by dilution in DMSO

and administered by dilution in growth media with no selection antibody, at final concentrations indicated in *Results* section.

3.2 Protein extraction and Western Blot

At the end of the treatment cells were trypsinized and centrifugated at 7,500 r.p.m, 4°C, for 5 min. to obtain pellets which were stored at -80°C or used immediately.

Cells were vigorously resuspended in lysis buffer [0.1 M KH₂PO₄ (pH 7.5), 1% Nonidet P-40 (Sigma-Aldrich) and 0.1 mM β-glycerophosphate supplemented prior use with 1X Complete protease inhibitor cocktail (Roche Diagnostics), 1mM NaF and 1mM Sodium Orthovanadate] and incubated for 30 min on ice. Then samples were centrifuged at 13,000 rpm, 4°C, for 15 minutes. The cleared supernatants were transferred in new tubes and stored at -80°C until use.

Protein quantification was performed using Bradford assay (Bio-Rad). Bovine serum albumin (BSA) was used to design standard curve. Samples (30-40 μg of protein) mixed with Leammli loading buffer [10% Glycerol, 0.2% SDS (Sigma-Aldrich), 1.5 mM Tris, 0.1% Bromophenol Blue, 1.5 M β-mercaptoethanol], boiled for 5 min and resolved on 8% or 10% SDS-PAGE, then transferred to nitro-cellulose membranes (Hybond C Extra, Amersham). Filters aspecific binding sites were blocked by incubation with 5% non fat powdered milk dissolved in TBS-T solution [20 mM Tris,

137 mM NaCl, 0.1% Tween 20 (Sigma-Aldrich), pH 7.4] for 1 h at room temperature (RT). After washing 3 times for 10 min. RT with TBS-T, filters were incubated overnight at 4°C with primary antibodies, diluted in 3.5% BSA TBS-T. The following antibodies were used:

Mouse monoclonal anti-E2F-1 (1:100, KH95, Santa Cruz), mouse monoclonal anti-total pRb (1:400, 1F8, NeoMarkers), mouse monoclonal anti-phospho pRb (1:200, Ser780, 51B7, NeoMarkers), rabbit polyclonal anti-cleaved PARP-1 (1:1000, Cell Signaling) mouse monoclonal anti-total MDM2 (1:100, clone 2A10 Calbiochem), rabbit polyclonal anti-phospho MDM2 (1:750, Sigma-Aldrich), mouse monoclonal anti-p53 (1:1000, BP53-12, Novocastra) and mouse monoclonal anti- β -actin (1:4000, A2228, Sigma-Aldrich Chemical Co.).

The next day filters were washed 3 times for 10 min. RT, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000 ECL, GE Healthcare) in 5% Milk-TBS-T for 1h at RT, and washed again. The horseradish peroxidase activity was detected using the appropriate enhanced chemiluminescence substrate (Cyanagen) on Hyperfilm enhanced chemiluminescence films (Amersham). Densitometric analysis was performed with GelPro analyzer 3.0 (Media Cybernetics software). Values were normalized on corresponding β -Actin expression and expressed as fold increase/decrease with respect to controls.

3.4 Co-immunoprecipitation

Cells were lysed in Co-IP buffer (50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.8% Nonidet P-40, 1mM DTT, 1mM EDTA supplemented with 1X Complete protease inhibitor cocktail (Roche Diagnostics), 1mM NaF and 1mM Sodium Orthovanadate) for 30 min on ice and cleared by centrifugation at 13,000 r.p.m, at 4°C for 15 min. Whole extracts were quantified using Bradford assay (Bio-Rad). Bovine serum albumin (BSA) was used to design standard curve. 1.5 mg of proteins for sample, were incubated overnight with 4-6 µg/mg of primary rabbit polyclonal anti-E2F-1 antibody (C-20, Santa Cruz Biotechnology). The next day Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were washed in Buffer Co-IP, incubated with 5% powdered Milk in Buffer Co-IP for 1h at 4°C to block aspecific binding sites, washed with Co-IP buffer and incubated in equal amount with each sample for 3h at 4°C in agitation, to allow them complex the antibody. After centrifugation Immuno-complexes were washed, resuspended in Leammli and resolved in 10% SDS PAGE. Subsequent step and antibody have been previously described in “Protein extraction and Western blot” section.

3.5 Isolation of total RNA, Reverse Transcription-PCR and Real Time PCR

At the end of the treatment cells were harvested and total RNA was extracted with TRI reagent (Ambion, Austin, TX, USA) according to manufacturer's instructions. Extracted RNAs were quantified with a Nanodrop spectrophotometer (ND1000) and 2µg of RNA for each sample were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. Real-time PCR analysis was performed in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR mastermix (Applied Biosystems) for the analysis of RB1, TP53, E2F-1, MDM2. Sybr Green Universal PCR MasterMix (Applied Biosystems) was used for RRM2 and MCM7. Probes were purchased from Applied Biosystems (Assay on Demand). Primers were used at a final concentration of 5 µM.

Primer sequences:

RRM2 Forward ATCGTCCACCGCAAATGCTTCTA

RRM2 Reverse AGCCATGCCAATCTCATCTTGTT

MCM7 Forward ATCGTCCACCGCAAATGCTTCTA

MCM7 Reverse AGCCATGCCAATCTCATCTTGTT

Experiments were carried out in triplicate for each data point. To normalize the expression levels of each gene, TaqMan human-β-

Glucuronidase (GUS) was used as an endogenous control gene. The relative amount of the gene in study was evaluated by the comparative $2^{-\Delta\Delta Ct}$ method. Briefly:

$$\Delta Ct = Ct_{\text{gene}} - Ct_{\text{GUS}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{control sample}}$$

$$2^{-\Delta\Delta Ct} = \text{gene expression in sample respect to control}$$

3.6 Crystalviolet assay

All cells were seeded in duplicate or triplicate in 12-well plates at different confluence depending on specific proliferation rate of each cell line:

wt- and p53DD HCT-116	10 • 10 ⁴ cells/well
HCT-116 p53 ^{-/-}	15 • 10 ⁴ cells/well
Saos-2	75 • 10 ⁴ cells/well

The day after plating, Nutlin-3a or Cdk 4/6 inhibitor were added at increasing concentrations to generate a dose-response curve. For combination treatment Nutlin-3a was used at 10 μM concentration and Cdk 4/6 inhibitor at 0.5 μM . An equal amount of vehicle (DMSO) was added in control wells. 96h after treatment cells were washed twice with PBS and fixed overnight in formalin at 4°C. The day after formalin was removed; cells were washed twice with bidistilled water, stained with a 0.1% w/v crystal violet and 20% v/v methanol water solution for 30

minutes in agitation, thus washed 4 times in double-distilled water and air dried for 15 min at room temperature. Densitometric quantification of crystal violet adsorbed was evaluated by densitometric quantification using ChemiDoc system (ChemiDoc XRS, Bio-Rad, Milan, Italy).

3.7 Clonogenic assay

HCT-116 p53^{-/-} cells were seeded in duplicate in a 6 wells plate at the concentration of 250 cells/well in normal medium. After 24h, 10 μ M Nutlin-3a, 0.5 μ M Cdk 4/6 or a combination of both were added at grow media and cells were maintained for 9 days at 37°C in humidified atmosphere containing 5% CO₂. Then cells were fixed in formalin, washed once with PBS and stained with a solution of 0,5% crystal violet and 25% methanol in water, for 20 min at room temperature. After washing and drying, the colonies were counted manually. Results are means of three counting reported as fold reduction respect to controls.

3.8 Cell Cycle analysis

For the cell cycle flow cytometry assay cells were collected 24 h after treatment, and fixed in 70% ethanol at 4 °C for 16 h. Fixed cells were then washed once with PBS, resuspended in 500 μ l PBS containing 10 μ g ml⁻¹ propidium iodide (Life Technologies) and 50 μ g ml⁻¹ RNase (Sigma-

Aldrich) and incubated for 30 min at room temperature in the dark. The cells were then centrifuged at 1200 r.p.m. for 5 min, resuspended in 500 μ l of PBS and analyzed with Fluorescent-Activated Cell Sorter (BD FACSaria cell sorter, BD Biosciences, San Jose, CA, USA). FlowJo analysis software was used for elaboration of FACS data.

3.9 Statistical analysis

Student's *t* test was used to test for statistical significance of the differences between the different group parameters. *p* values of less than 0.05 was considered statistically significant.

4. Results

4.1 Nutlin-3a treatment reduces cell proliferation and E2F-1 protein level in cells lacking functional p53 protein.

To study p53-independent effect of Nutlin-3a treatment in human cancer cells, I used HCT-116-derived HCT-116 p53^{-/-} cells, in which two promoter-less targeting vectors, each containing a geneticin- or hygromycin-resistance gene in place of genomic p53 sequences, sequentially disrupt the two p53 alleles (Bunz F. *et al.* 1998), and Saos2 cell line which are devoid of both *TP53* and *RB* expression (Chen X. *et al.* 1996; Wang LH. *et al.* 1998; Marcellus RC. *et al.* 1996).

To evaluate if Nutlin-3a downregulates E2F-1 protein, HCT-116 p53^{-/-} cells were treated with increasing doses of Nutlin-3a for 24h, and E2F-1 protein level was evaluated by Western blot. As expected, Nutlin-3a treatment down-regulated E2F-1 protein, and this effect was dose-dependent. In order to verify if E2F-1 down-regulation observed upon Nutlin-3a treatment was due to its increased proteasome-dependent degradation this experiment was carried out also in presence of 26S proteasome inhibitor MG-132. As shown in figure 1 a, MG-132 significantly rescued E2F-1 protein level from Nutlin-3a down-regulation both at 10 μ M and 20 μ M doses, but not when Nutlin-3a is used at 30 μ M concentration, the higher concentration tested. The involvement of proteasome was further confirmed by Real Time-RT PCR analysis of E2F-1 mRNA expression. In fact, consistent with Western blot data, no changes were observed in E2F-1 mRNA level, except for samples treated

with the higher concentration, demonstrating Nutlin-3a ability to induce E2F-1 protein degradation through the ubiquitin-proteasome pathway, in a p53-independent manner (Fig. 1 a).

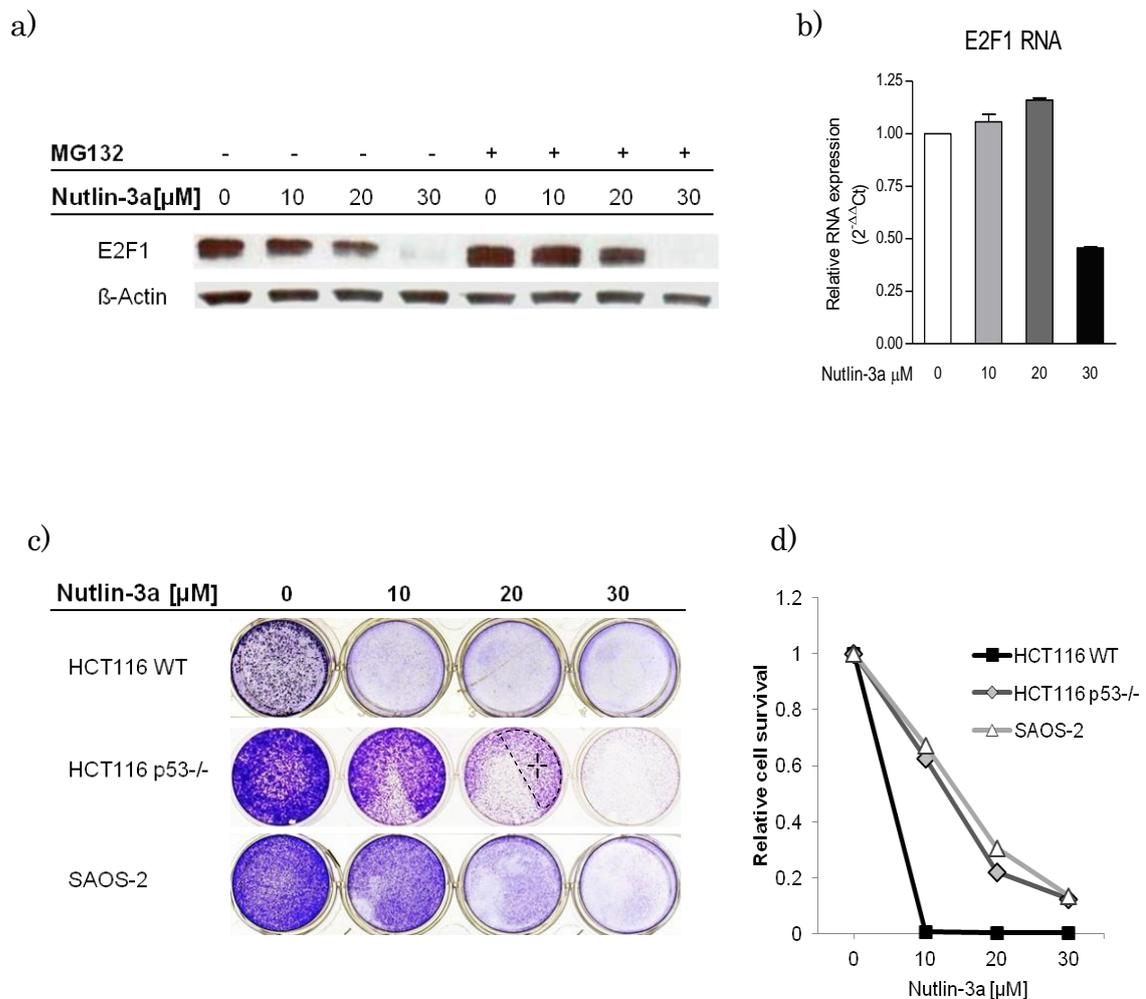


Fig. 1 Nutlin -3a induces E2F-1 proteasomal degradation and hinders cell proliferation in cells lacking functional p53 protein (p53-null tumoral cells). a) Representative Western blot of E2F-1 protein level in HCT-116 p53^{-/-} cells after 24h treatment with increasing doses of Nutlin-3a, in presence or not of 10 μ M proteasome inhibitor MG-132. Cells were exposed to MG-132 for last 2h of Nutlin-3a treatment. b) Real time RT-PCR analysis of E2F-1 mRNA in HCT-116 p53^{-/-} cells after 24h of treatment with increasing doses of Nutlin-3a. c) Crystal Violet assay and d) densitometric quantification in *wt*-HCT-116, HCT-116 p53^{-/-} and Saos-2 cells after 72h or 96h respectively of treatment with increasing doses of Nutlin-3a.

To investigate p53-independent effects of Nutlin-3a treatment on cell proliferation, HCT-116 p53^{-/-} and Saos-2 cells were treated with increasing doses of Nutlin-3a and proliferation rate was assessed by Crystal Violet staining and densitometric quantification.

In both cell lines, Nutlin-3a treatment resulted in a significant, dose-dependent, reduction of cell population, demonstrating that Nutlin-3a is able to regulate proliferation through mechanisms independent from p53 (Fig. 1 c, d). Furthermore, the observation that Crystal violet assay result reflects E2F-1 protein reduction revealed on Western blot, strongly suggests that Nutlin-3a reduces cell proliferation in p53-null cells down-regulating E2F-1 protein level.

Comparable results were obtained using HCT-116 p53DD cells, obtained by viral transduction of a retroviral vector expressing a double dominant-negative, truncated form of murine p53 (Donati G. et al., 2011). In addition HCT-116 and U2OS (human osteosarcoma) cells were transiently silenced for TP53 by RNA interference. Also in these cells Nutlin-3a significantly reduced E2F-1 protein level while I can't use them for 96h hours Crystal violet proliferation assay because after RNA interfering procedure I should wait at least 36-48h to obtain completely reduction of p53 protein and start the experiment, but after 96h from the end of the silencing procedure p53 start to raise up, giving results not completely independent from p53 activity (data not shown).

4.2 Cdk 4/6 inhibitor treatment causes a sustained inhibition of pRb phosphorylation, suppresses E2F-1 activity and reduce cell proliferation in p53-null cells.

As previously mentioned, it is well known that hypo-phosphorylated pRb, but not hyper-phosphorylated pRb, is able to bind E2F-1 and keep it inactive. Based on these data a Cdk 4/6 inhibitor was tested with the aim to obtain E2F-1 activity downregulation.

In order to verify the effect of the inhibitor on Cdk 4/6 activity, HCT-116 p53^{-/-} cells were treated for 24h with increasing doses of the drug, and phosphorylation level of pRb was assessed by Western blot. In both cell lines, the treatment significantly reduced pRb phosphorylation, showing a dose-dependent trend (Fig. 2, a). Then I performed a time course experiment using 1,5 μ M Cdk 4/6 Inhibitor, the concentration that lead to an almost complete absence of hypo-phosphorylated pRb, and I found that the shorter time-treatment that lead to a strong reduction of phosphorylated pRb was 8h. In order to verify if pRb phosphorylation reduction, observed after Cdk 4/6 inhibition, increased pRb binding to E2F-1, I isolated E2F-1 and associated proteins by Co-Immunoprecipitation. For this experiment HCT-116 p53^{-/-} cells were exposed to 1,5 μ M Cdk 4/6 Inhibitor for 8h. Western blot detection of pRb in E2F-1 Co-Immunoprecipitate, confirmed that an increased amount of pRb binds E2F-1 upon Cdk 4/6 inhibition (Fig. 2, b). This data were further confirmed by the reduced transcription of two E2F-1 target genes,

Ribonucleotide Reductase-2 (RNR2) and Minichromosome Maintenance Complex protein 7 (MCM7), assessed by Real Time RT-PCR analysis after 8h of treatment. This result demonstrates that E2F-1 transactivation activity is significantly reduced in response to Cdk 4/6 inhibitor treatment, in HCT-116 p53^{-/-} cells.

Finally, I investigated the impact of Cdk 4/6 Inhibitor treatment on cell proliferation in HCT-116 p53^{-/-}. Crystal violet assay showed that Cdk 4/6 inhibitor treatment significantly reduces cell population in a dose-dependent manner. Comparable results were obtained using HCT-116 p53DD cells (Fig 2, d).

Taken together, these data suggested that Cdk 4/6 inhibitor affects cell proliferation in both p53-null and p53-mutated cells by down-regulating E2F-1 activity, through the inhibition of pRb phosphorylation.

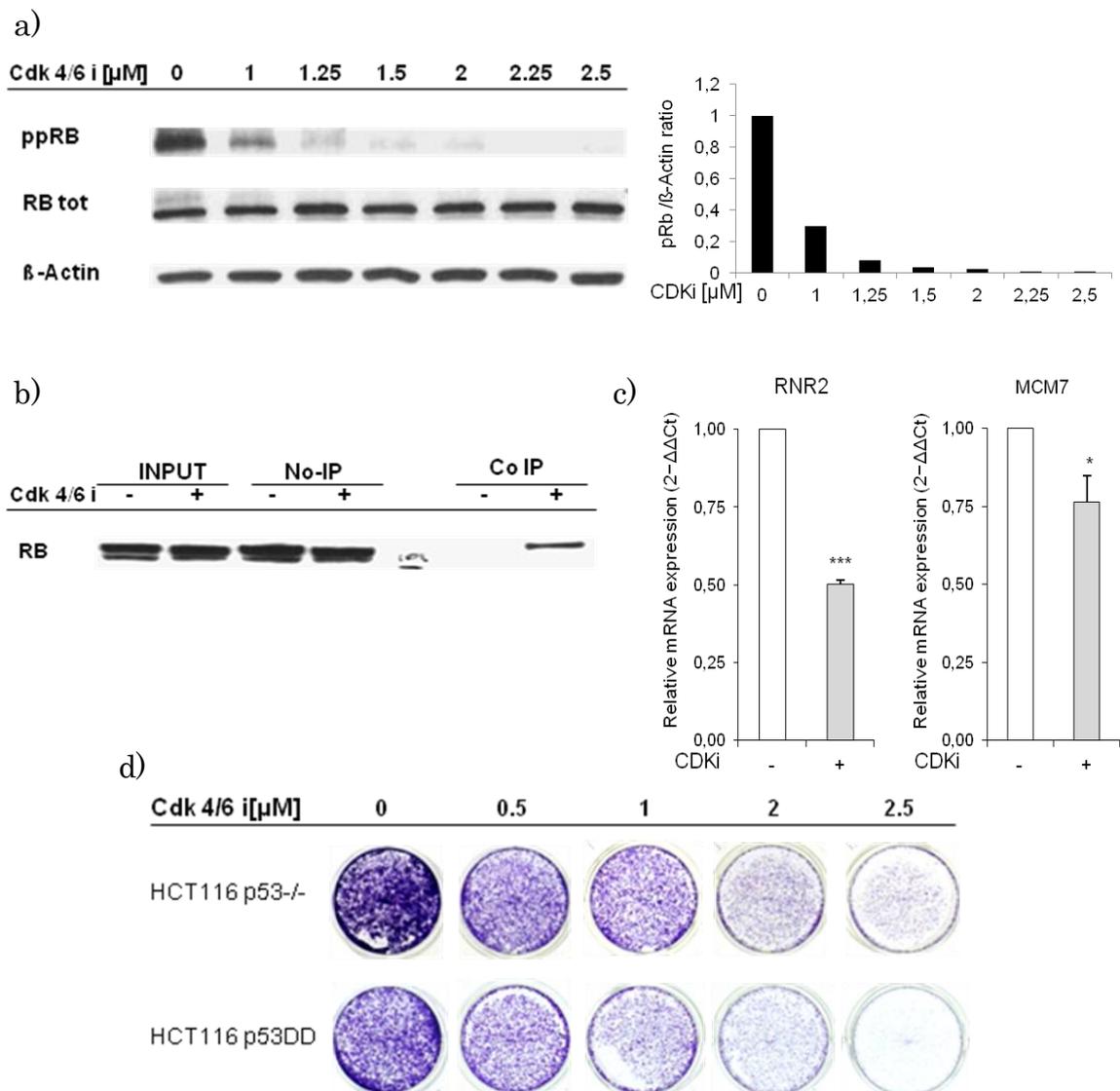


Fig. 2 Cdk 4/6 inhibitor inhibits cell proliferation in p53-null and p53-mutated cells, likely down-regulating E2F-1 activity, through the inhibition of pRb phosphorylation. a) Inhibition of pRb phosphorylation at Ser₇₈₀ on Western blot and densitometric quantification. HCT-116 p53^{-/-} cells were treated for 24 hours with increasing doses of Cdk 4/6 inhibitor (Cdk 4/6 i). b) Western blot detection of total pRb protein level in E2F-1 Co-Immunoprecipitate obtained from HCT-116 p53^{-/-} cells treated for 8h with 1,5 μ M Cdk 4/6 i. 10 μ M proteasome inhibitor MG-132 was added at last 2h of treatment to avoid changes in protein level due to proteasomal degradation. c) Real Time RT-PCR analysis of mRNA expression of E2F-1 target genes Ribonucleotide Reductase-2 (RNR2) and Minichromosome Maintenance Complex protein 7 (MCM7) in HCT-116 p53^{-/-} cells after 8h of 1,5 μ M Cdk 4/6 i treatment. Ct mean values of each triplicate were normalized to those obtained for β -Glucuronidase and expressed as fold reduction of 2^($\Delta\Delta$ Ct) values over control cells. d) Crystal Violet assay in p53^{-/-} and p53DD HCT-116 exposed for 96h to increasing doses of Cdk 4/6 i.

4.3 Cdk 4/6 inhibitor-Nutlin-3a drug combination exerts p53-independent antiproliferative activity arresting cells in G₁.

To study p53-independent effect of the Cdk 4/6 inhibitor-Nutlin-3a combination on proliferation, p53DD and p53^{-/-} HCT-116 cells were treated with the two drugs alone, or with a combination of them at the same concentrations used for single treatments. Cell proliferation rate was analyzed by crystal violet assay. As expected, the combination of the two compounds strongly reduce cell population in both p53-null and p53-mutated cell lines (Fig. 3, a). These data were supported by a clonogenic assay in p53^{-/-} that gave comparable result. The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. This cell is then said to be clonogenic. In this assay, the treatment with combined Cdk 4/6 inhibitor and Nutlin-3a resulted in a significative, almost complete, reduction of the number of colonies formed within a homogeneous initial cell population (Fig. 3, b). In both Crystal violet and Clonogenic assay, treatment with Cdk 4/6 inhibitor and Nutlin-3a combination showed a greater inhibitory effect with respect to either drug, when used alone (Fig. 3, a and b).

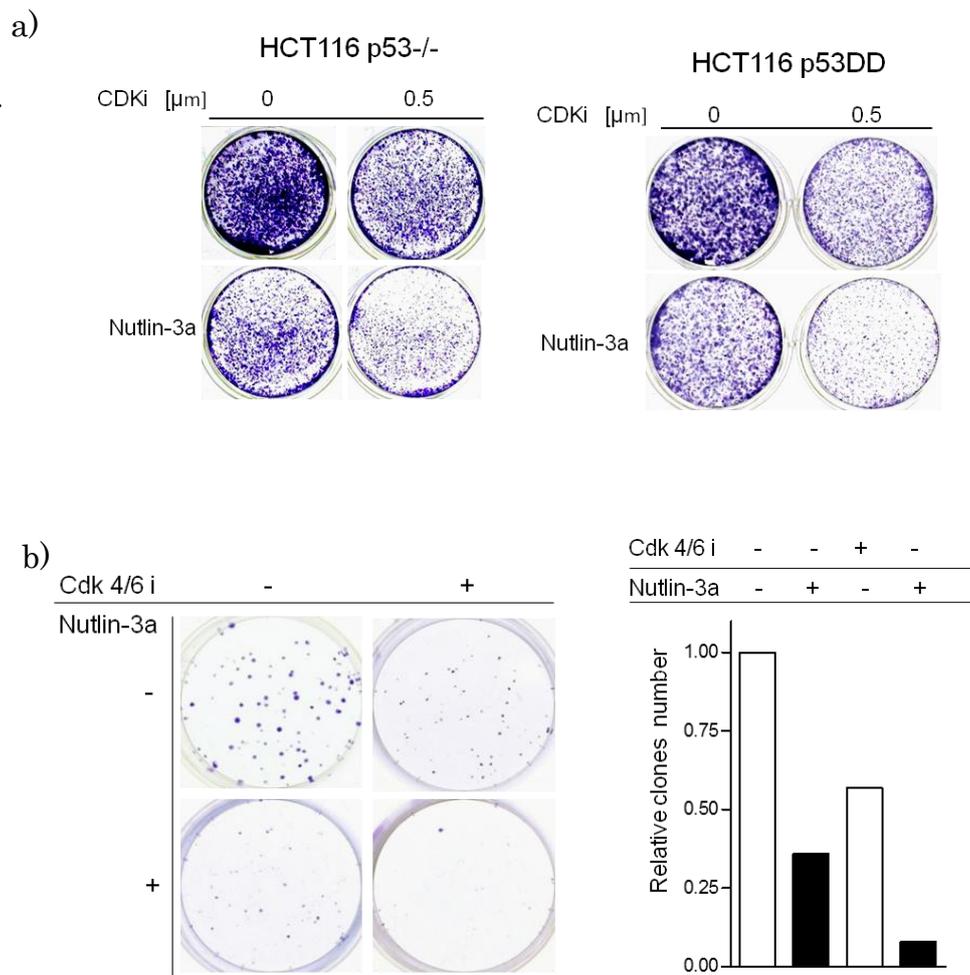


Fig. 3 Cdk 4/6 Inhibitor–Nutlin-3a combination treatment hinders cell proliferation in both p53-null and p53-mutated cell lines. a) Crystal Violet assay after 96h of treatment with 0.5 μ M Cdk 4/6 inhibitor (Cdk 4/6 i) and 10 μ M Nutlin-3a in HCT-116 p53^{-/-} and HCT-116 p53DDD cells. b) Clonogenic assay and quantification by counting of colony number after 9 days 0.5 μ M Cdk 4/6 and 10 μ M Nutlin-3a treatment in HCT-116 p53^{-/-}

Next I investigated if the reduction of cell population observed in p53^{-/-} and p53DD cells, upon drugs combination treatment, was due to an inhibitory activity on cell cycle progression, or to an increased death rate, as a result of the activation of apoptotic pathway. To evaluate the effect of the treatment on cell cycle progression, HCT-116 p53^{-/-} cells were exposed

to Cdk 4/6 inhibitor or to Nutlin-3a or to a combination of both drugs, at the same concentrations used for single treatments. After 24h of treatment, cells were stained with propidium iodide and cell cycle was studied through Flow cytometry. As shown in figure 4, Cdk 4/6 inhibitor and Nutlin-3a combination led to a significant increase in the percentage of cells in G1 phase with a concomitant decline in other phases of the cell cycle. Once again, the drug combination showed a greater effect with respect to treatment with single drugs (Fig. 4).

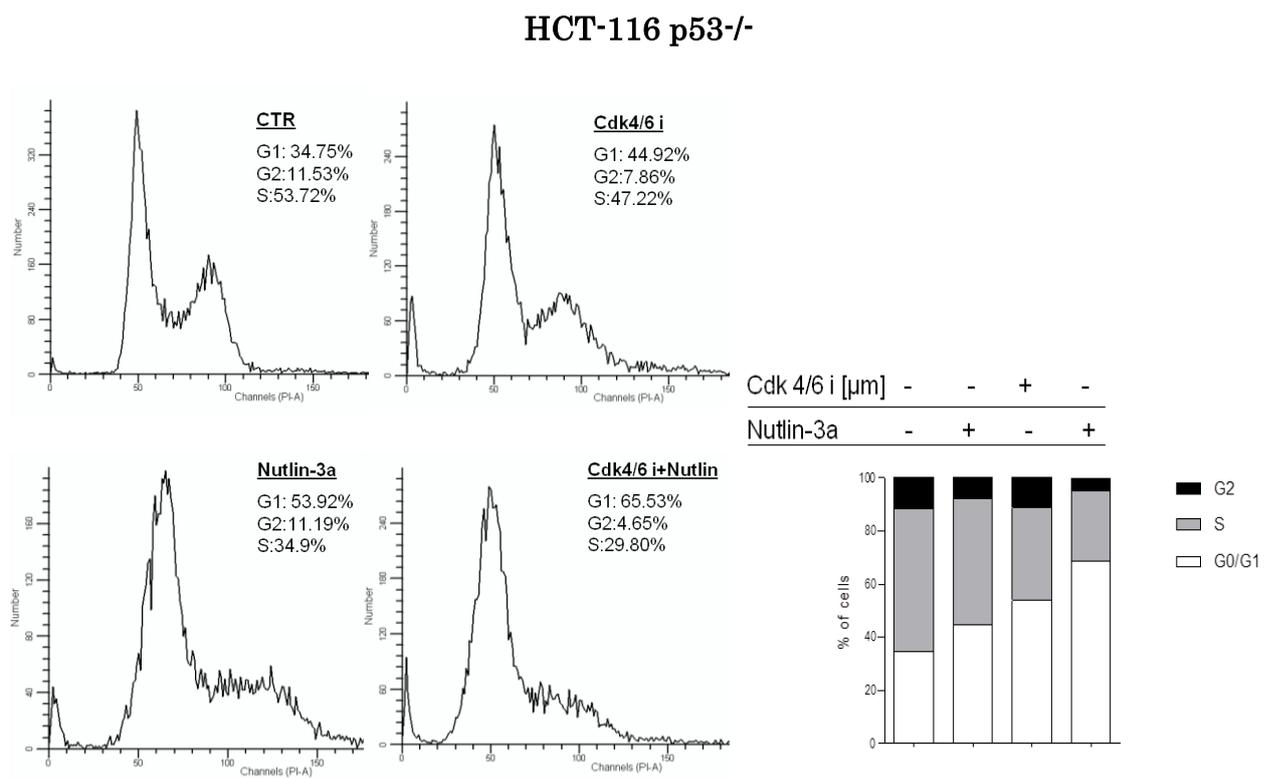


Fig. 4 Cdk 4/6 inhibitor-Nutlin-3a combination treatment arrest HCT-116 p53^{-/-} cells in G1. Flow cytometry analysis of HCT-116 p53^{-/-} cells exposed to 0,5 μ M Cdk 4/6 inhibitor (Cdk 4/6 i) or 10 μ M Nutlin-3a or to a combination of both drugs for 24 hours. Cells were harvested and fixed as described in Materials and Methods. Flow data generated by FACS (Fluorescence Activated Cell Sorting) machine were analyzed by FlowJo software to obtain DNA Histograms and percentage of cells in each phase.

Then, to further elucidate mechanisms underlying the reduction of cell population observed upon Cdk 4/6 inhibitor-Nutlin-3a combination treatment (Fig. 3), I investigated if the treatment exerts a cytotoxic effect on cells. Acute response to treatment was evaluated by Cristal violet assay. HCT-116 p53^{-/-} were exposed to the treatment for 24h, and then were left growing in normal medium for additional 72h. After Crystal violet staining, no differences were observed between treated and control cells, indicating that the treatment doesn't induce cell death within 24h. By contrast, the same treatment protocol induces a strong reduction of cell population in wt-p53 HCT-116 cells, presumably due to the activation of apoptotic pathway linked to the presence of functional p53 (Fig. 5, a). This hypothesis has been confirmed by Western blot detection of 85-kDa fragment of PARP-1, indicator of apoptosis pathway activation (Lazebnik Y.A. *et al.* 1994; Duriez P.J. *et al.* 1997; Martha A. *et al.*, 2001). In fact, after 24h of treatment no changes were observed PARP-1 cleavage in HCT-116 p53^{-/-} cells, while it significantly increased in wt-p53 HCT-116, accompanied by a strong stabilization of p53 protein (Fig. 5, b). These results confirm that Cdk 4/6 inhibitor-Nutlin-3a combination hinders cell proliferation in HCT-116 p53^{-/-} arresting cells in G1, and that the treatment can induce cytostasis or death depending on p53 status.

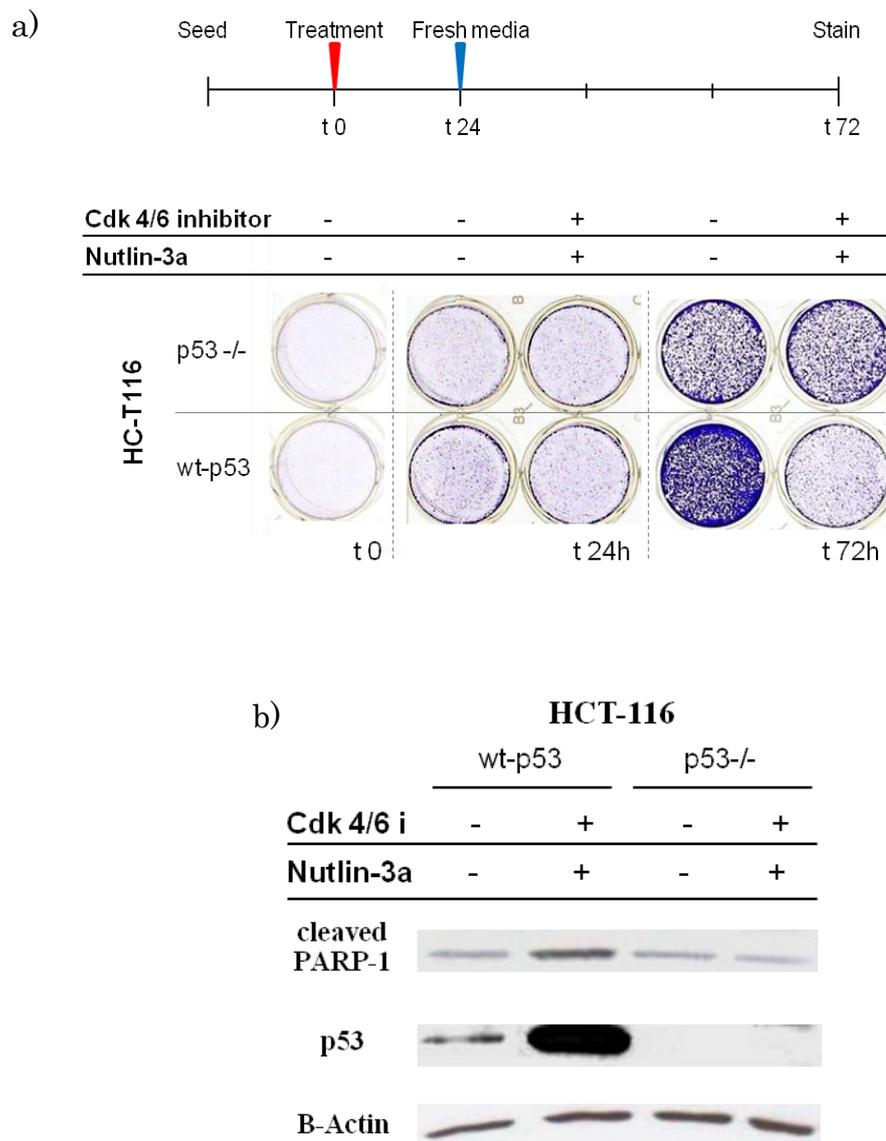


Fig. 5 Cdk 4/6 inhibitor-Nutlin-3a combination act as cytostatic or cytotoxic agent depending on p53 status. a) Crystal violet assay and representative scheme of experimental design. *wt* and *p53^{-/-}* HCT-116 cells were treated with a combination of 0.5 μ M Cdk 4/6 inhibitor (Cdk 4/6 i) and 10 μ M Nutlin-3a. After 24h the treatment was removed and cells were left in normal medium for additional 72h. b) 85-kDa fragment of cleaved PARP-1 and p53 protein level by Western blot. *wt* and *p53^{-/-}* HCT-116 cells were treated with a combination of 0.5 μ M Cdk 4/6 inhibitor and 10 μ M Nutlin-3a for 24h.

4.4 Cdk 4/6 inhibitor treatment hinders cell proliferation with a pRb-independent mechanism that induces E2F-1 degradation by proteasome machinery, and enhances Nutlin-3a ability to down-regulate E2F-1 protein level.

After I demonstrated that Cdk 4/6 inhibitor treatment down-regulates E2F-1 activity and hinders cell proliferation in p53-null cells, more strongly when combined with Nutlin-3a, I tested Cdk 4/6 inhibitor, alone or in combination with Nutlin-3a, on pRb⁻ and p53-null Saos-2 cell line, in order to verify that drug effects mentioned above, were the specific consequence of pRb phosphorylation inhibition, and thus were dependent on the status of pRb. For this purpose, as for HCT-116 p53^{-/-} cells, Saos-2 cells were exposed to drugs treatment for 96h, and Crystal violet staining was used to evaluate the effect on proliferation rate.

Unexpectedly, also in Saos-2 cells, Cdk 4/6 inhibitor treatment resulted in a dose-dependent reduction of cell population, showing a synergic/additive effect when combined with Nutlin-3a (Fig. 6, a and b). Surprisingly, western blot analysis revealed that Cdk 4/6 inhibitor treatment reduces E2F-1 protein level, showing also in this case a stronger effect when combined with Nutlin-3a (Fig. 6, c). On the other hand, the analysis of Real Time RT-PCR revealed no significant change in E2F-1 mRNA levels, upon Cdk 4/6 inhibitor treatment alone or in combination with Nutlin-3a (Fig. 6, d).

These observations drew the conclusion that Cdk 4/6 inhibitor acts not only down-regulating E2F1 activity, as initially hypothesized, but also

reducing E2F-1 protein level, through such post-transcriptional mechanism different from the inhibition of pRb phosphorylation.

Saos-2
p53^{-/-}, truncated pRb

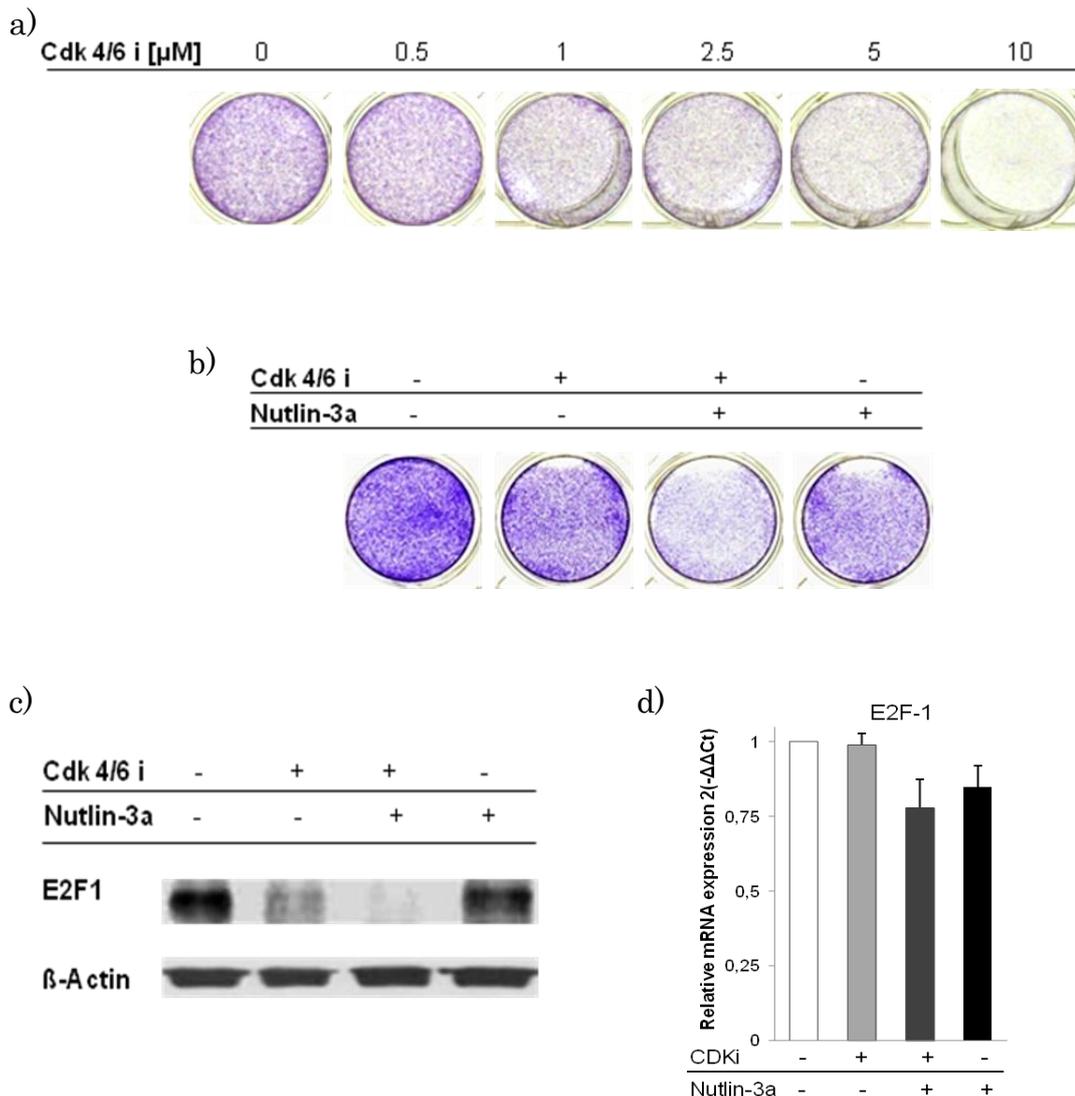


Fig.6 Cdk 4/6 inhibitor hinders cell proliferation in cells lacking functional pRb protein. This effect is stronger when Cdk 4/6 inhibitor is combined with Nutlin-3a. Crystal violet assay in Saos-2 cells after 96h of treatment with a) increasing doses of Cdk 4/6 inhibitor or b) 0,5 μ M Cdk 4/6 inhibitor or 10 μ M Nutlin-3a or to a combination of both drugs. c) E2F-1 protein level and d) E2F-1 mRNA expression in Saos-2 cells treated as in (b). Ct mean values of each triplicate were normalized to those obtained for β -Glucuronidase and expressed as fold reduction of 2^{-ΔΔCt} values over control cells.

4.5 Cdk 4/6 inhibitor downregulates E2F1 increasing its proteasome-dependent degradation, likely as a result of a lesser interaction with MDM2

In the second part of the study, I focused my attention on searching the possible alternative mechanism by which Cdk 4/6 inhibitor reduces E2F-1 protein level.

To investigate if E2F-1 protein reduction was actually due an increased degradation by proteasome machinery, I exposed HCT-116 p53^{-/-} cells to the drug adding MG-132 proteasome inhibitor at last two hours of treatment. As Western blot image in figure 7 shows, MG-132 rescued E2F-1 from down-regulation observed upon Cdk 4/6 inhibitor treatment, confirming that Cdk4/6 inhibitor increases E2F-1 degradation by proteasome 26 S.

Since data in the literature show that MDM2 is able to bind E2F-1 protecting him from degradation (Zhang Z *et al.* 2005), and that the down-regulation of MDM2 phosphorylation reduces its protein-protein interaction ability (Moll UM. and Petrenko O, 2003) I hypothesized that following Cdk 4/6 inhibition a reduction of MDM2 phosphorylation could occur, compromising the interaction of MDM2 with E2F-1 with a consequent increase in E2F-1 degradation rate. To investigate on this idea I carried out a Western blot detection of MDM2 phosphorylation.

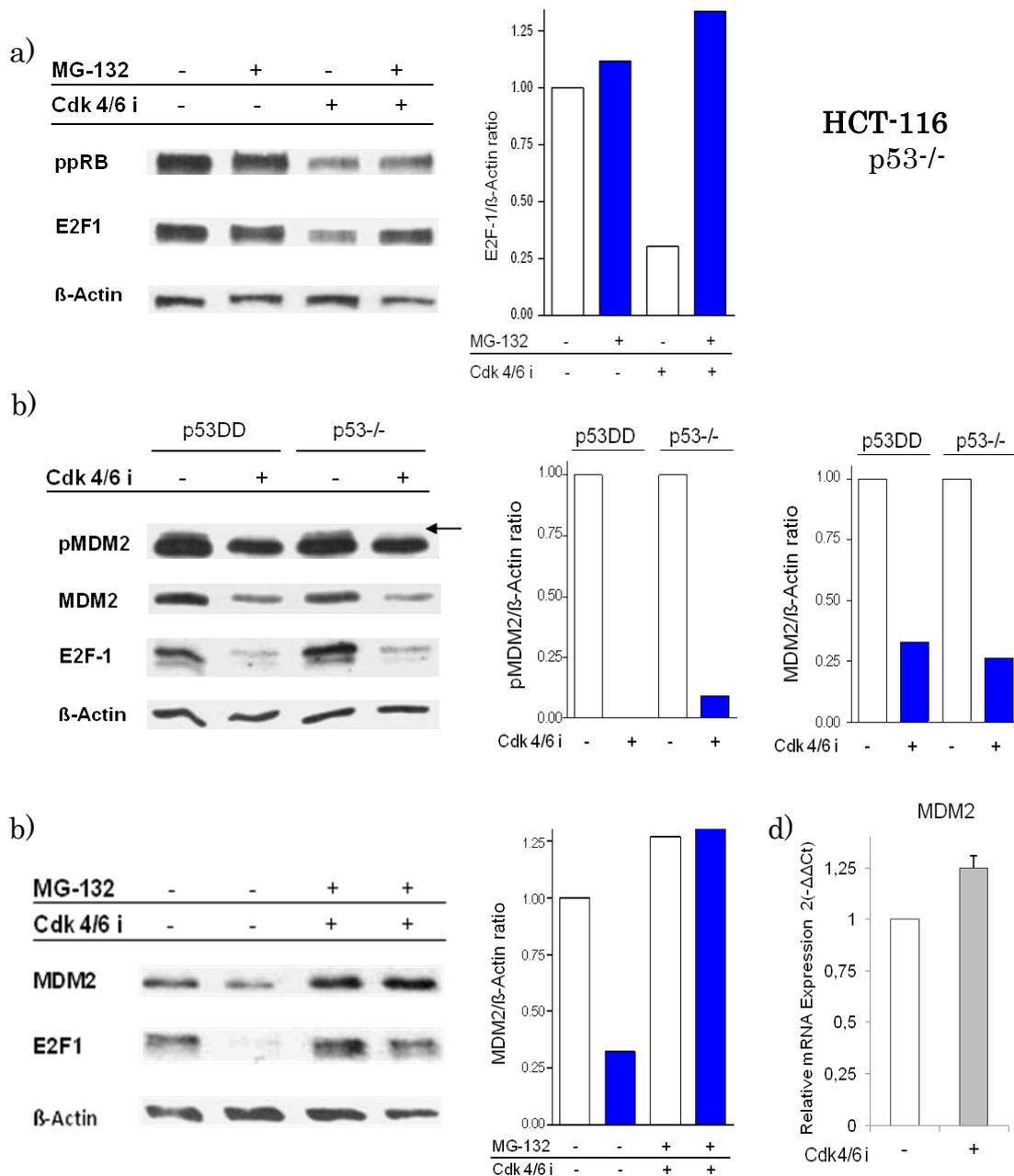


Fig. 7 Cdk 4/6 inhibitor induces E2F-1 and MDM2 protein degradation by the 26S proteasome likely by reducing MDM2 phosphorylation. a) Representative Western blot detection of E2F-1 level and respective densitometric quantification in HCT-116 p53^{-/-} upon 8h of 1,5 μ M Cdk 4/6 inhibitor treatment. MG-132 proteasome inhibitor 10 μ M was added at last 2h of Cdk 4/6 treatment. b) Total and phosphorylated MDM2 protein level by Western blot and respective densitometric quantification in p53^{-/-} and p53DD cells treated with 1,5 μ M Cdk 4/6 inhibitor for 8h. c) Representative western blot detection of MDM2 and E2F-1 protein level in HCT-116 p53^{-/-} treated as in (a). d) MDM2 mRNA level in HCT-116 p53^{-/-} upon 8h of 1,5 μ M Cdk 4/6 inhibitor treatment. Ct mean values of each triplicate were normalized to those obtained for β -Glucuronidase and expressed as fold reduction of $2^{-\Delta\Delta Ct}$ values over untreated cells.

As hypothesized, Western blotting analysis revealed a reduced MDM2 phosphorylation in treated cells with respect to control cells. To exclude that this reduction was due to a reduction of MDM2 protein, I looked also to total MDM2 protein level. I found that Cdk 4/6 inhibitor treatment reduce total levels of MDM2. To understand which mechanism was responsible of MDM2 reduction, I checked mRNA level by Real Time RT.PCR, and no significant changes were observed, leading to the conclusion that MDM2 protein reduction occurs via a post-transcriptional mechanism. These data prompt me to investigate if Cdk 4/6 inhibitor causes MDM2 proteasome-dependent degradation. When MG-132 proteasome inhibitor was added to medium for last two hours of Cdk 4/6 inhibitor treatment, I didn't observed any reduction of total MDM2 level with respect to control cells.

Taken together these data indicate that Cdk 4/6 inhibitor increase proteasome-dependent degradation of both E2F-1 and MDM2 protein through p53-independent mechanism.

5. Discussion

E2F-1 belongs to the E2F family of transcription factors that are situated downstream of growth factor signaling cascades, where they play a central role in cell growth and proliferation through their ability to regulate genes involved in cell cycle progression (Chen C and Wells AD 2007). E2F-1 exerts its transcriptional control on cell cycle by regulate the timely expression of many target genes whose products are required for S phase entry and progression (Müller H *et al.* 2001; Polanger S, Ginsberg D. 2009). For this reason, E2F-1 transcription activity regulation appears to be one among the most important mechanisms controlling cell cycle progression at G1/S check-point level.

At this regard, in previous study conducted in my laboratory, it was noticed that E2F1 was down-regulated upon inhibition of ribosomal RNA synthesis and that this down-regulation slows proliferation rate in p53-mutated cell lines (Donati G, *et al.* 2011). These results brought to light a p53-independent mechanism to control proliferation of tumor cells, underling the impact that E2F-1 regulation could have in the control of proliferation of tumor that don't express p53 or harbor p53 mutations with complete loss of function. These observations prompt me to investigate on mechanisms able to modulate E2F-1 activity, in order to obtain a suppression of TP53^{-/-} tumor proliferation.

It has been demonstrated that MDM2 direct binding to E2F-1 prolongs the half-life of E2F1 protecting him from ubiquitination (Zhang Z. *et al.*,

Oncogene. 2005). In fact in the study by Donati et al. 2011, it was showed that inhibition of MDM2 by binding of ribosomal protein, no longer used for ribosome building after rRNA synthesis reduction, down-regulates E2F-1 by increasing its proteasomal degradation. Since it has been reported that the region of p53, necessary for interaction with MDM2, is well conserved in E2F-1, and that both p53 and E2F-1 bind the same region of MDM2 (Marti K *et al.* 2001), I wonder if the MDM2 inhibitor Nutlin-3a, that has been extensively demonstrate to inhibit p53-MDM2 interaction, could reduce also MDM2 binding to E2F-1 and lead to its degradation, with the same mechanism previously described (Donati et al. 2011).

In my experiments, Nutlin-3a effectively reduced E2F-1 protein level, and this effect was rescued by the proteasome inhibitor MG132 demonstrating that Nutlin-3a increases E2F-1 degradation by the 26S proteasome machinery (Fig. 1 a). Accordingly no changes were observed in E2f-1 mRNA, except when Nutlin-3a was used at 30 μ M concentration, that is the higher dose tested, at which some additional mechanism likely occurred. Moreover, I found that Nutlin-3a inhibits proliferation in both HCT-116 p53^{-/-} and p53^{-/-} / pRb-null Saos-2 cells (Wang LH. *et al.* 1998; Marcellus RC. *et al.* 1996) showing a dose-dependent trend (Fig. 1 b). The fact that this reduction reflected E2F-1 protein reduction rate, let me conclude that Nutlin-3a inhibitory effect on proliferation is a consequence of E2F-1 down-regulation, which could represent an important

mechanism to block proliferation of tumor lacking of functional p53 protein. My results are in line with study in literature reporting that the Mdm2 stabilizing activity on E2F-1 is independent from the status of both p53 and pRb (Martin *et al.* 1995; Xiao *et al.*, 1995). On the contrary, Wunderlich M. and Berberich S.J. established that this MDM2 ability was mediate by p53, coming to this conclusion through experiments involving mutagenesis of Mdm2 in the region involved in interaction with p53 (Wunderlich M and Berberich SJ 2002). Nevertheless, this MDM2 region is necessary also for its interaction with E2F-1 (Martin *et al.*, 2001), thus, maybe, in their experiments Wunderlich M. and Berberich S.J disrupted MDM2 interaction not only with p53, but also with E2F-1, and this could be one possible explanation for these data discrepancy.

The interaction of pRb with E2F correlates with the capacity of pRb to arrest cell growth in G1 phase. Conversely, phosphorylation of the pRb protein, mediated by the G1 cyclins and associated Cyclin-dependent kinases, inactivates pRb and allows progression through the cell cycle (Johnson DG *et al.* 1994). Considering that in my experiments a sufficient amount of E2F-1 protein was left to promote cell proliferation after Nutlin-3a treatment, I expected that drug activation of pRb, should greatly increase the Nutlin-induced cytostatic effect by allowing the residual E2F-1 amount to be completely linked to pRb. Since pRb is active only in its hypo-phosphorylated state, its activation can be reached by

inhibiting its phosphorylation, and this provided the rationale for testing inhibitors of Cdk4 and Cdk6, two Cyclin-dependent kinases that associated with Cyclin G1, trigger the massive pRb phosphorylation necessary for S phase entry of proliferating cells (ref)

First I verified the efficacy of the drug on Cdk 4/6 activity by Western blot detection of phosphorylated pRb at Serine 780, the pRb residue target by Cdk 4/6, in p53^{-/-} cells (Fig 2 a). In line with several study in literature, I observed a strong reduction of pRb phosphorylation upon Cdk 4/6 inhibitor treatment. Then, I investigate the effect on E2F-1 activity and I found that after only 8h of treatment Cdk4/6 inhibitor increases the amount of pRb bound to E2F-1, and contemporary down-regulates mRNA expression of two E2F-1 target genes that are necessary for entry in S phase of cell cycle. Therefore, as expected, Cdk 4/6 inhibitor inhibits E2F-1 trans-activation activity blocking pRb phosphorilation. More importantly, Cdk 4/6 reduced HCT-116 p53^{-/-} cells population in a dose-dependent way presumably due to E2F-1 down-regulation. These data represent a valid rationale to combine Cdk 4/6 inhibitor to Nutlin-3a to obtain a drug with strong, p53-independent, anti-proliferative activity by targeting two main signaling pathways that regulate E2F-1:

- Nutlin-3a: to reduce E2F-1 protein increasing its degradation
- Cdk 4/6 inhibitor: to down-regulate E2F-1 activity by increasing inhibitory binding of pRb

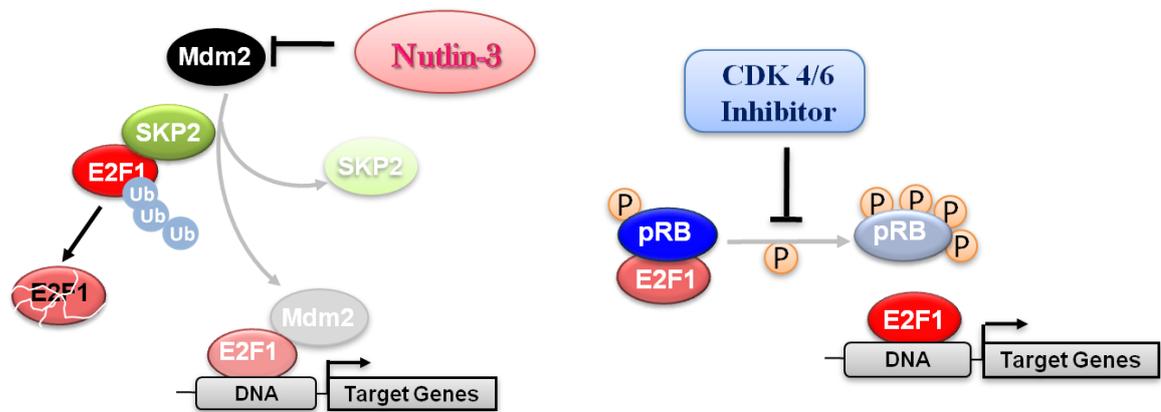


Fig. c. Down-regulation of E2F-1 by a double mechanism.

After I demonstrated the ability of both Nutlin-3a and Cdk 4/6 inhibitor to hinder cell proliferation through a p53-independent manner, I investigated how they affect proliferation when administered together, using the lower doses which exhibited an appreciable inhibitory effect in single treatments (Fig 1c, d; 2 d). On Crystal violet assay, the treatment allowed to a strong reduction of cell proliferation in both p53^{-/-} and p53DD cells, thus showing a good efficacy in both p53-muted and p53-null tumor cells. Worth of note, when the drugs were administered together they inhibit cell proliferation at a greater extent with respect to single treatment (Fig. 3 a). Accordingly, flow cytometry analysis showed an accumulation of the percentage of single cells in G1 phase, with a concomitant decrease of cell number in the other phases. Considering that E2F-1 activity is required for expression of proteins necessary for

transition from G1 to S phase of cell cycle, this result is consistent with the previous results showing the ability of both the drugs to down-regulate E2F-1.

In recent years, an increasing number of studies showed E2F-1 dual role in controlling both cell growth and apoptosis, and E2F1 mediated apoptosis is described to be associated with both p53-dependent and-independent mechanisms (Saha A *et al.* 2012; Wu Z *et al.* 2009; Engelmann D *et al.* 2010; Pediconi N *et al.* 2003). Moreover, it has been showed that Nutlin-3a induces apoptosis in p53-null cell, by activating p73 (Ramesh M. *et al.* 2011). Thus, to further clarify the mechanism by which drugs combination reduced cell population in Crystal violet assay, I evaluated acute death response and apoptosis pathway activation upon drugs combination treatment. After 24h exposure I didn't observe neither cell death, neither activation of apoptotic pathway in p53^{-/-} cells (Fig. 5). On the contrary, in wt-p53 HCT-116 cells cell population was strongly reduced after 24h treatment, and I observed a significant increase of 85-kDa fragment of Poly (ADP-Ribose) Polymerase (PARP-1) resulting from Caspase cleavage, used as indicator of apoptosis pathway activation (Lazebnik Y.A. *et al.* 1994; Duriez P.J. *et al.* 1997; Martha A. *et al.*, 2001). These results are in line with, and confirm that the Cdk 4/6 inhibitor-Nutlin-3a combination hinders cell proliferation in HCT-116 p53^{-/-} arresting cells in G1, and that the treatment can act as cytostatic or cytotoxic agent depending on p53 status. Further supporting this data,

western blot analysis showed a strong stabilization of p53 protein accompanying the increase PARP-1 cleavage in wt-p53 cells, upon combination treatment.

It has been recognized, through numerous studies, that deficiency in pRb is associated with a resistance to Cdk 4/6 inhibition (Lukas J *et al.* 1994). Consistent with this phenomenon is the finding that pharmacological inhibitors such as PD332991 fail to effect pRb-negative tumor cell lines (Rivadeneira DB *et al.* 2010).

To specifically define the influence of pRb on the response of p53-null cells to Cdk 4/6 inhibition, I used Saos-2 human osteosarcoma cells that are devoid of both p53 and pRb expression. Unexpectedly, and in contrast with data in literature, Saos-2 cells showed to be just a little less susceptible to Cdk 4/6 inhibition, respect to p53-null wt-pRb cells. In fact, also in these cells Cdk 4/6 inhibitor reduced proliferation in a dose dependent manner, and this effect was enhanced by addition of Nutlin-3a (Fig. 6 a, b). These results are consistent with a study where pRb-independent effect of Cdk4/6 on proliferation where observed in pRb-deficient cell line derived from human hepatocarcinoma (Rivadeneira DB *et al.* 2010), but not elucidating mechanism was found to explain this effect. Due to the critical role of E2F-1 in cell cycle control, and since all Cdk 4/6 inhibition effects observed in previous results, where

attributed to E2F-1 downregulation, I checked if E2F-1 was affected in these pRb-null cells, at both protein and mRNA level. Surprisingly, I found that Cdk4/6 inhibitor reduces E2F-1 protein level in Saos-2 (Fig. 4) and also in p53^{-/-} and p53-mutated HCT-116 cells (data not shown). This finding let me to reconsider the initial hypothesis that Cdk 4/6 effect were mediated only by specific inhibition of pRb phosphorylation, and conclude that such other mechanism occur to reduce E2F-1 protein level. The fact that no changes were observed in E2F-1-mRNA level, let me to investigate among post-transcriptional mechanisms of E2F-1 regulation, and I found that E2F-1 reduction, was due to its increased degradation through proteasome pathway.

Since it is known that MDM2 bind E2F-1 protecting him from degradation (Zhang *et al.* 2005), and it has been reported that inhibition of MDM2 phosphorylation reduce MDM2-p53 interaction (Zhou *et al.* 2001), we hypothesized that the interaction MDM2-E2F1 could be compromised by this mechanism following Cdk 4/6 inhibition, leading to a greater degradation of E2F-1. As hypothesized, Western blotting analysis revealed a reduction in the level of phosphorylation of MDM2 at Ser166 and Ser186, that are reported to promotes the nuclear

import of Mdm2 and binding to p53 (Feng J. *et al.* 2004). Moreover, Cdk 4/6 inhibition resulted in a reduction of total levels of MDM2, and this reduction was canceled by inhibiting proteasome. It was in fact reported that phosphorylation of Mdm2 by Pkb leads to inhibition of Mdm2 self-ubiquitination and stabilization of Mdm2 by protecting him from proteasome-dependent degradation (Feng J. *et al.* 2004). Thus, my results allowed the hypothesis to be suggested that the inhibitor was able to induce E2F-1 proteasome-dependent degradation reducing MDM2-stabilisind binding, as a result of a reduced MDM2 phosphorylation by Pkb, that in turn increases MDM2 degradation, thus reducing MDM2 protein level.

Accordingly with this hypothesis, we found that MDM2 was reduced after Cdk 4/6 inhibition, due to its increased degradation (Fig. 7 c, d). However, subsequent experiments have shown that reduction of the phosphorylation and the levels of MDM2 are not tied to a reduced activity of Pkb.

6. Conclusion

The data presented provide evidence that Nutlin-3a-PD0332991 association could represent a potent anti-proliferative chemotherapeutic treatment to block proliferation in cells lacking not only functional p53 but also lacking functional pRb, by modulating at least two distinct mechanisms leading to a synergistic downregulation of E2F-1.

Consistent with the mechanism described by Donati (2011), my results show that the inhibition of MDM2 by Nutlin-3a lead to a down-regulation of E2F-1 protein level by increasing its proteasomal degradation, and inhibits proliferation in cells lacking functional p53.

Furthermore, I demonstrated that Cdk4/6 inhibition by PD0332991 reduces E2F-1 activity by increasing hypo-phosphorylated pRb-E2F-1 binding, and enhances Nutlin-3a ability to inhibit progression from G1 to S phase of cell cycle in p53-null and p53-mutated cells.

Unexpectedly, I found that PD0332991 reduced proliferation also in cells lacking functional pRB, and this effect is stronger when PD0332991 is co-administered with Nutlin-3a. This result was explained by the observation that PD0332991 not only reduces E2F-1 activity, but also reduce E2F-1 levels increasing its degradation. Even if the mechanism by which PD0332991 reduce E2F-1 protein level still remains unclear, these result suggest that the combined treatment with inhibitors of MDM2 and inhibitors of Cdk4/6 may represent a valid chemotherapeutic approach to inhibit proliferation in tumors independently of p53 and pRb function.

7. Bibliography

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