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The expression of the ΔNp73β isoform of p73 leads to tetraploidy

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ABSTRACT

The p73 locus gene has a complex structure encoding a plethora of isoforms. The different ΔN truncated isoforms of p73 may exert different activities depending on the cellular context. The β isoform of ΔNp73 seems to have a particular pattern of action even if its role in cell cycle and mitosis is still under investigation. To gain further knowledge of ΔNp73β's function, we investigated the effects of its over-expression in tumour cellular models, using the tetracycline-inducible expression system. In the human lung carcinoma cell line H1299, ΔNp73β over-expression resulted in suppression of cell growth and in cell death. Surprisingly stable over-expression of ΔNp73β impaired the genomic stability of tumour cells, leading to the formation of tetraploid cells. The cells become enlarged and multinucleate, with incorrect mitotic figures, and died by apoptotic-independent pathways. Our data suggest that ΔNp73β-induced aberrant mitosis evades the control of the mitotic spindle assay checkpoint, leading to tetraploidy and cell death through mitotic catastrophe rather than apoptosis. The various C-terminal regions of ΔNp73 may influence the final cellular phenotype and we assume that the β one in particular could be important in both cell growth control and regulation of mitosis.

1. Introduction

TP53 is the prototype tumour suppressor gene in human cancers on account of its vital role in deciding the destiny of cells in response to cellular stresses and DNA damage. Inactivation of the p53 pathway occurs in more than half of human sporadic cancers, and germline mutation of p53 results in Li-Fraumeni syndrome.[1,2]

For almost two decades, p53 was considered unique in its structure and function, but recently the discovery of two TP53-related genes, TP73 and TP63, derived probably from a common ancestor, has outlined a new gene family with similar, but not overlapping, functions.[3] p53 and p73 share architectural homologies in their typical structure of transcription factors, with an N-terminal transactivation (TA) domain, a central DNA binding domain (DBD) and a C-terminal oligomerisation domain (OD). In addition, these genes express an array of isoforms predicting a complex set of transcription.[4] Despite these similarities, they have different functions. p53 is an ubiquitous protein mostly regulated at the post-transcriptional level and strongly specialised in stress response, while p73 is a tissue-specific protein regulated both at the

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transcriptional and at the post-transcriptional level with roles both in response to genotoxic stress and in epithelial and neurological differentiation.[5]

The TP73 gene (1p36,33) gives rise to various N- and C-terminal isoforms generated through either alternative exon splicing or using a second promoter. The N-terminal p73 isoforms can be grouped in two classes: the transactivation-competent TAp73 proteins and the transactivation-defective, N-terminally truncated ΔTAp73 proteins (ΔN'p73, Ex2p73, Ex2/3p73 and ΔNp73).[5] The ΔNp73 isoforms lack 62 residues that constitute the TA domain, but possess 13 unique residues in their N-terminus which constitute a novel TA domain.[6] Despite the differences in N- and C-termini, all p73 isoforms conserve the DBD and two N- and one C-terminal proline-rich motifs (PXXP motif), which are indispensable for the TA function.[6,7]

The TAp73 isoform mimics p53, inducing cell cycle arrest and apoptosis in response to DNA damage.[8–10] However, it also transactivates a unique set of target genes,[8–10] suggesting that it has a distinct role from p53. TAp73 also appears to play a physiological role in the conclusive stages (late anaphase/telophase) of mitosis because the abrogation of TAp73 expression affects mitotic completion and exit to the subsequent interphase.[11,12] These findings suggest that TAp73 also has an important function in cell growth control.

The function of ΔNp73 isoforms is still controversial since in specific circumstances they may behave differentially. Early studies provided evidence that ΔNp73, lacking the TA domain, was transcriptionally inactive and acted as a dominant negative regulator of both wild type p53 and TAp73, inhibiting their pro-apoptotic effect in response to chemotherapeutic agents through hetero-oligomerisation with p73 or p53, or competitive inhibition in p53 binding to its DNA responsive elements.[13,14] It was also reported that ΔNp73 promotes immortalisation in primary fibroblasts and cooperates with the oncogene Ras to drive their transformation in *in vitro* and *in vivo*.[15,16] Thus, in primary human tumours ΔNp73 may display some 'oncogenic' features. On the other hand, recent studies suggest that ΔNp73α and β may be regulators of signal transduction because they influence the expression of various genes in a p53-independent fashion (despite the lack of the TA domain).[6,17,18] In addition, in both p53 null and wild type cells, over-production of ΔNp73α does not seem to affect cell growth or response to chemotherapy,[19–21] while over-expression of ΔNp73β suppressed cell growth.

These data suggest that ΔNp73 isoforms may have variable or even some opposing biological functions in different cellular contexts and that the various C-terminal regions of ΔNp73 may influence the final cellular phenotype.[22] The evidence that cells have multiple p73 isoforms with multiple activities and that there is a functional cross-talk among all family members, endowing them with both tumour suppressor and oncogenic roles, ruled out the notion that all these variables need to be considered in defining the role of p53 family genes in tumourigenesis and cell responses to genotoxic stresses.

Among all C-terminal ΔNp73 variants, we studied the role of ΔNp73β isoform in regulating the cell cycle in tumour cell models. *In vitro* ΔNp73β over-expression induces mitotic defects and impinges the genomic stability of tumour cells, leading to tetraploidy. Additionally, we found that the tetraploid status was associated with correct S-phase entry but the suppression of entry in subsequent mitosis.

2. Materials and methods

2.1. Cell culture

The H1299 cell line (human lung carcinoma $p53^{-/-}$) was routinely maintained in RPMI1640 medium supplemented with 10% foetal calf serum (FCS).

We generated different stable ΔNp73β over-expressing clones using a tetracycline-regulated expression system (T-Rex System, Invitrogen). Briefly, ΔNp73β cDNA (kindly provided by Dr. De Laurenzi) was subcloned in the tetracycline-inducible plasmid pcDNA4/TO and was used to transfect a H1299 clone expressing the tetracycline repressor (pcDNA6/TR). Clones were screened for the inducible expression of the ΔNp73β and two of them (H1299/ΔNβ18 and H1299/ΔNβ20) were selected to grow in medium supplemented with 10% TET System-approved foetal bovine serum (BD biosciences) with 5 μg/mL of blasticidin (InvivoGen) and 10 μg/mL zeocin (Invitrogen). We used the H1299/Mock clone transfected with the pcDNA4/TO empty vector as internal control. All cells were grown at 37 °C in a 5% CO_2 atmosphere.

2.2. Real time RT-PCR

Real time RT-PCR was used for the relative quantification of ΔNp73. Three hundred nanograms of total RNA purified with the SV40 Total RNA Isolation System (Promega) were retrotranscribed in 20 μl with Archive Kit (Applied Biosystem) and 2 μl further amplified by *Real Time* PCR (ABI Prism 7900 Sequence Detection System, Applied Biosystem). Primers and probe sequences to detect the levels of ΔNp73 were 5'-GGATTCCAGCATGGACGTCTT-3' as forward primer and 5'-CGCCTACCATGCTGTACGT-3' as reverse primer and 5'-GGCTGCTCATCTGGTCCAT-3' as TaqMan probe (Assay by Design, Applied Biosystems). Primers and TaqMan probe sequences to detect the 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).

2.3. Western blot

For Western blot analysis, doxy-untreated and treated cells were washed twice with ice-cold PBS, removed by scraping and collected by centrifugation. Cells were lysed in ice-cold lysis buffer (50 mM tris pH8, 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 10% glycerol, 1 mM $MgCl_2$ and 1% Triton X-100) containing protease inhibitors (Sigma) and incubated on ice for 30 min. Samples were centrifuged at 13,000x*g* for 10 min at 4 °C and the protein content of the supernatant was determined using a Bio-Rad Protein assay (Bio-Rad).

Forty micrograms of total cellular protein was resolved by SDS-PAGE on an 8% polyacrylamide gel and electrotransferred to PVDF membrane. Immunoblot analysis was done using the following antibodies: rabbit anti-p73 (1:1000, Oncogene

Research), goat anti-actin (1:500; Santa Cruz Biotechnology). Membranes were then reacted with secondary antibodies (1:3000, Santa Cruz Biotechnology) and developed using the ECL kit (Amersham Biosciences).

2.4. Cell proliferation

Growth rate was analysed by seeding cells in six-well plates (Iwaki) at 25,000 cells/mL in selection medium. After 24 h, protein expression was induced by adding 200 pg/mL doxy (Sigma) in three wells for each plate. At the time points indicated, cell from a six-well plate were rinsed twice with PBS, trypsinised and collected separately. Cells from each well were counted using a cell culture counter (Coulter Channelyser® 256, Beckman Coulter). The average number of cells from three wells was used for growth rate evaluation. The mean ± SD is shown.

2.5. Clonogenic assay

Long-term cell growth was evaluated in a clonogenic assay. The three different clones were plated at 200–300 cells/well in six-well plates; 24 h after seeding, doxy was added to half the plates. The colonies formed were stained with 10% crystal violet in 20% ethanol.

2.6. Cell viability

The propidium iodide exclusion assay was used to evaluate cell viability. Briefly, the three different clones were plated at 25,000 cells/mL in selection medium and treated with doxy (200 pg/mL) for 72 h. Floating cells in the medium and adhering cells on the plates were collected and concentrated by centrifuging. Cell pellets were resuspended in 0.5 mL of a solution containing 1.25 μg/mL propidium iodide (PI) in PBS and stained for 2 min in the dark at 4 °C. Then cells were analysed for a positive reaction to PI using a FACS Calibur instrument (Becton Dickinson, Sunnyvale, CA, USA), with a 488 nm excitation laser beam, and fluorescence pulses were detected at 620 nm.

2.7. DNA histogram analysis

The three different clones were seeded at 25,000 cells/mL in T75 flasks. Twenty-four hours after plating, cells were induced with doxy (200 pg/mL), and floating and adhering cells were collected after 72 h, fixed in 70% ethanol and kept at 4 °C before staining. The cells (1.5×10^6) were washed with PBS and stained with 1 mL of a solution containing 10 μg/mL of PI and 12.5 μl of RNAse 10,000 U (1 mg/mL in water) overnight at 4 °C in the dark. Flow cytometric analysis was done on at least 20,000 events using a FACS Calibur instrument (Becton Dickinson, Sunnyvale, CA, USA) with a 488 nm laser beam, and fluorescence pulses were detected at 620 nm.

2.8. Morphological analysis

EosinG-thiazin staining was done to determine cellular morphology. The three clones were plated at 25,000 cells/mL in 24-well plates on glass coverslips and induced with doxy (200 pg/mL) after 24 h; 72 h after doxy treatment, cells were washed twice with PBS and slides were stained with Diff-Quick Rapid Differential Staining (Baxter® & Dade®). Slides were incubated at room temperature with Fixative Solution (Fast green in methanol 0.002 g/L) for 30 s, with Stain Solution I (EosinG in phosphate buffer, pH 6.6) for 30 s and with Stain solution II (Thiazin dye in phosphate buffer, pH 6.6) for 20 s. Slides were washed twice with PBS and twice with ultra-pure water. Finally, they were mounted with Entellan reagent and examined at a magnification of 40× by light microscopy.

2.9. Immunofluorescence microscopy

In vitro growing cells were seeded at 25,000 cells/mL in 24-well plates on glass coverslips and treated with doxy (200 pg/mL) after 24 h. At the time point indicated, cells were fixed for 20 min in 4% paraformaldehyde solution, permeabilised with 0.01% Triton X-100 and blocked in 1% BSA in PBS for 20 min at room temperature. All subsequent antibody incubations were in PBS containing 1% BSA-0.01% Triton X-100. Antibody reagents were rabbit anti-p73 (1:1000, Oncogene Research), rat anti-α-tubulin (1:100, Cell Signalling) and mouse anti-phospho-histone H3 (Ser10) (1:100, Cell Signalling). Incubations with primary antibodies were kept overnight at 4 °C, followed by three washes with 1% BSA-PBS solution. Secondary reagents were Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes™, Invitrogen), anti-rat IgG FITC-conjugated (1:100, Sigma) and Alexa Fluor® 594-conjugated rabbit anti-mouse IgG (1:700, Molecular Probes™, Invitrogen). Secondary antibodies were incubated for 1 h at room temperature. After PBS washing, Hoechst 33258 counterstaining was done (1:1000, 10 min). Finally, coverslips were mounted with FluorSave Reagent (Calbiochem). Immunofluorescence was examined using a fluorescent microscope (Olympus FV 500). An additional sample was incubated with the secondary antibody alone as negative control.

2.10. BrdU incorporation

In vitro growing cells were seeded at 25,000 cells/mL in 24-well plates on glass coverslips, treated with doxy (200 pg/mL) for 24 h, then exposed to BrdU (Sigma) 20 μM for 24 h. After fixation in ethanol 96% for at least 4 h, cells were treated with 2 N HCl for 30 min, incubated with 0.5% Tween-20 (Sigma) in PBS and 1% (v/v) normal goat serum (NGS) for 15 min and incubated with the monoclonal antibody anti-BrdU (Becton Dickinson) diluted 1:10 in 0.5% (v/v) Tween-20 in PBS and 1% (v/v) NGS for 1 h in the dark. Then, the cells were incubated with 0.5% Tween-20 (sigma) in PBS and 1% (v/v) normal goat serum (NGS) for 15 min and labelled with Alexa Fluor 488 F(ab')2 fragment goat anti-mouse IgG (Molecular Probes, Invitrogen) diluted 1:50 in 0.5% (v/v) Tween-20 in PBS and 1% (v/v) NGS for 1 h in the dark. Nuclei were visualised by staining with Hoechst 33258 1:1000 for 10 min. All incubations were kept at room temperature. Finally, coverslips were mounted with FluorSave Reagent (Calbiochem). Immunofluorescence was examined using a fluorescent microscope (Olympus FV 500). An additional sample was incubated without the primary antibody as negative control.

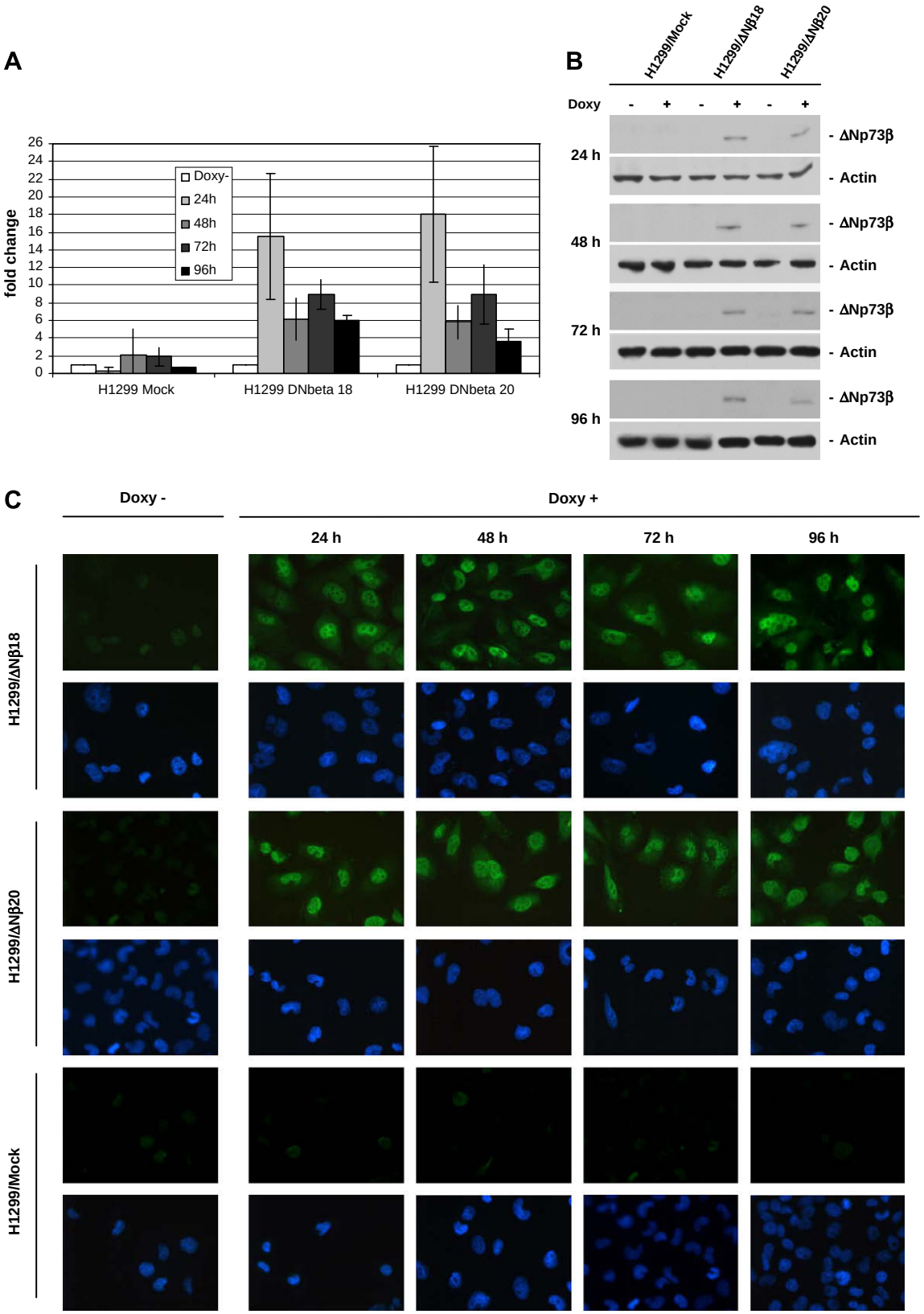

Fig. 1 – Characterisation of ΔNp73β expressing clones. (A) Relative ΔNp73 mRNA expression levels in H1299/Mock, H1299/ΔNβ18 and H1299/ΔNβ20 clones were determined by *real time* RT-PCR after doxy exposure for the times indicated. ΔNp73 expression in the absence of doxy was arbitrarily set at 1. Values represent the mean of three samples ± SD. (B) Western blot analysis of uninduced (–) and induced (+) clones at different doxy treatment times. Actin was used as homogeneous gel loading. (C) Immunofluorescence staining of ΔNp73 (in green) in clones untreated (–) and treated (+) with doxy for different times. Cells were further stained with Hoechst 33258 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. *Generation of stable clones expressing inducible ΔNp73β protein*

A tetracycline-inducible system was used to generate cellular clones over-expressing ΔNp73β isoform in the human lung cancer cell line H1299 (p53 null). For further characterisation, we selected two representative clones that inducibly express ΔNp73β (H1299/ΔNβ18 and H1299/ΔNβ20) and a control clone (H1299/Mock). As seen in Fig. 1A, ΔNp73β mRNA levels rose rapidly in H1299/ΔNβ18 and H1299/ΔNβ20 clones 24 h after doxy treatment, with 14- and 18-fold increases in the ΔNp73 transcript levels compared to the uninduced counterpart. ΔNp73 mRNA levels remained up-regulated in both ΔNp73β-expressing clones up to 96 h of exposure to doxy. As expected, in the control clone there was no change in ΔNp73 mRNA levels. Western blot analysis confirmed this strong induction also at protein level (Fig. 1B). Interestingly, ΔNp73β levels already rose at 6 h of doxy treatment (data not shown), remaining elevated up to 96 h, and were barely detectable at 120 h (data not shown).

To determine the subcellular localisation of the ΔNp73β isoform, cells were immunostained with anti-p73 antibody and examined using a fluorescent microscope. There was a predominant nuclear localisation of ΔNp73β protein in both ΔNp73β-expressing clones up to 96 h of doxy induction (Fig. 1C). As expected, in the control clone no ΔNp73β-FITC protein signal was detectable.

3.2. *In vitro ΔNp73β is active in suppressing cell growth and in cell death*

To characterise the activity of ΔNp73β, we analysed the effects of ΔNp73β over-expression on the clonogenic potential of these clones, finding that ΔNp73β expression suppressed cell growth (Fig. 2). In the absence of doxy cells grew normally, whereas with the drug the growth rates of both ΔNp73β-expressing clones were substantially reduced up to six days after exposure (Fig. 2A and B). The addition of doxy did not

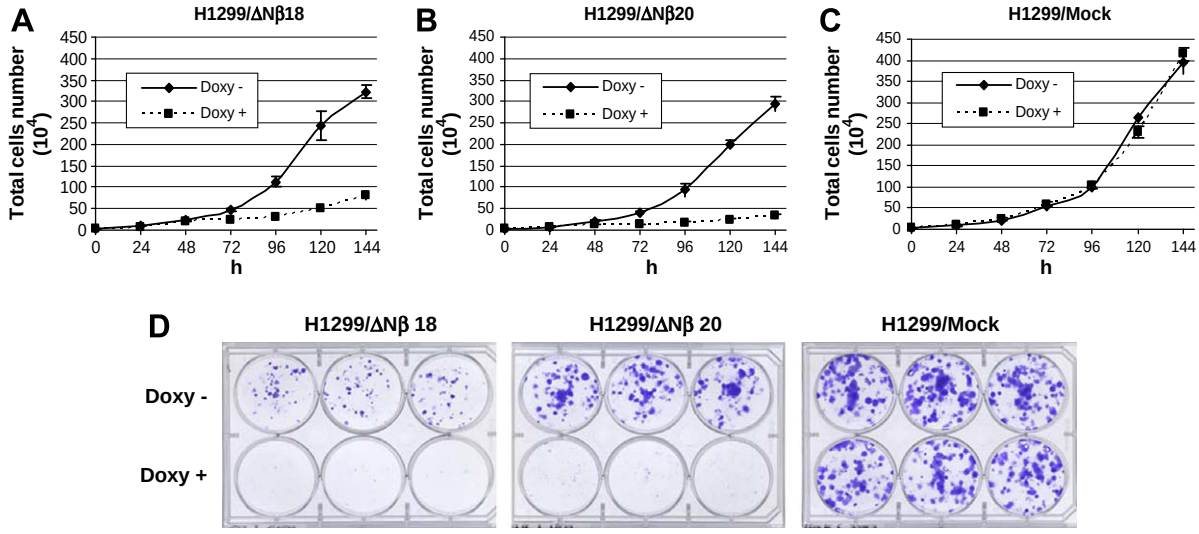

Fig. 2 – *In vitro* growth of clones. Growth curves of H1299/ΔNβ18 (A), H1299/ΔNβ20 (B) and H1299/Mock (C) clones under both uninduced (Doxy –) and induced (Doxy +) conditions. Values represent the mean of three samples ± SD. ΔNp73β expression inhibited cell growth. Panel D: Long-term cell growth. Selected clones were seeded in six-well plates and doxy added to half the plates. Ten days after induction, plates were stained with 10% crystal violet in 20% ethanol. ΔNp73β expression greatly reduced the cells colony-forming ability.

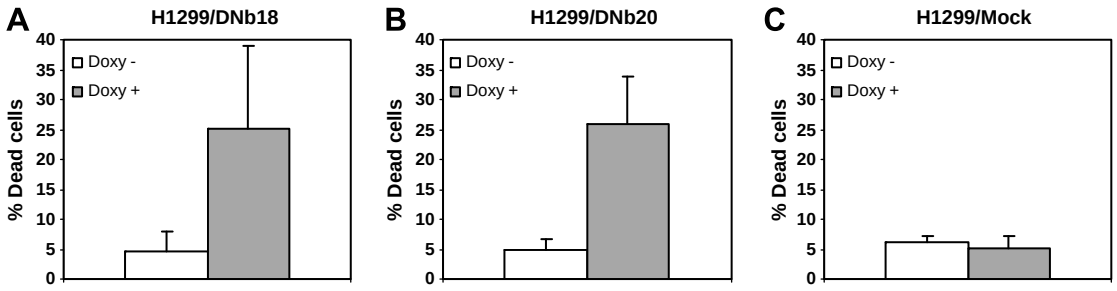

Fig. 3 – Cell viability. Percentage of dead cells in uninduced (Doxy –) and induced (Doxy +) cells of H1299/ΔNβ18 (A), H1299/ΔNβ20 (B) and H1299/Mock (C) clones. ΔNp73β expression induced cell death.

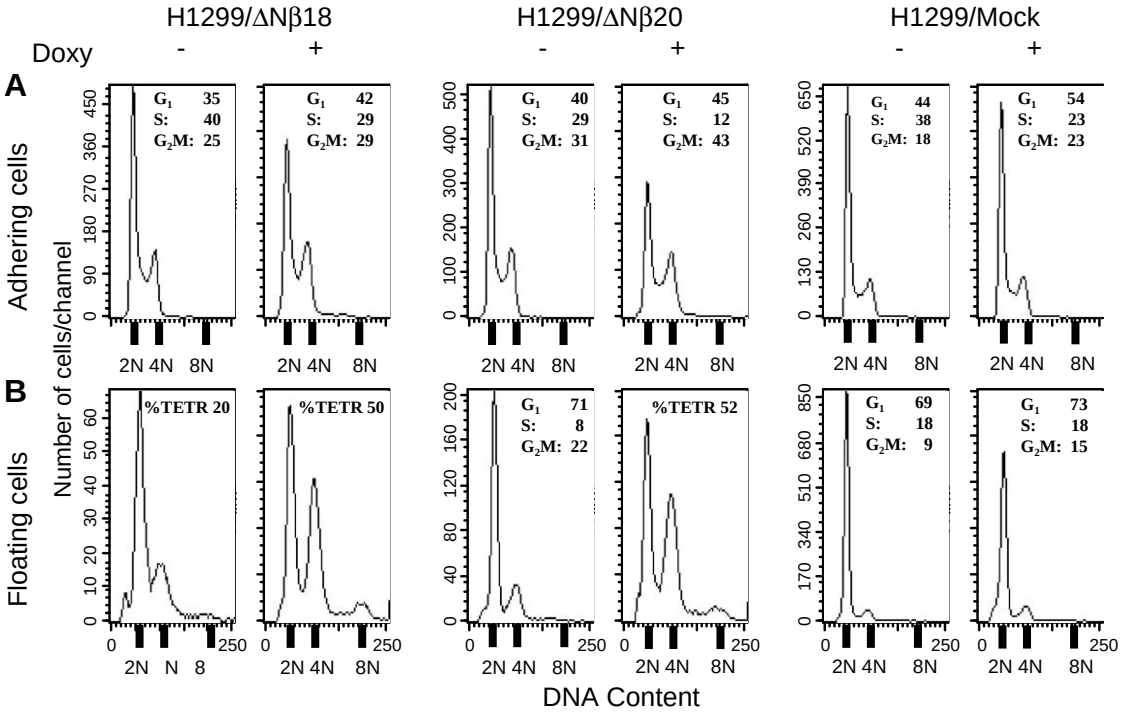

Fig. 4 – DNA histogram analysis. DNA content of adhering (A) and floating cells (B) of the three selected clones under uninduced (Doxy –) and induced (Doxy +) conditions. Percentage of cells in G_1, S and G_2M phases of the cell cycle are reported in each box. When the presence of DNA tetraploid cells did not allow a proper estimation of cell cycle phase distribution, the percentage of DNA tetraploid cells (%tetr) was reported.

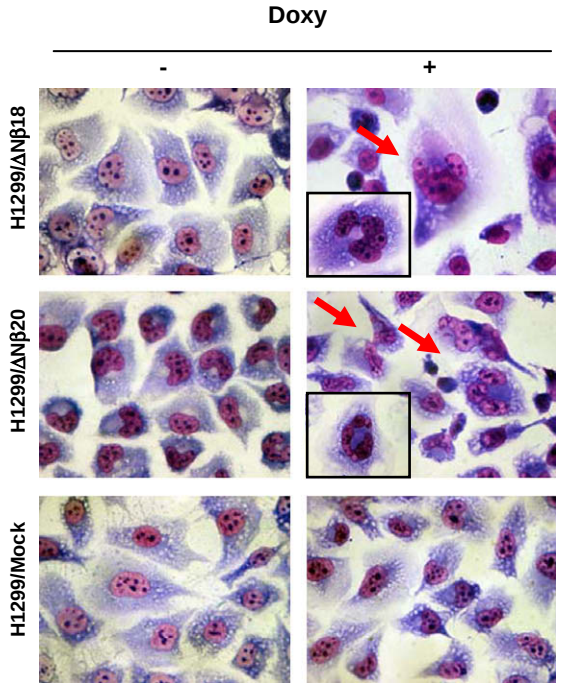

Fig. 5 – Morphological analysis. EosinG-thiazin staining of doxy-untreated (–) and treated (+) selected clones. Arrows indicate the typical large, multinucleate cells characteristic of ΔNp73β-expressing clones.

affect the growth rate of the control clone (Fig. 2C). The same results were obtained in the clonogenic assay, which showed severely diminished colony-forming ability in both ΔNp73β-expressing clones compared to the uninduced counterpart (Fig. 2D). We found a significant increase in the floating cells in the two ΔNp73β-expressing clones after doxy treatment, but not in the H1299/Mock clone.

Next, we investigated whether the anti-proliferative effect of ΔNp73β was due to cell death. In the PI exclusion assay, an over-expression of ΔNp73β resulted in an increase in the cell death (Fig. 3). The percentage of PI-positive cells increased from 4.6 to 25.1 in the H1299/ΔNβ18 clone and from 4.8 to 25.8 in the H1299/ΔNβ20 clone when ΔNp73β was induced (Fig. 3A and B). In the control clone, the number of dead cells was nearly comparable under both conditions (Fig. 3C).

3.3. *In vitro ΔNp73β induces tetraploidisation and abnormal mitosis*

We investigated the DNA content of ΔNp73β-expressing clones using cell cycle flow cytometric analysis. As showed in Fig. 4A, adhering cells of all selected clones maintained a similar cell cycle phase distribution and DNA content both with and without doxy. Surprisingly, floating cells of both ΔNp73β-expressing clones presented a significant subpopulation with tetraploid DNA content after doxy treatment (Fig. 4B). In contrast, none of the few doxy-untreated floating cells had tetraploid DNA content. These results suggest that ΔNp73β induced tetraploidisation and that DNA tetraploid cells preferentially detach from the surface.

Morphological analysis of eosinG-thiazin stained cells indicated many large cells containing multiple nuclei in both ΔNp73β-expressing clones under induced conditions. By counting the multinucleated cells in several fields, we found that 72 h following doxy treatment, 29 and 32% of cells showed multinuclei in H1299/ΔNβ18 and H1299/ΔNβ20 clones, respectively, while no cells with this feature were observable in uninduced conditions. H1299/Mock maintained a normal mononucleate population (Fig. 5).

Mitotic failure of ΔNp73β-expressing clones was confirmed using α-tubulin immunostaining to visualise the cytoskeleton and the spindle apparatus (Fig. 6). Many induced ΔNp73β-expressing cells had a normal cytoskeleton but abnormal mitosis characterised by centrosome triplication, multipolar spindle and cytokinesis failure. H1299/Mock showed a normal mitotic phenotype both with and without doxy. Thus, a substantial proportion of ΔNp73β-expressing cells had much larger morphology, containing multiple nuclei and mitotic defects.

To characterise the cell cycle progression of the tetraploid population of ΔNp73β-expressing cells, we assayed S phase entry by BrdU incorporation (Fig. 7). Both doxy-untreated and treated clones had comparable BrdU incorporation, indicating that multinucleate doxy-treated cells had an active S phase and continued to cycle. The fact that multinucleate cells, however, incorporated BrdU suggests that the tetraploid population does not arrest in G1 and prompted us to investigate whether these cells entered mitosis.

We employed histone H3 phosphorylation at Ser-10 immunostaining, which has been widely used as a specific marker

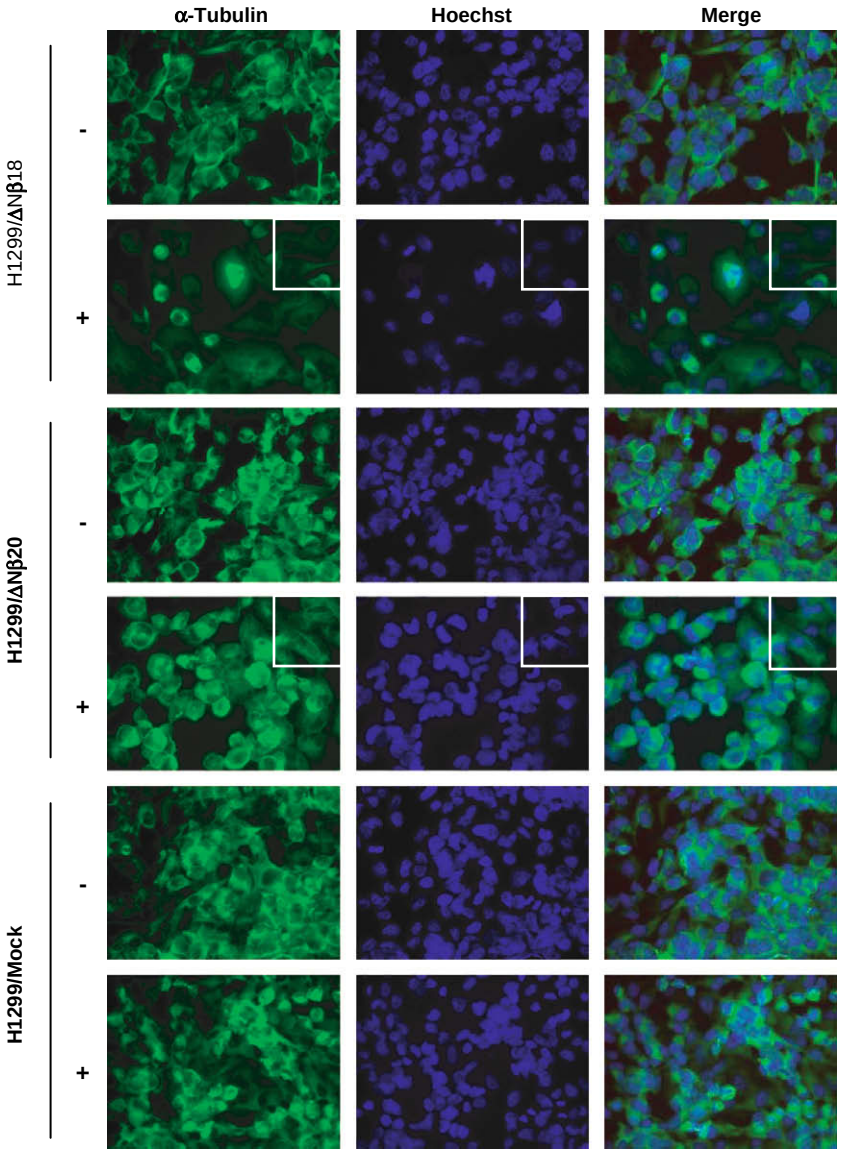

Fig. 6 – Expression of α-tubulin. Doxy-untreated (–) and treated (+) cells were immunostained with anti-α-tubulin (green) and further stained with Hoechst 33258 (blue). Both H1299/ΔNβ18 and H1299/ΔNβ20 clones showed abnormal mitosis in the presence of doxy. The control clone H1299/Mock showed a normal mitotic phenotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

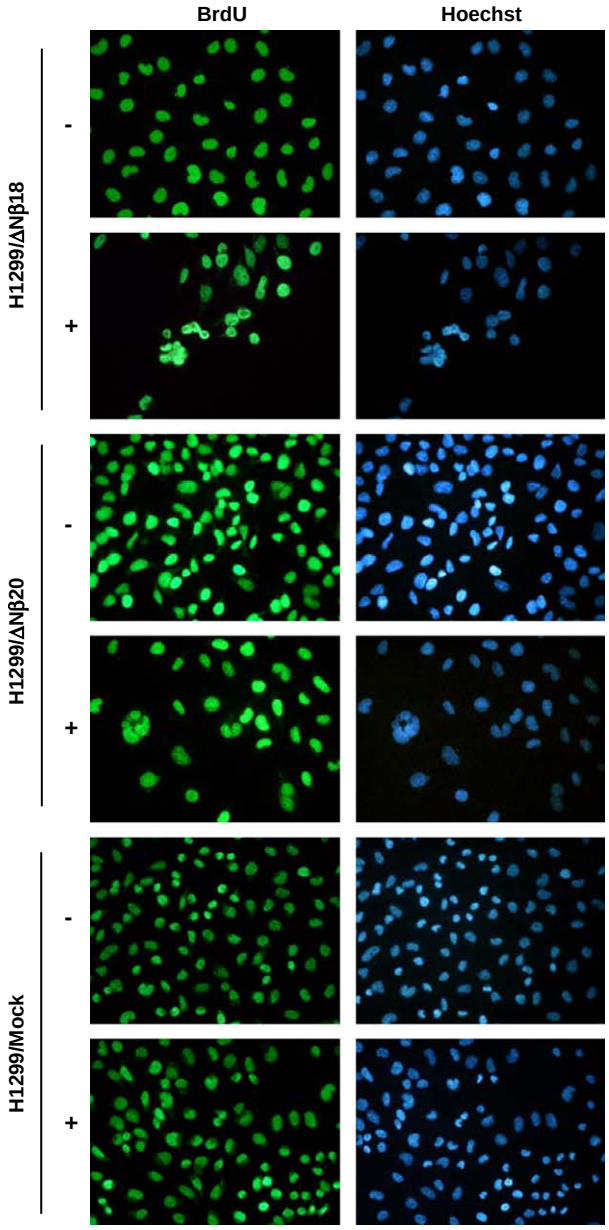

Fig. 7 – S-phase entry assay. Doxy-untreated (–) and treated (+) cells were cultured for 24 h with BrdU. After fixation, cells were immunostained with anti BrdU (green) and nuclei counterstained with Hoechst 33258 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of mitosis because H3 phosphorylation is associated with chromatin condensation, that is observed when cells transverse from G2 into M-phase.[23] H3 phosphorylation was completely suppressed in multinucleate cells, whereas mononucleate cells normally entered mitosis. A field of cells was shown for ΔNp73β-expressing clones after doxy exposure, with only mononucleate cells H3-P positive and all multinucleate cells H3-P negative. The control clone and doxy-untreated H1299/ΔNβ18 and H1299/ΔNβ20 clones only had mononucleate cells positive for H3-P (Fig. 8).

Our data provide evidence that the tetraploid status of ΔNp73β-expressing cells inhibited subsequent entry in mitosis.

4. Discussion

Of all the ΔNp73 isoforms, the β one clearly has contrasting functions in different circumstances (primary or transformed cells). ΔNp73β is the predominant isoform expressed in the developing murine brain and antagonises p53-dependent apoptosis in neurons.[24] In hepatocellular carcinoma ΔNp73β gives resistance to chemotherapy by the inhibition of apoptosis.[25] On the other hand, the over-expression of ΔNp73β in tumour cell lines is functional in transactivation and growth suppression, and the ΔNp73β activity appears to depend on a novel activation domain, formed from the N-terminal 13 unique residues and the N-terminal PXXP motifs.[6] However, many concerns remain. In this study, we addressed the role of ΔNp73β in cell cycle progression and, interestingly, found that the expression of ΔNp73β in tumour cell lines resulted in defects in cell division and altered genomic integrity, with the acquisition of tetraploid DNA content. Our results are not restricted to clone selection since in this cellular context both isogenic ΔNp73β-expressing clones behaved the same way.

In agreement with Liu and colleagues[6], we confirmed the role of ΔNp73β in cell proliferation. The inducible ΔNp73β expression in the H1299 cell line (p53-/-*) results in both growth suppression and cell death. The cell cycle phase distribution evaluated by monoparametric flow cytometric DNA analysis showed a new cell population with tetraploid DNA content and showed no evident cell cycle phase perturbations. However, the different growth rates up to 144 hours after doxy exposure might be related to the fact that crossing the G_1 phase for both H1299/ΔNβ18 and H1299/ΔNβ20 clones could be delayed, but not completely arrested, as shown by the presence of BrdU-positive S-phase cells. This lack of cell cycle arrest in a specific phase correlates with the lack of change in either total protein levels of same cyclins (e.g. cyclin E, cyclin A and cyclin B) or some cell-cycle specific kinases (e.g. cdk2 and cdc2) (see Supplementary Information Table S1).

These features appear to be independent from p53 status since ΔNp73β-inducible clones selected in another cell line of different origin expressing a wild type p53 (the osteosarcoma cell line U2OS) showed similar cell growth suppression (see Supplementary Information Fig. S1).

We constructed a plasmid that allows constitutive expression of ΔNp73β and stably introduced this into several human cancer cell lines with different p53 statuses (H1299, A2780, Saos-2, SKOV-3 and U2OS). In all these cells, no stable clones were selected, and the constitutive expression of ΔNp73β was not tolerated, probably, because of its strong inhibitory effect on cell growth (data not shown).

Differently from Liu and colleagues[6], we found that ΔNp73β induced cell death with mechanisms different from apoptosis. The involvement of apoptosis was ruled out by TUNEL flow cytometric assay (see Supplementary Information Fig. S2) and we assumed that ΔNp73β-induced catastrophic cell death might provide a second layer of

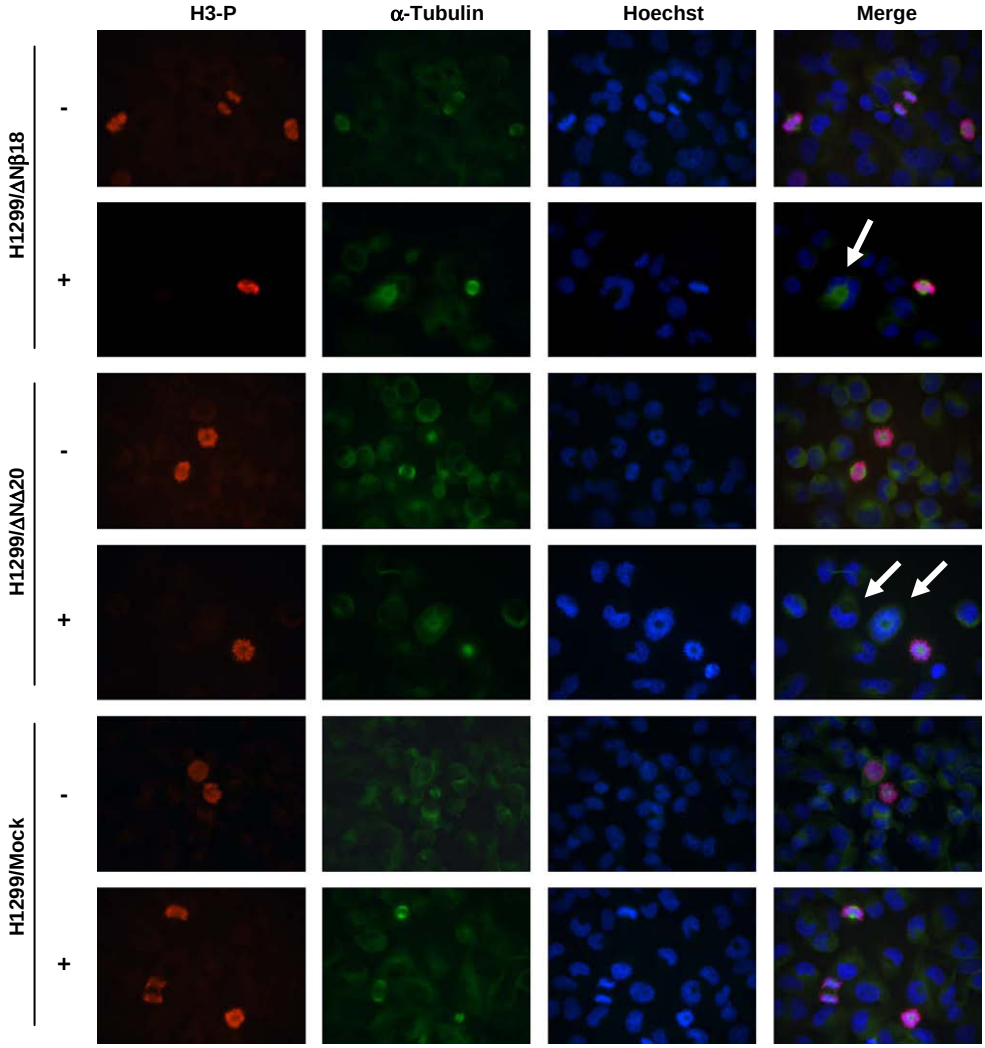

Fig. 8 – Expression of histone H3 phosphorylation. Doxy-untreated (–) and treated (+) cells were immunostained with anti-H3-P (red), anti-α-tubulin (green) and further stained with Hoechst 33258 (blue). White arrows indicate multinucleate H3-P negative cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protection in cells that displayed inherent resistance to apoptosis. In fact, in many solid tumours the absence of apoptosis may be compensated by other cellular mechanisms, such as permanent growth arrest with phenotypic features of cell senescence, or cell death through mitotic catastrophe.[26]

Senescence in tumour cells could be a result of the inhibition of telomerase.[27,28] p73 is an important transcriptional regulator of a key component of telomerase activity, the human telomerase reverse transcriptase (hTERT).[29,30] The expression of hTERT in our cell models did not change in the presence of ΔNp73β (see Supplementary Information Fig. S3A). We also tend to exclude a senescence-like phenotype because the ΔNp73β-induced growth suppression was not accompanied by the expression of a specific senescent marker, lysosomal senescence-associated β-galactosidase (see Supplementary Information Fig. S3B).

The abnormal mitosis, polyploidy and large-multinucleated cells in ΔNp73β-expressing clones were consistent with a cell death mechanism involving mitotic catastrophe in a p53-independent way. Mitotic catastrophe can result in cell death by either caspase-dependent or independent routes.[31] In our models, we did not have caspase activation (data not shown) indicating that cell death occurs by mechanisms that are not caspase-dependent. Nevertheless, whether caspase-dependent or independent, mitotic catastrophe may be considered an important safeguard to prevent the proliferation of polyploid cells and reduce the risk of aneuploidy.

There may be several reasons why cells become tetraploid, including exit of mitosis following failures of mitotic spindle assembly, of chromosome segregation or of cytokinesis.[32] During a normal cell cycle, genomic integrity is ensured by distinct checkpoint control systems that monitor mitotic spindle assembly, proper sister chromatid segregation, cytokinesis and mitotic exit.[33] The mitotic spindle assembly checkpoint and the post-mitotic G1 checkpoint are particularly important to prevent polyploidisation and subsequent aneuploidy.

The post-mitotic G_1 checkpoint is p53-dependent and arrests tetraploid cells in G1, independently of the cause of the mitotic defect, preventing entry into S phase and the propagation of errors to daughter cells. The mitotic spindle assembly checkpoint is controlled by numerous molecular players such as Bub1, BubR1, Bub3, Mad1, Mad2 and Mps1.[33] Treatment with nocodazole induced a G_2-M phase block (data not shown) supporting the idea that our models had a functional mitotic spindle checkpoint. Anyway, we did not see any change in the levels of expression of Mad2 and BubR1 in ΔNp73β-expressing cells compared to the non-induced counterpart suggesting that ΔNp73β did not target these checkpoint proteins.

It has been reported that the cdki Kip2/p57 is one specific target of p73, important for coordination and completion of mitosis and for the M-G_1 transition.[12] We ruled out the involvement of this factor in the mitotic alteration observed because the transcriptional level of cdki Kip2/p57 did not change in the presence of the isoform ΔNp73β (manuscript in preparation).

On the basis of this evidence, we speculate that ΔNp73β-induced cell damage evades the mitotic spindle assay checkpoint so aberrant mitosis is bypassed, favouring a transition to the tetraploid state and leading to catastrophic cell death.

Our finding that the tetraploid cell population preferentially detaches from the surface may be due to a subtle cytoskeletal disorganisation that could perturb cell interactions with the substrate. However, immunofluorescence did not show up any obvious differences in microtubule polymerisation between mononucleate and multinucleate cells. In addition, when we cultured these cells on matrigel-coated plates, induced cells showed no growth suppression suggesting that there were no changes in cell adhesion to the substrate (data not shown). Anyway, the detachment of the tetraploid population from the surface makes it harder to understand the role of ΔNp73β.

The current study is the first, to our knowledge, to shed light on an involvement of ΔNp73β in tetraploidisation. ΔNp73β-expressing cells displayed aberrant mitosis, escaped from checkpoint-mediated mitotic arrest becoming tetraploid, and finally died through mitotic catastrophe rather than apoptosis. Considering that ΔNp73β is transcriptionally active, investigating the gene expression profile of these cells might help decipher the molecular mechanisms of ΔNp73β-induced tetraploidy or cell death. Elucidation of these molecular changes in tumour cells could suggest new anticancer strategies acting by crossfire with tumour growth.

Moreover, the evidences reported here together with the previously published manuscripts,[19–21] suggest that inside the different ΔNp73 isoforms there could be substantial differences in their role in cancer development. The different isoforms, (and particularly the α and β ones) could have opposite effects, at least in vitro. It is therefore important to have data on the expression of the different C-terminal isoforms in human tumours in order to determine their role in tumour progression and response to treatment.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.09.024.

REFERENCES

1. Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. *Cancer Res* 2000;**60**(24):6788–93.
2. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000;**408**(6810):307–10.
3. Strano S, Rossi M, Fontemaggi G, et al. From p63 to p53 across p73. *FEBS Lett* 2001;**490**(3):163–70.
4. Moll UM, Erster S, Zaika A. P53, p63 and p73 – solos, alliances and feuds among family members. *Biochim Biophys Acta* 2001;**1552**(2):47–59.
5. Moll UM, Slade N. P63 and p73: roles in development and tumor formation. *Mol Cancer Res* 2004;**2**(7):371–86.
6. Liu G, Nozell S, Xiao H, Chen X. DeltaNp73beta is active in transactivation and growth suppression. *Mol Cell Biol* 2004;**24**(2):487–501.
7. Nozell S, Wu Y, McNaughton K, et al. Characterization of p73 functional domains necessary for transactivation and growth suppression. *Oncogene* 2003;**22**(28):4333–47.
8. Chen X. The p53 family: same response, different signals? *Mol Med Today* 1999;**5**(9):387–92.
9. Harms K, Nozell S, Chen X. The common and distinct target genes of the p53 family transcription factors. *Cell Mol Life Sci* 2004;**61**(7–8):822–42.
10. Fontemaggi G, Kela I, Amariglio N, et al. Identification of direct p73 target genes combining DNA microarray and chromatin immunoprecipitation analyses. *J Biol Chem* 2002;**277**(45):43359–68.
11. Fulco M, Costanzo A, Merlo P, et al. P73 is regulated by phosphorylation at the G2/M transition. *J Biol Chem* 2003;**278**(49):49196–202.
12. Merlo P, Fulco M, Costanzo A, et al. A role of p73 in mitotic exit. *J Biol Chem* 2005;**280**(34):30354–60.
13. Kartasheva NN, Contente A, Lenz-Stoppler C, Roth J, Dobbelstein M. P53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene* 2002;**21**(31):4715–27.
14. Grob TJ, Novak U, Maisse C, et al. Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell Death Differ* 2001;**8**(12):1213–23.
15. Petrenko O, Zaika A, Moll UM. ΔNp73 facilitates cell immortalization and cooperates with oncogenic Ras in cellular transformation in vivo. *Mol Cell Biol* 2003;**23**(16):5540–55.
16. Stiewe T, Zimmermann S, Frilling A, Esche H, Putzer BM. Transactivation-deficient DeltaTA-p73 acts as an oncogene. *Cancer Res* 2002;**62**(13):3598–602.

17. Kartasheva NN, Lenz-Bauer C, Hartmann O, Schafer H, Eilers M, Dobbelstein M. DeltaNp73 can modulate the expression of various genes in a p53-independent fashion. *Oncogene* 2003;**22**(51):8246–54.
18. Tanaka Y, Ota K, Kameoka M, Itaya A, Yoshihara K. Up-regulation of NFkappaB-responsive gene expression by DeltaNp73alpha in p53 null cells. *Exp Cell Res* 2006;**312**(8):1254–64.
19. Marabese M, Marchini S, Sabatino MA, Polato F, Vikhanskaya F, Marrazzo E, et al. Effects of inducible overexpression of DNp73[alpha] on cancer cell growth and response to treatment in vitro and in vivo. *Cell Death Differ* 2005;**12**(7):805–14.
20. Marrazzo E, Marchini S, Previdi S, Broggini M. Questioning the oncogenic role of DeltaNp73alpha in different cell lines expressing p53 or not. *Cancer Biol Ther* 2006;**5**(7):794–803.
21. Sabatino MA, Previdi S, Broggini M. In vivo evaluation of the role of DNp73alpha protein in regulating the p53-dependent apoptotic pathway after treatment with cytotoxic drugs. *Int J Cancer* 2007;**120**(3):506–13.
22. Irwin MS. DeltaNp73: misunderstood protein? *Cancer Biol Ther* 2006;**5**(7):804–7.
23. Norbury C, Nurse P. Animal cell cycles and their control. *Ann Rev Biochem* 1992;**61**:441–70.
24. Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD. An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. *Science* 2000;**289**(5477):304–6.
25. Muller M, Schilling T, Sayan AE, Kairat A, Lorenz K, Schulze-Bergkamen H, et al. TAp73/DeltaNp73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. *Cell Death Differ* 2005;**12**:1564–77.
26. Roninson IB, Broude EV, Chang BD. If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat* 2001;**4**(5):303–13.
27. Shammas MA, Koley H, Batchu RB, et al. Telomerase inhibition by siRNA causes senescence and apoptosis in Barrett's adenocarcinoma cells: mechanism and therapeutic potential. *Mol Cancer* 2005;**4**:24.
28. Herbert BS, Pongracz K, Shay JW, Gryaznov SM. Oligonucleotide N3' → P5' phosphoramidates as efficient telomerase inhibitors. *Oncogene* 2002;**21**(4):638–42.
29. Racek T, Mise N, Li Z, Stoll A, Putzer BM. C-terminal p73 isoforms repress transcriptional activity of the human telomerase reverse transcriptase (hTERT) promoter. *J Biol Chem* 2005;**280**(49):40402–5.
30. Beitzinger M, Oswald C, Beinoraviciute-Kellner R, Stiewe T. Regulation of telomerase activity by the p53 family member p73. *Oncogene* 2006;**25**(6):813–26.
31. Mansilla S, Priebe W, Portugal J. Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. *Cell Cycle* 2006;**5**(1):53–60.
32. Andreassen PR, Martineau SN, Margolis RL. Chemical induction of mitotic checkpoint override in mammalian cells results in aneuploidy following a transient tetraploid state. *Mutat Res* 1996;**372**(2):181–94.
33. Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer* 2005;**5**(10):773–85.