Cancer Biology & Therapy

ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: http://www.tandfonline.com/loi/kcbt20

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To cite this article: Eleonora Marrazzo, Sergio Marchini, Sara Previdi & Massimo Broggini (2006) Questioning the oncogenic role of ΔNp73α in different cell lines expressing p53 or not, Cancer Biology & Therapy, 5:7, 794-803, DOI: 10.4161/cbt.5.7.2753

To link to this article: http://dx.doi.org/10.4161/cbt.5.7.2753

Published online: 01 Jul 2006.

[Cancer Biology & Therapy 5:7, 794-803, July 2006]; ©2006 Landes Bioscience

Research Paper

Questioning the Oncogenic Role of ΔNp73α in Different Cell Lines Expressing p53 or Not

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Received 01/18/06; Accepted 03/28/06

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=2753

KEY WORDS

ΔNp73α, tumor biology, p53

ABBREVIATIONS

cDDP	cis-diamminedichloroplatinum
Doxy	doxycycline
DX	doxorubicin
h	hour
HSF	heat shock factors
MIN	microsatellite instability nucleotide
SCLC	small cell lung cancer
SD	standard deviation
TET	tetracycline
UV	ultraviolet
wt	wild type

ACKNOWLEDGEMENTS

The generous contribution of the Italian Association for Cancer Research is gratefully acknowledged. This work was partially supported by Fondazione Cariplo. Eleonora Marrazzo is recipient of a fellowship from the Nerina and Mario Mattioli foundation.

ABSTRACT

The recent finding that the 1p36.3 locus gene encodes an array of different p73 isoforms with apparently distinct and sometimes opposing cellular functions, might explain the difficulty in establishing the protein's role as tumor suppressor. Therefore we need to investigate the roles of each of these splicing variants in cellular functions when expressed alone or in combination with other family members, as well as the genetic background on which the proteins are expressed. We investigated, in two p53 null cell lines, the human SCLC line H1299 and a subline derived from the human colon carcinoma cell line HCT116 (HCT116/379.2), the effects of ΔNp73α overexpression on cell growth and the response to anticancer treatment. We generated three different clones overexpressing ΔNp73α under a tetracycline inducible promoter. Immunofluorescent staining and luciferase reporter assays confirmed that clones HCT116/ΔNA and H1299/ΔN7 and H1299/ΔN11 did express a functional, nuclear localized ΔNp73α protein. The stable overexpression of ΔNp73α protein did not confer any cell growth advantage. Doubling time of clones overexpressing ΔNp73α were comparable to counterparts not expressing it. Clonogenic assays showed that the cytotoxic activity of different DNA damaging agents, such as cDDP, UV light and doxorubicin, were comparable in clones expressing ΔNp73 or not. The overall data argue against an oncogenic role for this isoform. These findings are independent of the p53 status since they overlap with those previously obtained by our group in HCT116 cell lines, wild type for p53.

INTRODUCTION

For more than 25 years, p53 has been considered one of the most important tumor suppressor genes.[1] The p53 protein is an effective barrier against the process of carcinogenesis,[2] suppressing tumor formation by direct or indirect activation of a plethora of genes involved in apoptosis, growth arrest, cytoskeleton rearrangement, growth factor regulation and cell adhesion, and then as repression of genes involved in metabolism.[3] In view of its central role in tumor prevention, it is not surprising that p53 is often a target of genetic alterations in cancer. More than half of human cancers have mutations in the p53 gene and in the majority of the remainders its function is impaired by aberrations in proteins that act either upstream or downstream of the p53 pathway.[2,4]

The p53 gene homologous p73 encodes a protein with significant structural homology to p53.[5] p73 shares more than 60% amino acid identity within the p53 DNA binding domain, 38% with the p53 oligomerization domain and 29% with the p53 transactivation domain.[6] p73 also shows many p53-like features: it can bind to p53 DNA binding sites, transactivate p53 responsive genes, induce irreversible cell cycle arrest and promote apoptosis.[6] However, unlike p53, p73 mutations are extremely rare in human cancer and in contrast to p53 knockout mice, p73 deficient mice lack a cancer phenotype, but present neurological and immunological defects.[7-11]

The expression of the p73 gene is also complicated by the presence of multiple isoforms that may exert distinct or overlapping functions. Alternative splicing at the 3' portion of the p73 gene leads to the expression of nine carboxy-terminal isoforms (α to ϕ), which can be combined with five different amino-terminal portions. The amino-terminal isoforms consist of two classes of proteins: the transactivation-proficient TAp73 proteins and the transactivation-deficient ΔTAp73 proteins. The N-terminally truncated p73 forms are generated through either alternative exon splicing of the P1 promoter transcript (producing ΔN'p73, Ex2p73 and Ex2/3p73)[12-14] or alternative use of a second promoter located in intron 3 (producing ΔNp73).[15]

Bourdon et al. discovered that p53 also has a complex gene structure similar to the p73 gene and the p53 gene encodes at least nine different p53 isoforms, resulting from alternative splicing and the use of an internal promoter in intron 4.[16]

ΔTAp73 isoforms have a dominant-negative effect on both wild-type p53 and full-length TAp73 by blocking their target gene transactivation, apoptosis, and growth suppression functions. This is thought to be mediated either by competition through its DNA binding domain, and/or by protein-protein interaction through its oligomerization domain.[17] Ectopic expression of ΔTAp73 in mice is reported to inhibit p53-induced apoptosis and to protect $p73^{-/-}$ neurons from death induced by nerve growth factor (NGF) withdrawal.[18]

Among ΔTAp73 isoforms, the ΔNp73 protein is particularly interesting since in neuroblastoma, gliomas, ovarian carcinomas, tumors of the prostate and lung cancers, its expression could even be correlated with advanced tumor stage or poor prognostic parameters.[19-23] The balance between TAp73 and N-terminally truncated p73 isoforms might be involved in cancer progression.[17] Thus, the p73 locus gene would encode both a tumor suppressor (TAp73) and a putative oncogene (ΔNp73). Nevertheless it is still controversial whether ΔNp73 is involved as oncogene during tumorigenesis. ΔNp73 promotes immortalization in primary cells and cooperates with oncogene *Ras* to drive their transformation in vivo.[24] On the other hand, recent results from our laboratory have shown that at least in cancer cells expressing p53 and p73 functional pathways, ΔNp73α is not associated, either in vitro or in vivo, with a more malignant phenotype or a more aggressive and resistant tumor.[25]

To gain further knowledge of ΔNp73α's role in cancer cell growth and response to treatment, we investigated the effects of its overexpression in human cellular models with a $p53^{-/-}$ and $p73^{+/+}$ genetic background.

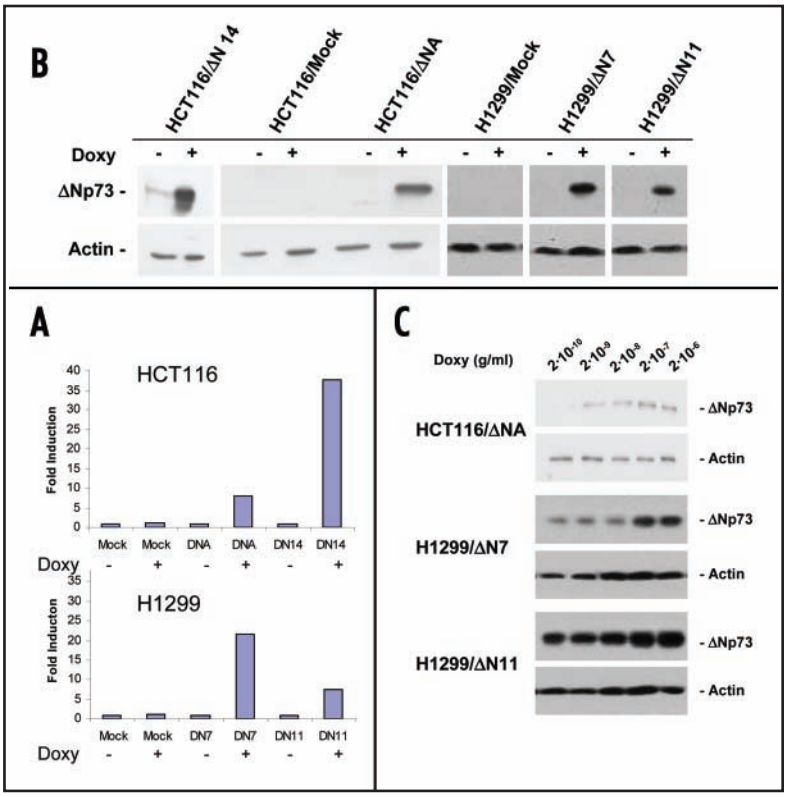


Figure 1. Expression of ΔNp73α in HCT116 and H1299 clones. (A) Relative mRNA expression of ΔNp73α in HCT116 (upper) and H1299 (lower) clones following doxy treatment. mRNA levels were determined by real time RT-PCR 24h following the addition of doxy. Expression of clones in absence of doxy was arbitrary set to 1. (B) Western blot analysis of ΔNp73α expression with and without doxy for 24 h. Actin was used as homogeneous gel loading. Clone HCT116/ΔN14 was used as wt p53 internal control and HCT116/Mock and H1299/Mock as negative internal controls (C) Western blot analysis of doxy-dependent expression of ΔNp73α in HCT116/ ΔNA, H1299/ΔN7 and H1299/ΔN11 clones; 24 h after seeding, cells were treated with increasing concentrations of doxy for 24 h and proteins were extracted. Actin was used as homogeneous gel loading.

MATERIALS AND METHODS

Cell culture. The HCT116 clone 379.2 ($p53^{-/-}$), derived from a human colon carcinoma, is routinely maintained in Iscove medium supplemented with 10% foetal calf serum. The H1299 cell line ($p53^{-/-}$), derived from a human lung carcinoma, is routinely maintained in RPMI1640 medium supplemented with 10% foetal calf serum. For each cell line a clone transduced with a tetracycline repressor (pcDNA6TR, Invitrogen, CA, USA) was used to generate ΔNp73α expressing clones. ΔNp73α cDNA (kindly supplied by Dr. De Laurenzi) was subcloned in the tetracycline-inducible plasmid pCDNA4/TO and different clones were picked up. Three of them, namely HCT116/ΔNA, H1299/ΔN7 and H1299/ΔN11, were selected and allowed to grow in medium supplemented with 10% of TET System approved foetal bovine serum (BD biosciences, USA) with 5 μg/mL of blasticidin (Invitrogen), and 10 μg/mL zeocin (Invitrogen). Clone HCT116 and an H1299 clone transfected with the empty vector (HCT116/Mock and H1299/Mock, respectively) were used as internal controls. Doxycyclin (doxy) was purchased from Sigma (Milan, Italy). Transient transfection with wild type p53 was cloned by the Lipofectamine 2000 method (Invitrogen). Evaluation of growth rate was performed by counting cells using a cell culture counter (Coulter Channelyser® 256, Beckman Coulter, Milan-Italy) and the rate measured on the exponential phase.

Real Time RT-PCR. Two hundred ng of total RNA purified with the Trizol protocol (Invitrogen) were retrotranscribed in 20 μL with TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and 2 μL were further amplified by Real Time PCR. Real Time PCR was used for relative quantification of ΔNp73α, using actin as internal control.

Primers and probe sequences to detect the levels of ΔNp73α were 5'-GGATTCCAGCATGGACGTCTT-3' as forward primer and 5'-CGCCTACCATGCTGTACGT-3' and 5'-GGCTGCTCATCT-GGTCCAT-3' as TaqMan probe (Assay by Design, Applied Biosystems). Primers and TaqMan probes sequences to detect the TAp73, mdm2, p21 and actin mRNA levels were supplied as ready to use solution (Assay on Demand, Applied Biosystems). Reactions were run in a total volume of 25 μL with TaqMan PCR Master Mix, following the manufacturer's instructions (Applied Biosystems).

Western blot. Cell extracts from untreated and drug treated cells were prepared by lysing cells in 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.1% Nonidet NP-40, 5 mM EDTA, 50 mM NaF with aprotinin, leupeptine and phenyl-methyl-sulfonyl-fluoride (PMSF) as protease inhibitors, for 30 min on ice. Insoluble material was pelleted at 13000 x g for 10 min at 4°C and the protein concentration was determined using a Biorad assay kit (BioRad, Italy). Forty micrograms of total cellular proteins were separated on SDS-PAGE and electrotransferred to PVDF membrane. Immuno-blotting was carried out with p73 monoclonal antibodies (Oncogene Research,

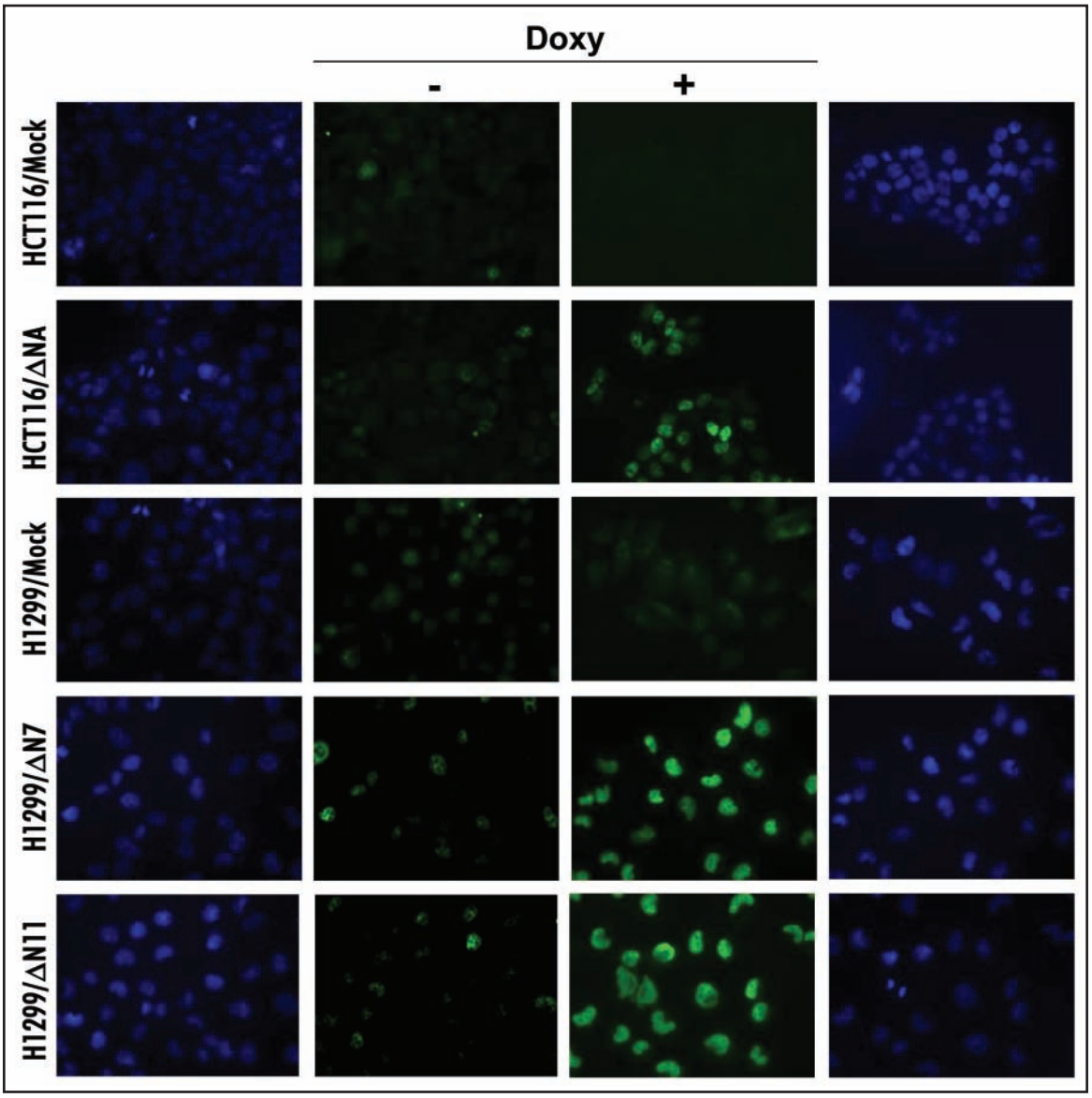


Figure 2. Expression of the ΔNp73α isoform in HCT116 and H1299 intact cells. Doxy-untreated (-) and treated (+) cells were fixed, permeabilized, immunostained with anti-ΔNp73α (green) and further stained with Hoechst 33258 (blue). Fluorescence was visualized by fluorescent microscopy (original magnification × 40).

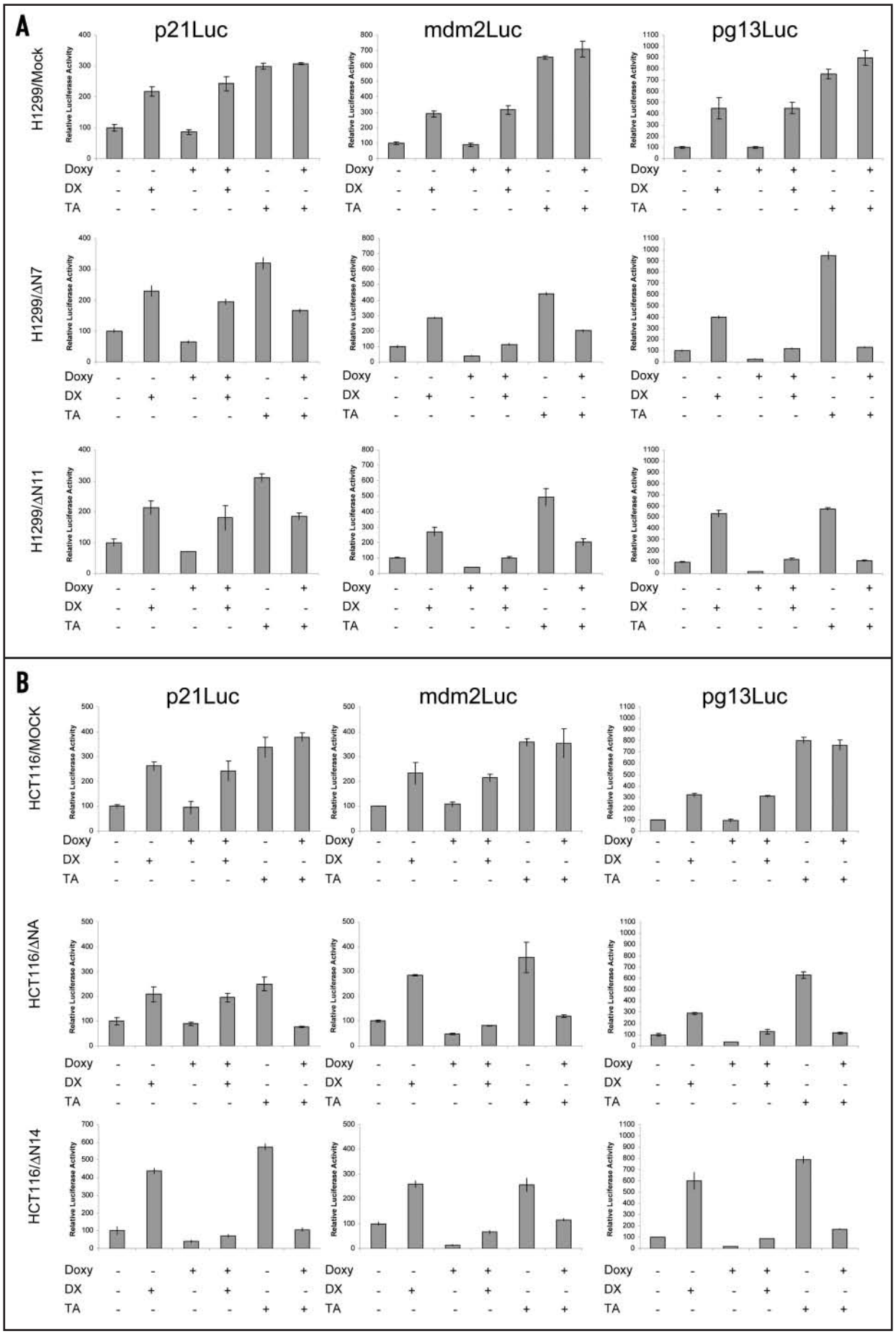
CA, USA) and actin was detected by an anti-actin polyclonal antibody (Santa Cruz Biotechnology, CA, USA). Antibody binding was revealed by peroxidase labelled secondary antibodies and visualized using enhanced chemioluminescence (Amersham, Italy).

Immunofluorescence. In vitro growing cells (25.000 cells/well) were seeded in 24-well plates on glass coverslips; 24 h after doxy treatment, cells were fixed for 20 min in cold methanol-acetone solution (1:1), permeabilized with 0.01% Triton X-100 and blocked in 1% BSA in PBS for 20 min at room temperature. Cells were then incubated overnight at 4°C with monoclonal p73 antibody (Oncogene Research, CA, USA) diluted 1:1000 in 1% BSA-0.01% Triton X-100 in PBS. After washing with 1% BSA-PBS solution, cells were incubated with an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes ^{TM}, Invitrogen, CA, US), for 1h, at 1:500 dilution in 1% BSA-PBS. After PBS washing, Hoechst 33258 counterstaining was done (10 min, 1:1000).

Finally, coverslips were mounted with FluorSave Reagent (Calbiochem). Immunofluorescence staining was examined using a fluorescent microscope (Olympus FV 500, Milan, Italy). An additional sample was incubated with the secondary antibody alone as negative control.

Luciferase Assay. On 12-well plates, 6 x 10^6 cells were seeded per well. After 24h doxy was added to half the plates and cells expressing the ΔNp73α protein or not were transfected in triplicate with

Figure 3 (Next page). Functional activity of ΔNp73α in HCT116 (B) and H1299 (A) clones with or without doxy. 0.4 μg of p21Luc, mdm2Luc or pG13Luc or pcDNA3/TAp73α vectors are cotransfected into selected clones with 15 ng of Renilla Luciferase Assay Vector (PRL-SV40) as internal normalization control. Luciferase activity is determined 24 h after transfection and after exposing transfected cells to 1 μM DX treatment for 6 h. Values are percentages of luciferase activity normalized with renilla activity. Luciferase activity for each clone determined in the absence of doxy is set to 100%. Bars are ± SD of at least three determination. TA, pcDNA3/TAp73α.



0.4 μg/well of purified plasmid (p21Luc, mdm2Luc, pG13Luc or pcDNA3/TAp73α) and 15 ng/well of PRL-SV40 (Promega, Italy) as internal normalization control, using the Lipofectamine 2000 method (Invitrogen). In addition, 18h after transfection cells were treated with 1 μM doxorubicin (DX) for 6 h. Reporter gene activities were evaluated 24 h after transfection using the Dual Luciferase System (Promega, Italy). Results are expressed as the percentage of the control luciferase reported activity normalized by the renilla activity value. The mean ± SD of three independent experiments is shown.

In vitro cytotoxicity. For clonogenic assay, the five different clones were plated at 200–300 cells/well in six-well plates; 24 h after seeding, doxy was added to half the plates and cells were then treated for 2 h with different concentrations of cis-diamminedichloroplatinum (cDDP, Sigma) or DX or UV doses. After washing with PBS, plates were incubated for 8-10 days in drug-free medium. The colonies formed were stained with 10% crystal violet in 20% ethanol and automatically counted on an image analyzer (Immagini & Computer, Italy). The survival curves were plotted as percentages of untreated controls. Each experiment consisted of three replicates. The mean ± SD is shown.

RESULTS

To investigate the effects of ΔNp73α overexpression in the absence of a functional p53 pathway, two different cell lines expressing not wt p53 but wt p73, the human colon carcinoma cell line HCT116 clone 379.2 and the lung adenocarcinoma cell line H1299, were selected and transfected with a doxy-inducible promoter. Different clones were allowed to grow in vitro and the H1299/ΔN7, H1299/ΔN11 and HCT116/ΔNA were selected for further studies. Clones HCT116/Mock and H1299/Mock, transfected with pCDNA4/TO empty plasmid, were used as negative controls, and the previously characterised clone HCT116/ΔN14 (obtained in the parental HCT116 $p53^{+/+}$ cell line) was used as an internal positive control.

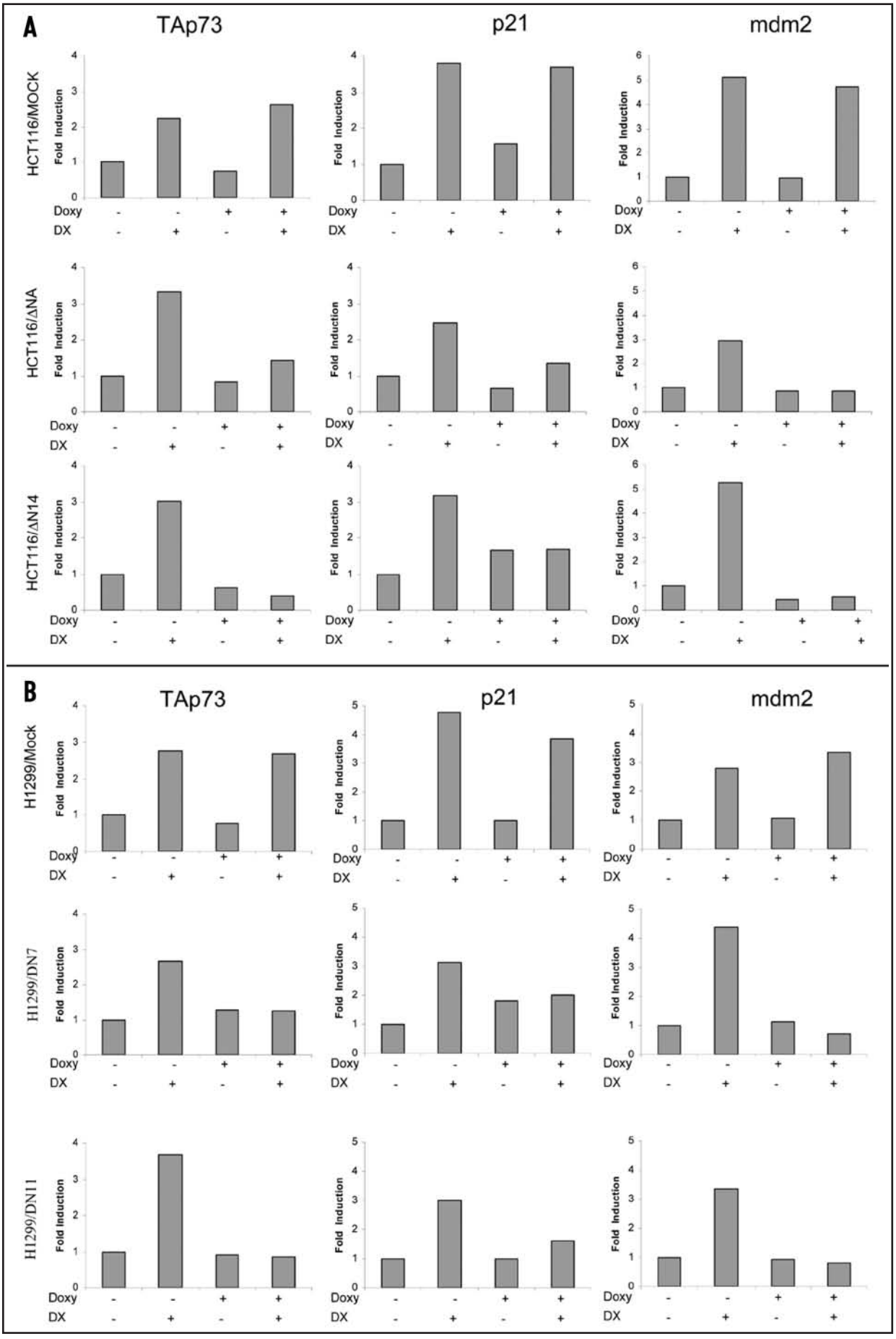
Real time-RT-PCR and western blot analysis reported in Figure 1A and B showed the ΔNp73α expression level after 24 h exposure to doxy in comparison to untreated cells. These clones showed a strong doxy dependence with a 7-to 20-fold increase in the ΔNp73α mRNA expression if compared with the not induced cells (21-, 7,5-, 8-fold for H1299/ΔN7, H1299/ΔN11 and HCT116/ΔNA, respectively). As expected, the HCT116/ΔN14 clone showed a more than 30 fold increase in the expression of ΔNp73α mRNA compared to the untreated counterpart. In HCT116/Mock or H1299/Mock cells, we did not observe any change in ΔNp73α mRNA levels after doxy exposure. This strong induction was also observable at protein level (Fig. 1, panel B). In the absence of doxy, ΔNp73α levels were almost undetectable in all selected clones. Homogeneous gel loading was tested by actin probing. Tetracycline-dependent expression of ΔNp73α protein was assayed by adding different doxy concentration in the medium 24h after cells were seeded (Fig. 1C). The two cell lines respond differently to doxy, H1299 being much more sensitive to induction than the HCT116 clones. The doses of 200 pg/mL for H1299 and 2 μg/mL for HCT116 were therefore selected for further studies.

To visualize the subcellular localization of ΔNp73α, HCT116 and H1299 cells were stained with Hoechst 33258 and anti-ΔNp73α antibodies and analysed by fluorescent microscopy (Fig. 2). Images obtained by optical slicing of HCT116/ΔNA cells, 24 h after doxy treatment (2 μg/mL), showed a higher fluorescent intensity than untreated cells. Hoechst 33258 staining confirmed that ΔNp73α distribution was mainly within the nucleus. The results were similar with H1299/ΔN7 and H1299/ΔN11 (doxy 200 pg/mL for 24 h). As expected, in the control clones HCT116/Mock and H1299/Mock no fluorescent signal was detectable.

To verify whether the high nuclear levels of ΔNp73α were also functionally active, we checked their ability to inhibit p73 transcriptional function by luciferase assay. p21Luc, mdm2Luc and pG13Luc plasmids containing different p53/p73 responsive elements 5' to the luciferase gene, were transiently transfected in the different clones and cells were further treated or not for 24 h with previously selected concentrations of doxy. Figure 3A clearly showed that in H1299/ΔN7 and H1299/ΔN11, ΔNp73α competes with endogenous TAp73 in the binding and activation of the transcription of the genes. This effect was seen both in a "pure" artificial p53-responsive promoter (containing only 13 copies of the consensus p53 binding element, pG13Luc) and in two "natural" p53-responsive promoters isolated from p21 and mdm2 genomic sequences respectively. In clones H1299/ΔN7 and H1299/ΔN11, the effect on promoter activity after exposure to doxy, was marked compared to the parental untreated cells (more than 50%) with the mdm2Luc and pG13Luc plasmids but only 30–35% of untreated controls for the p21Luc plasmid. In the HCT116/ΔNA clone (Fig. 3B), ΔNp73α inhibited the luciferase activity measured after transfection of the mdm2 and pG13Luc plasmid but no differences were found between doxy treated and untreated cells for the p21Luc plasmid. The effect observed in clone HCT116/ΔN14 was as previously reported, while in the HCT116/Mock and H1299/Mock, the three tested promoters showed overlapping luciferase activity in doxy treated and untreated cells. To verify the ability of ΔNp73α to specifically compete with TAp73 activity, we cotransfected the TAp73α cDNA with the luciferase promoter of the previously selected genes and the luciferase activity measured when cells were induced or not to express ΔNp73α. The same experiment was run after exposing cells to a well known TAp73α stabilising agent like DX (1 μM, for 6 h). The overall data reported in Figure 3A and B showed that ectopic TAp73α overexpression or DX exposure were able to increase the transcription of the reporter gene subcloned downstream of the three previously selected promoter. ΔNp73α overexpression following doxy exposure competed with TAp73 in the transcription of luciferase genes when sub-cloned downstream of the p21, mdm2 and pG13 promoter. Similar results were obtained after DX treatment for the mdm2 and pG13 promoter (Fig. 3A and B). For all the tested HCT116 and H1299 clones after DX treatment, we could observe that ΔNp73α, when overexpressed, was, however, not able to restore the p21 promoter activity to the basal levels.

To strengthen our results, we measured by real time RT-PCR the levels of endogenous TAp73α, p21 and mdm2 after exposure to DX (1 μM for 6 h). Figure 4A and B showed that in our experimental models DX is able to increase the mRNA levels of both TAp73, p21 and mdm2 while ΔNp73α, when overexpressed, competed and

Figure 4 (next page). Real time RT-PCR of HCT116 (A) and H1299 (B) ΔNp73α overexpressing clones. mRNA levels of the TAp73α, p21 and mdm2 gene were measured by RT-PCR after DX treatment to verify the ability of doxy inducible DNp73α to interfere with the transcription of the three selected genes. In each graph the basal levels were arbitrary set to 1.



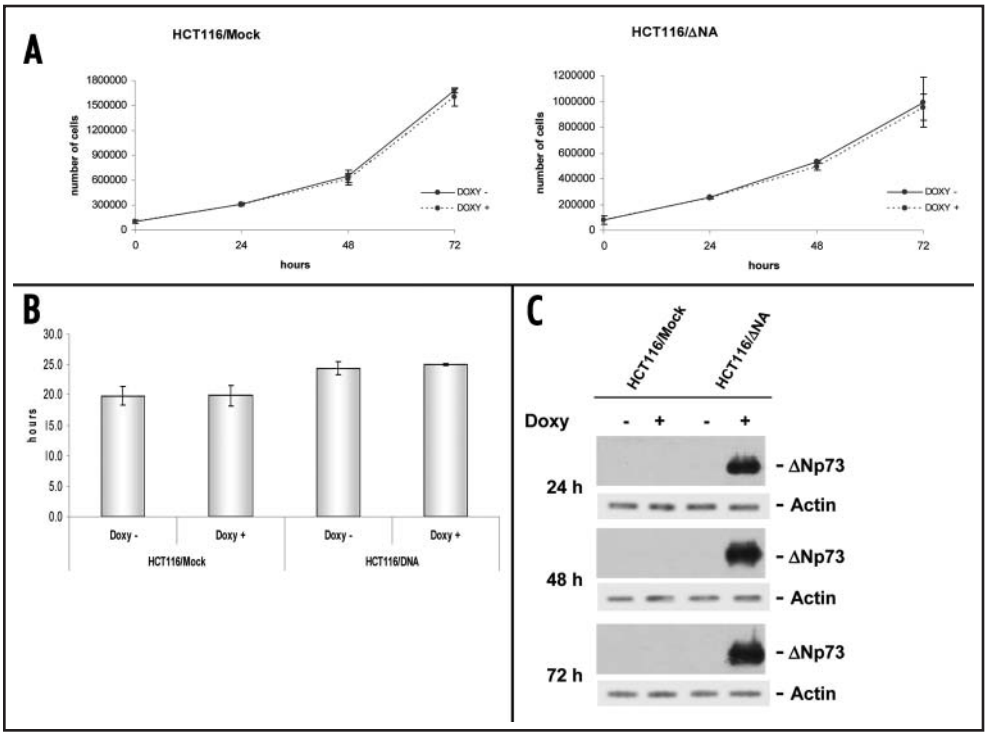


Figure 5. In vitro growth of HCT116 clones. (A) Growth curves of HCT116/Mock and HCT116/ΔNA clones without (DOXY -) and with doxy (DOXY +). Cells are seeded in six-well plates and counted using a Coulter Cell Counter every 24 h after the addition of doxy. (B) Doubling time plot in hours for each clone with or without doxy. (C) Western blot analysis of ΔNp73α expression in HCT116/Mock and HCT116/ΔNA clones is done using protein extracts from cells used to determine the growth curves reported in (A). Actin is used as homogeneous gel loading. Bars are ± SD.

reduced their expression to the basal levels of untreated cells. No effect was observable in the H1299/Mock and HCT116/Mock after doxy treatment.

Having found that upon induction the three ΔNp73α clones expressed a functional ΔNp73α, we analysed the growth of these clones in the absence and presence of doxy. Figure 5A and Figure 6A, show that in the two cell lines the addition of doxy did not modify the growth of either mock transfected or ΔNp73α expressing clones. Experiments were performed by counting cells at different times after doxy exposure. Doubling times analysis showed that no significant differences in their growth rate (Figs. 5 and 6, panel B). In these experiments the cells were seeded and doxy added after 24 h, as previously reported. The same results were obtained by clonogenic assay, which did not show any difference in the growth rate of cells expressing or not the ΔNp73α protein (data not shown). The expression of ΔNp73α in the selected clones after doxy induction was checked by Western blotting at all time points used for determining cell growth and the ΔNp73α protein was clearly detectable till the end of the experiment in only doxy-treated clones. (Figs. 5 and 6 panel C).

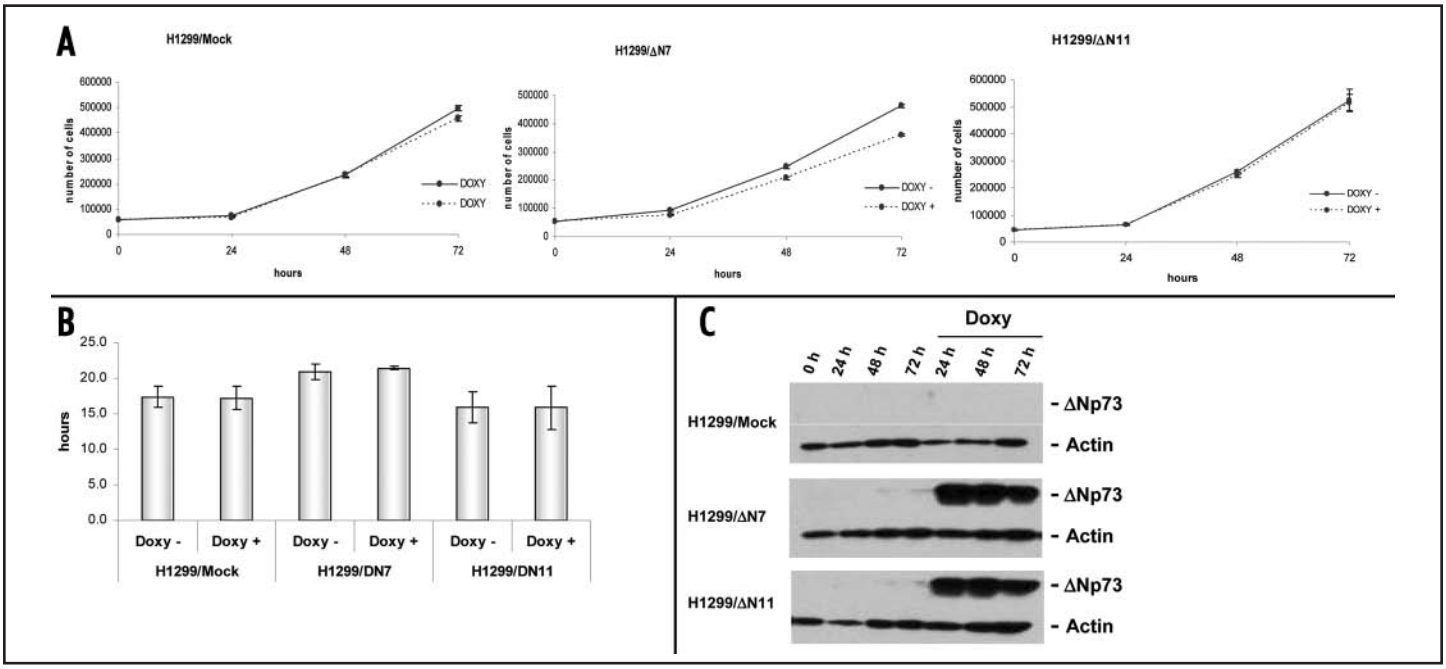


Figure 6. In vitro growth of H1299 clones. (A) Growth curves of H1299/Mock, H1299/ΔN7 and H1299/ΔN11 clones without (DOXY -) with doxy (DOXY +). Cells are seeded in six-well plates and counted using a Coulter Cell Counter every 24h after the addition of doxy. Bars are ± SD. (B) Doubling time plot in hours for each clone with or without of doxy. (C) Western blot analysis of ΔNp73α expression in H1299/Mock, H1299/ΔN7 and H1299/ΔN11 clones is done by using protein extracts from cells used to determine the growth curves reported in (A). Actin is used as homogeneous gel loading.

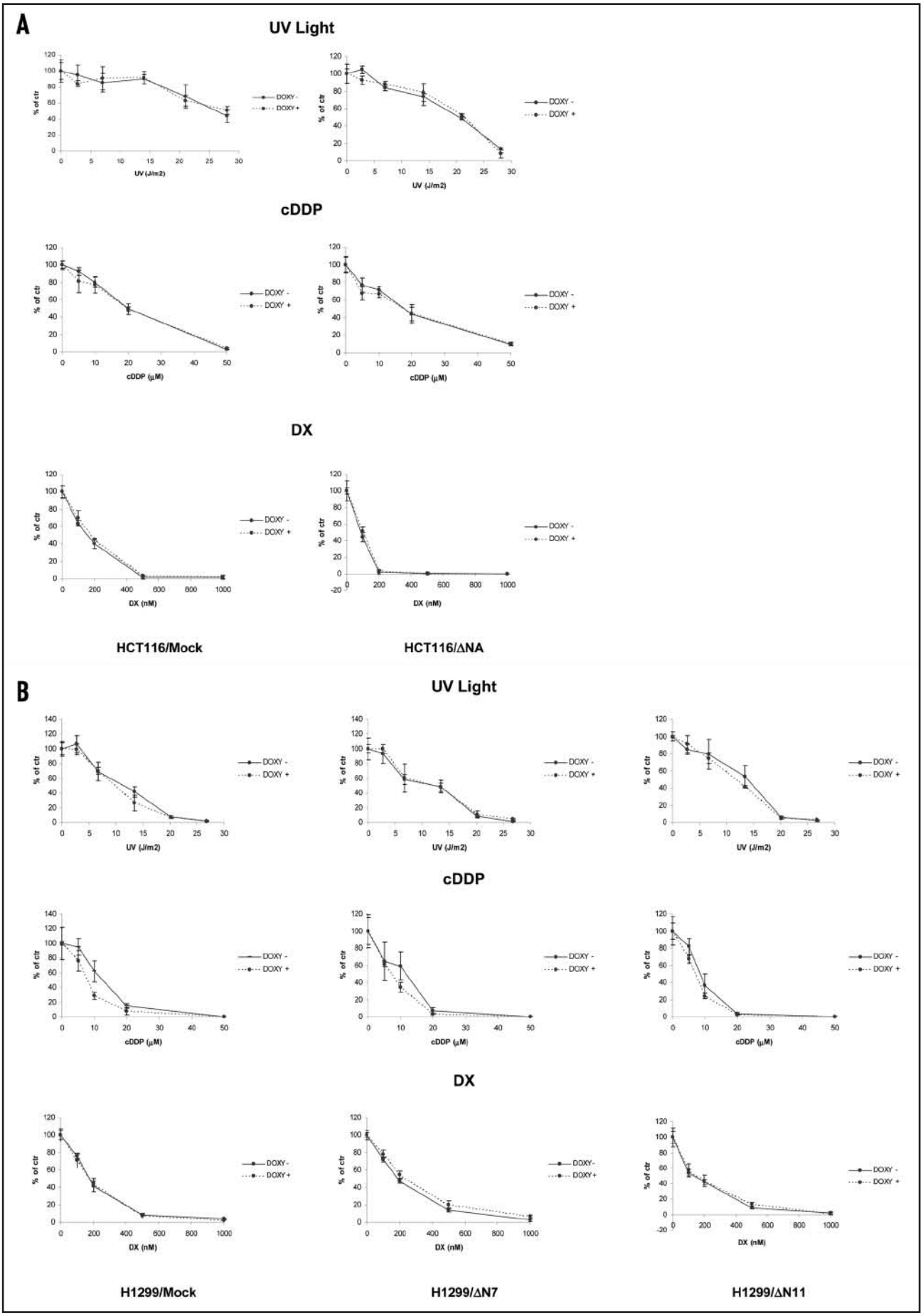


Figure 7. In vitro cytotoxicity. Response of HCT116 clones (A) and H1299 clones (B) to treatment with UV, cDDP or doxorubicin. The survival curves are plotted as percentages of untreated values. Bars are ± SD.

We further investigated the three clones' ability to respond to DNA damage's induction with and without doxy. Three different kinds of damage, i.e., exposure to UV light, treatment with the alkylating agent cDDP or with the intercalating agent DX, were selected. All these treatments were given when the ΔNp73α form was already expressed and Figure 7 results for the two HCT116 clones (Fig. 7A) and the three H1299 clones (Fig. 7B). Inhibition of the growth was dose dependent for UV, cDDP or doxorubicin. However, for all the clones, cell growth inhibition plots obtained with or without doxy almost overlapped, suggesting that, at least in these models, high levels of ΔNp73α do not cause any evident modification of the cellular response to damage.

DISCUSSION

In recent years, the growing knowledge of the human genome structure has disclosed the fine molecular architecture of many genes, particularly of those at the crossroads of important cellular functions. The findings that the p73 (and recently p53) locus gene, gives rise to multiple protein isoforms, generated by alternative mRNA splicing or by the use of different promoters, shed new lights on their role in cancer progression and responses to anticancer treatment.[11,16,17]

The amino-terminally truncated ΔTAp73 isoforms lack the transactivation domain and, despite being transcriptionally inactive, they play an important regulatory role in cellular functions. They exert a dominant-negative effect over wild type p53 and p73 pathways by blocking their transactivation activity through hetero-oligomerization with p73 or p53 or competitive inhibition in the binding of p53 to its DNA responsive elements.[13,26-28]

The increasing interest in the role of ΔNp73 in driving cellular responses to anticancer agents and tumor growth control is also justified by the finding that several amino-terminally deleted versions of TAp73 were expressed in certain human cancers but not in their surrounding normal tissues.[15,29] ΔNp73 was proposed as an adverse prognostic marker in neuroblastoma patients.[23] Studies are still necessary to assess the attractive oncogenic role of ΔNp73 in clinical setting, but ΔNp73 isoforms overexpression might reflect inactivation of the tumor suppressor activities of p53 and, with bona fide, of the TAp73 isoforms.

The complexity of this "transcriptional puzzle" ruled out three interesting considerations: first, p53 or p73 status cannot be considered the sole predictor of clinical outcome and response to anticancer therapy since the TA:ΔN ratio seems essential to confer a tumor growth advantage.[17] Second, it is possible to mutate and inactivate gene function by selectively deregulating the alternative promoter activity of these dominant negative isoforms. Third, it is important to know the function of each individual isoform of TAp73 and ΔNp73 variants when a specific isoform is expressed alone or in combination with other isogenic forms within a specific genetic cellular background.

On the basis of these considerations, we investigated the role of one of these amino-terminally truncated p73 isoforms, ΔNp73α, in cellular activity and the response to some genotoxic stresses. Data previously obtained by our laboratory in in vitro and in vivo models using the colon-carcinoma cell lines HCT116 (wt for both p53 and p73) sharply contrasted with the general view that ΔNp73α per se might be the main actor in controlling cellular response to genotoxic stresses or malignant phenotype. The open question was whether the lack of any ΔNp73α activity was related to the presence of functionally active p53 and p73 pathways, as well as the peculiar genetic background of the HCT116 cell line ($hMLH1^{-/-}$). To further address the question whether the data obtained were not restricted to clone selection or were not cell-type-dependent, we generated isogenic cell clones in two different human cancer cell lines not expressing p53 but wt for p73. The un-induced and induced cells are isogenic and therefore ideal for examining ΔNp73α functions, especially the long term effects on cell growth.

The overall in vitro data presented herein overlapped an earlier finding.[25] Our cellular models argued against a role of the ΔNp73α isoform as a potent pro-survival protein since ΔNp73α overexpression did not rescue cell lines from cell death or did not confer any growth advantage. These data were confirmed both in models with a $p53^{-/-}$ or $p53^{+/+}$ genetic background and with a $MIN^{+/+}$ (H1299) or $MIN^{-/-}$ (HCT116) phenotype.

In our cellular models, tetracycline concentrations in the medium were titrated to achieve high stable levels of functional ΔNp73 over the endogenous TAp73 in order to force the system towards long-term effects of stable ΔNp73 expression (72 h). In these conditions, functional ΔNp73α overexpression had no significant effects on cell growth or sensitivity to DNA damaging agents: doubling time was almost comparable within each clone (around 22 h). In addition, the immuno-fluorescence staining and luciferase assay results argued against the hypothesis that data were related to abnormal ΔNp73α sub-cellular localization or expression of a functionally defective protein. The low inhibition of p21 promoter activity by ΔNp73α in the two H1299 clones and in the HCT116 one might be explained by transcription factors other than p73 being involved in the transcriptional regulation of the p21 gene.[30-32]

In several human cell lines, wt or not for p53, expression of DNp73α, but not β, was reported to act as an anti-apoptotic transcriptional factor in the light of its ability to activate HSF-responsive genes like HSp70 in in vitro.[33] However, in our models, when cells were exposed to different genotoxic stresses like UV light, cDDP or DX, the presence of ΔNp73α did not protect them against cell death since cells from clone H1299/ΔN7 or H1299/ΔN11 and those from clone HCT116/ΔNA, when expressing ΔNp73α, shared the same sensitivity as the parental cell lines not expressing ΔNp73α. In addition, ΔNp73α overexpression does not act as a pro-survival factor for cells exposed to a low O_2 concentration (data not shown), as previously reported for sympathetic neurons.[34]

The in vitro data reported here support the notion that among the different dominant negative p73 isoforms, the α one does not seem to act per se as a pro-oncogenic factor. This is not in contrast with the possibility that ΔNp73α may cooperate with other oncogenic signals, not strikingly p53-dependent, to drive cellular transformation and abnormal cell growth. Clearly, more detailed clinical studies are still needed to see whether ΔNp73α expression can really be considered a negative prognostic factor.

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