

# Effects of Delta Np73 beta on cisplatin treatment in colon cancer cells

Jasmine Loof, Daniella Pfeifer, Zhen-Yu Ding, Xiao-Feng Sun and Hong Zhang

**Linköping University Post Print**

N.B.: When citing this work, cite the original article.

This is the authors' version of the following article:

Jasmine Loof, Daniella Pfeifer, Zhen-Yu Ding, Xiao-Feng Sun and Hong Zhang, Effects of Delta Np73 beta on cisplatin treatment in colon cancer cells, 2012, *Molecular Carcinogenesis*, (51), 8, 628-635.

which has been published in final form at:

<http://dx.doi.org/10.1002/mc.20835>

Copyright: Wiley-Blackwell

<http://eu.wiley.com/WileyCDA/Brand/id-35.html>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-79660>

## Effects of $\Delta$ Np73 $\beta$ on Cisplatin Treatment in Colon Cancer Cells

Jasmine Lööf<sup>1</sup>, Daniella Pfeifer<sup>2</sup>, Zhenyu Ding<sup>2</sup>, Xiao-Feng Sun<sup>2</sup> and Hong Zhang<sup>1</sup>

<sup>1</sup>Division of Tumor Biology, Systems Biology Research Centre, University of Skövde, Sweden

<sup>2</sup>Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Sweden

**Corresponding authors:** Xiao-Feng Sun, Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Science, Linköping University, SE-58185 Linköping, Sweden. Tel: +46-10-1032066, Fax: +46-10-1033090. E-mail: xiao-feng.sun@liu.se;

Hong Zhang, Division of Tumor Biology, Systems Biology Research Centre, University of Skövde, SE-54128 Skövde, Sweden, Tel: +46-500-448482, Fax: +46-500-448499, E-mail: hong.zhang@his.se

**Grant support:** This study was supported by grants from the Swedish Cancer Foundation, Swedish Research Council and the Health Research Council in the South-East of Sweden.

**Abbreviated title:** p73 in colon cancer cells

**Keywords:** Cell death; HCT116 cells; HT29 cells; p73 protein; p53

**ABSTRACT**

p73 can activate transcription of p53-responsive genes, thereby inhibiting cell growth. An alternative promoter in the *TP73* gene gives rise to an N-terminally truncated isoform of p73,  $\Delta Np73$ , which lacks the transactivation domain of the full length TAp73 protein. TAp73 is considered pro-apoptotic, and  $\Delta Np73$  anti-apoptotic. In this study, we overexpressed  $\Delta Np73\beta$  in p53 wild type and p53 mutant colon cancer cell lines and further exposed the cells to cancer therapeutic drug cisplatin. The results showed that cisplatin decreased the protein expression levels of  $\Delta Np73\beta$  in a dose-dependent manner, and both TAp73 and p53 were upregulated after cisplatin treatment. Further, clonogenic potential and cell viability were decreased, and apoptotic cells increased, in p53 mutant and in p53 wild type cells. Cellular viability was significantly higher in  $\Delta Np73\beta$ -cells than mock-transfected cells. However,  $\Delta Np73\beta$  overexpression did not affect the cellular susceptibility to cisplatin. In conclusion, the overexpression of  $\Delta Np73\beta$  increases viability in p53 wild type and p53 mutant colon cancer cells, and cisplatin induces the degradation of  $\Delta Np73\beta$  in a dose-dependent manner.

## INTRODUCTION

p73 protein is encoded by the *TP73* gene located to chromosome 1p36, a region frequently deleted in various cancers [1]. The structure and function of the p73 protein are homologous to p53, and it activates the transcription of p53-responsive genes and inhibits cell growth in a p53-like manner by inducing apoptosis [2]. It has been speculated that p73, like p53, is a tumor suppressor. However, initial genetic studies showed that p73-deficient mice do not develop spontaneous tumors [3]. Inactivation of p53 has been shown in the majority of human malignancies [4], while p73 mutations are rare in primary tumors [5]. Overexpression of p73 has been found in various tumor types, such as colorectal, breast, ovarian and lung cancers [6-9], suggesting that p73 plays an oncogenic role in tumorigenesis. Further, overexpression of the p73 protein has been associated with a poor prognosis in several types of cancers [6,10].

*TP73* gene gives rise to several different mRNAs, and thus a number of different isoforms of the protein differing both N- and C-terminally. There are two N-terminal isoforms of p73, the full length proapoptotic variant of the protein, TAp73, and the N-terminally truncated form,  $\Delta$ Np73. The  $\Delta$ Np73 isoform arises through the use of an alternative promoter located in intron 3 [3,11]. There are also several C-terminal splice variants of the protein termed  $\alpha$ ,  $\beta$ , etc [1,12-14].

The TAp73 isoform is homologous to p53; harboring the N-terminal transactivating (TA) domain responsible for gene activation [2], while the  $\Delta$ -isoforms lack the TA domain [3].  $\Delta$ Np73 shares the DNA-binding and oligomerisation domains with TAp73 and inhibits p53 and TAp73, either by competing for DNA-binding sites or by oligomerizing with the full length proteins [14-15]. Interestingly, the alternative

promoter in intron 3 contains a p53/TAp73 responsive element, and p53, TAp73 and  $\Delta$ Np73 regulate each other through a negative feedback loop where  $\Delta$ Np73 displays dominant negative behavior [11,16,17]. This indicates that the oncogenic properties of p73 are attributed to the  $\Delta$ -isoforms.  $\Delta$ Np73 is involved in development of the brain in mouse, where it protects the neuronal cells from apoptosis [18].

Furthermore, knock-out mice lacking the TA-isoforms of p73 are prone to developing tumors [19]. This supports the idea that full length p73 has tumor suppressor functions which are balanced by the anti-apoptotic  $\Delta$ -isoforms, and the ratio of the different isoforms determines p73 functions. Further complicating the scenario is the differential behavior of the C-terminal isoforms. The anti-apoptotic role of  $\Delta$ Np73  $\beta$  is not clearly established, as results from studies of the  $\beta$ -isoform seem ambiguous [20-21].

Cisplatin (*cis*-diammine-dichloro-platinum) is widely used as DNA-damaging drug in cancer therapy. It interacts with DNA, resulting in the formation of DNA adducts, primarily intrastrand crosslinks [22]. Subsequently, it induces DNA damage recognition proteins to signal to downstream effectors such as p53, resulting in cell cycle arrest and apoptosis. It is well-known that p53 plays an important role in response to DNA-damaging agents, with p53<sup>-/-</sup> cells being resistant to drug-induced apoptosis [23]. Also, p73 is activated by DNA damaging drugs, including cisplatin [24-25], and silencing of p73 with siRNA results in drug resistance in various cancer cells [24,26]. In this study, we attempted to clarify the mechanisms behind the effects of  $\Delta$ Np73 $\beta$  on cisplatin treatment in colon cancer cells.

## MATERIALS AND METHODS

### Cell Culture and Transfection

Human colon carcinoma cell lines, HCT116 with wild type p53 (HCT116<sup>p53+/+</sup>) and truncated p53 missing 40 amino acid residues (HCT116<sup>p53-/-</sup>) [27], as well as HT29 were cultivated in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (GIBCO, Invitrogen, Carlsbad, CA), 1.5 mM L-glutamine (GIBCO) and 1X PEST (GIBCO) at 37°C in a 5% CO<sub>2</sub> incubator. The HCT116<sup>p53-/-</sup> cells are considered functionally p53 negative. The cells (1.5×10<sup>4</sup> cells/cm<sup>2</sup>) were seeded in 6-, 12-, or 96- well plates under standard cell culture conditions as described above for 24 h. HCT116 cells were then transfected with a pCMV6-XL5 vector (OriGene, Rockville, MD) containing transfection-ready cDNA for ΔNp73β (NM\_001126241.1, OriGene) using transfection reagent FuGENE® 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. HT29 cells were transfected using the calcium precipitation method. Briefly, plasmid DNA was mixed with HEPES buffer and calcium chloride, and added with equal volume of 2 times HeBs buffer in a dropwise fashion. The mixture was incubated for 20 min at room temperature and added to the culture medium. The pCMV6-XL5 vector lacking the cDNA insert was used as a negative control. The cells were then treated with various concentrations of cisplatin (Sigma-Aldrich) 24 h after transfection.

### Colony Forming Assay

HCT116 cells and HT29 were transfected in six-well plates as described above. After 24 h cells were trypsinized with TrypLE (Invitrogen, Carlsbad, CA) and

$1 \times 10^3$  cells were seeded in six-well plates with or without cisplatin (0.6  $\mu\text{M}$ ). The cells were grown under standard cell culture condition for seven days, fixed in 4% buffered formaldehyde and stained with 5% Giemsa. The cell colonies were counted and a survival fraction between cisplatin treated and control cells was calculated.

### **XTT Viability Assay**

Cellular viability was determined at 48 and 72 h after the transfection using TACS™ XTT assay in 96-well plates according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The assay is based on the cleavage of the yellow tetrazolium salt XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carbox-anilide) into a soluble orange formazan dye. This reaction is attributed mainly to the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells. The absorbance measured at 450 nm is proportional to the number of viable cells.

### **Western Blot**

The cells were lysed in RIPA buffer (150 mM NaCl, 2% Triton, 0.1% SDS, 50 mM Tris pH 8.0) containing 1% Protease Inhibitor Cocktail (Sigma) at 48 and 72 h after the transfection. Protein concentrations were determined by BCA Protein Assay (Pierce, Woburn, MA). The samples (20  $\mu\text{g}$  protein) were subjected to electrophoresis (35 min, 200V) on precast Criterion Tris-HCl gels, 4-15% (Bio-Rad, Hercules, CA) and electrotransferred on to polyvinylidene difluoride membranes (Amersham Biosciences/GE healthcare, Piscataway, NJ). The membranes were incubated with mouse monoclonal antibodies against  $\Delta\text{Np73}$

(1:500), TAp73 (1:500) and p53 (1:1000) (Abcam, Cambridge, MA) at 4°C overnight, then with a secondary HRP-conjugated goat-anti-mouse antibody (1:1000, DAKO Cytomation, Glostrup, Denmark) at room temperature for 1 h. The proteins were detected using the Amersham ECL Plus Western Blot detection system (Amersham biosciences/GE Healthcare) and visualized in a Fujifilm LAS-1000 CCD camera (Fujifilm, Tokyo, Japan). Equal loading of protein samples was verified using a primary polyclonal rabbit anti- $\beta$ -actin antibody (1:1000, Cell Signalling Technology, Danvers, MA) and a secondary polyclonal goat anti-rabbit antibody (1:2000, DAKO).

### **M30-Apoptosense ELISA and DAPI Assay**

Apoptosis was quantitatively detected by using M30-Apoptosense® ELISA kit (Peviva, Bromma, Sweden) at 72 h after the transfection according to the manufacturer's instructions. Absorbance was measured at 450 nm and normalized against total protein concentration in each sample.

The plates with cells were centrifuged to spin down apoptotic cells. Cells were trypsinized, resuspended in PBS and centrifuged on to glass slides using a Shandon Cytospin® 2 Cytocentrifuge (Thermo Scientific, Waltham, MA), then fixed with 4% buffered formaldehyde and mounted with VECTASHIELD® HardSet™ Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA). The cells were visualized in a UV-light fluorescence microscope, and apoptotic cells were examined.

**Statistical Analysis**

All experiments were performed in triplicates on three different occasions. Student's t-test was performed to evaluate statistical significance. Data are represented as means  $\pm$  standard error of mean (SEM). Two-sided p-values below 0.05 were considered as statistically significant.

## RESULTS

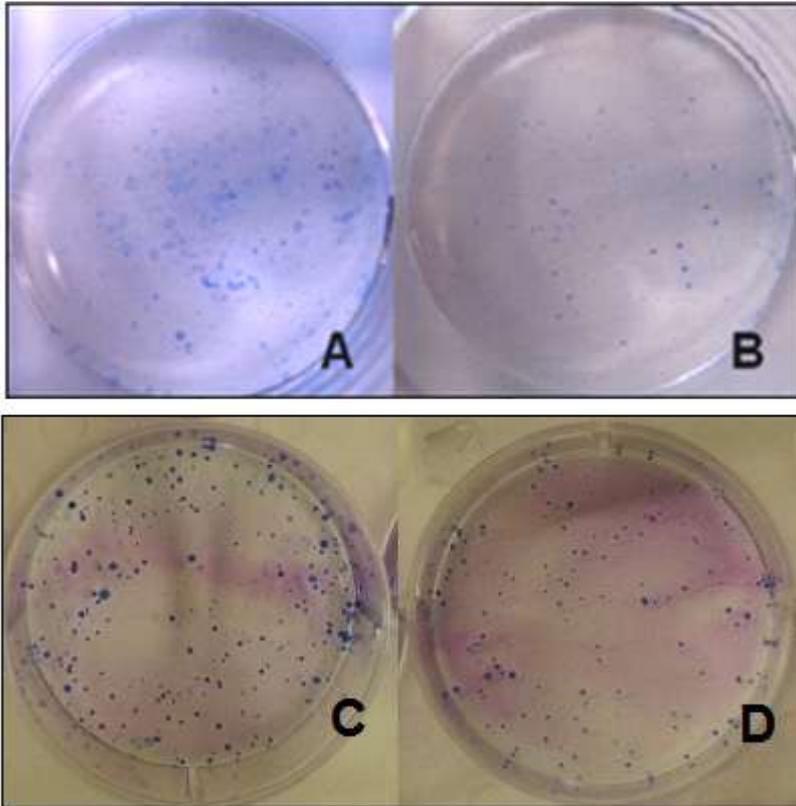
### Colony Forming Assay

Transfected cells were treated with 0.6  $\mu\text{M}$  cisplatin for 7 d, and cell colonies were counted (Figure 1). Cisplatin markedly reduced the number of colonies, and a quota between cisplatin treated samples and controls, the survival fraction, was calculated.  $\Delta\text{Np73}\beta$ -transfected HCT116<sup>p53+/+</sup> cells had a survival fraction of  $0.53\pm 0.08$  and mock-transfected cells had a fraction of  $0.44\pm 0.1$ . The survival fractions were not significantly different ( $p=0.13$ ). The survival fraction for  $\Delta\text{Np73}\beta$ -transfected HCT116<sup>p53-/-</sup> cells was  $0.49\pm 0.07$  and for mock-transfected cells  $0.43\pm 0.06$ . The survival fractions did not significantly differ ( $p=0.07$ ). HT29 cells were highly resistant to cisplatin, as shown by results both from our experiments and other reports [28-29]. The survival fraction for  $\Delta\text{Np73}\beta$ -transfected HT29 cells was  $0.95\pm 0.20$  and for mock-transfected cells  $0.91\pm 0.22$ . There was no significant difference ( $p=0.80$ ).

### Cellular Viability

Cellular viability was examined using the XTT assay after the treatment with increasing concentrations of cisplatin (0-60  $\mu\text{M}$ ). The HCT116 cells were found to respond to 10-20  $\mu\text{M}$  of cisplatin, therefore these concentrations were chosen for subsequent experiments.

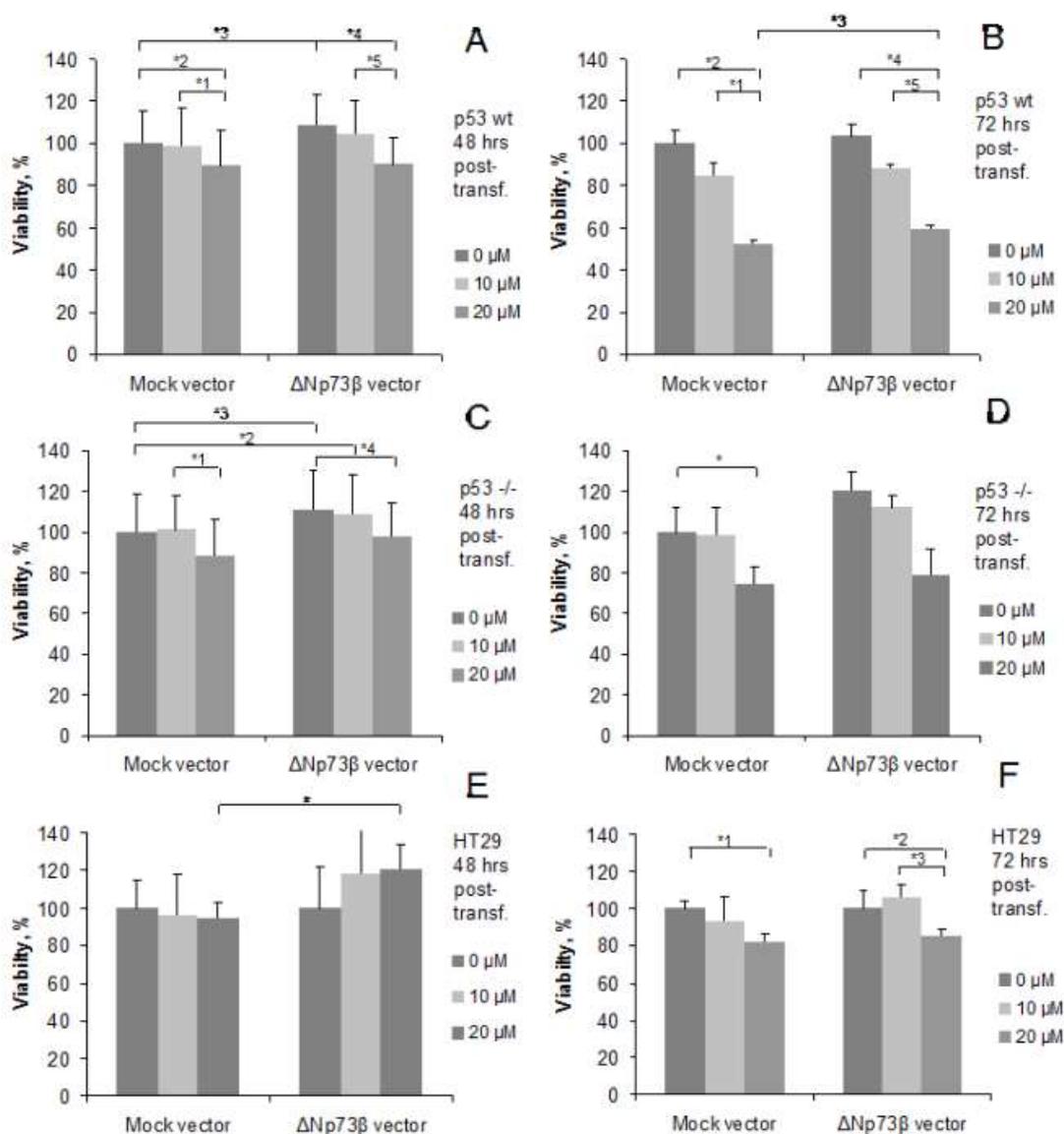
Cellular viability was determined at 48 and 72 h after transfection.  $\Delta\text{Np73}\beta$ -transfected HCT116<sup>p53+/+</sup> cells were significantly more viable than mock-transfected cells at 48 h after the transfection ( $p=0.005$ , Figure 2A). The cellular viability of HCT116<sup>p53+/+</sup> cells was significantly decreased after treatment with 20



**Fig 1.** Colonies of HCT116<sup>p53+/+</sup> colon cancer cells fixed and stained with Giemsa 7 d after seeding the cells without (A) or with 0.6  $\mu$ M cisplatin (B). Colonies of HCT116<sup>p53-/-</sup> cells appeared similar under the same conditions. Colonies of HT29 cells were treated without (C) or with 0.6  $\mu$ M cisplatin (D).

$\mu$ M cisplatin compared to treatment with 0 and 10  $\mu$ M cisplatin, both in  $\Delta$ Np73 $\beta$ -transfected cells ( $p=0.026$  and  $0.034$  for 0 and 10  $\mu$ M, respectively) and mock-transfected cells ( $p=0.038$  and  $0.023$  for 0 and 10  $\mu$ M, respectively, Figure 2A). There was no difference between HCT116<sup>p53+/+</sup> cells treated with 0 and 10  $\mu$ M cisplatin, neither in  $\Delta$ Np73 $\beta$ -transfected cells ( $p=0.40$ ) nor in mock-transfected cells ( $p=0.86$ ). Cellular viability was decreased after treatment with 20  $\mu$ M cisplatin compared to treatment with 0 and 10  $\mu$ M, both in  $\Delta$ Np73 $\beta$ -transfected cells at 72 h after the transfection ( $p=0.008$  and  $0.0007$  for 0 and 10  $\mu$ M, respectively) and in mock-transfected cells ( $p=0.015$  and  $0.024$  for 0 and 10  $\mu$ M, respectively, Figure 2B). Further, cellular viability of  $\Delta$ Np73 $\beta$ -transfected

HCT116<sup>p53+/+</sup> cells were significantly higher than mock-transfected cells treated with 20  $\mu$ M cisplatin ( $p=0.006$ , Figure 2B).



**Fig 2.** Cellular viability measured using XTT assay in A) HCT116<sup>p53+/+</sup> cells at 48 h after the transfection B) HCT116<sup>p53+/+</sup> cells at 72 h after the transfection C) HCT116<sup>p53-/-</sup> cells at 48 h after the transfection D) HCT116<sup>p53-/-</sup> cells at 72 h after the transfection E) HT29 cells at 48 h after the transfection and F) HT29 cells at 72 h after the transfection. All cells were transfected with either  $\Delta$ Np73 $\beta$ - or mock-vector (control), treated or not treated with cisplatin. Untreated mock-transfected cells are set as 100% viable. The p-values from two-sided t-tests are in A) \*<sup>1</sup>=0.023, \*<sup>2</sup>=0.038, \*<sup>3</sup>=0.005, \*<sup>4</sup>=0.026 and \*<sup>5</sup>=0.034 B) \*<sup>1</sup>=0.024, \*<sup>2</sup>=0.015, \*<sup>3</sup>=0.006, \*<sup>4</sup>=0.008 and \*<sup>5</sup>=0.0007 C) \*<sup>1</sup>=0.005, \*<sup>2</sup>=0.02, \*<sup>3</sup>=0.01 and \*<sup>4</sup>=0.048 D) \*=0.028 E) \*=0.001 F) \*<sup>1</sup>=0.001, \*<sup>2</sup>=0.045 and \*<sup>3</sup>=0.002.

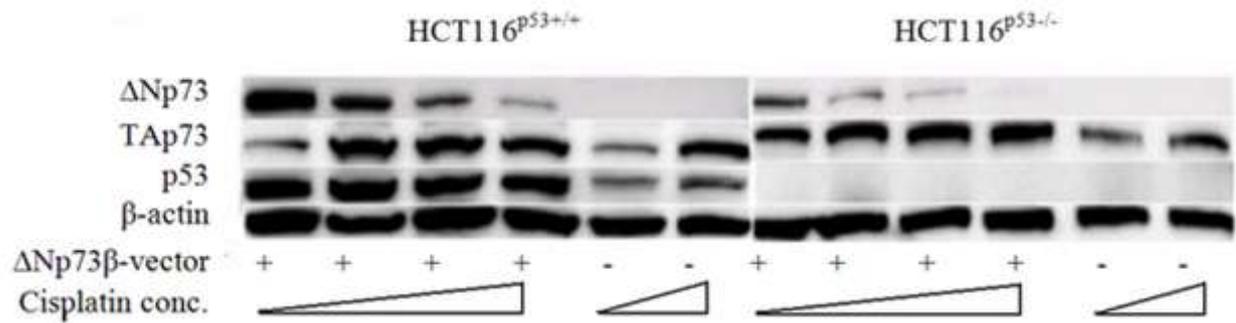
$\Delta$ Np73 $\beta$ -transfected HCT116<sup>p53<sup>-/-</sup></sup> cells were significantly more viable than mock-transfected cells at 48 h after transfection ( $p=0.01$ , Figure 2C). The viability of  $\Delta$ Np73 $\beta$ -transfected cells treated with 10  $\mu$ M cisplatin was also higher than untreated mock-transfected cells ( $p=0.02$ , Figure 2C). The treatment with 20  $\mu$ M cisplatin decreased cellular viability in  $\Delta$ Np73 $\beta$ -transfected HCT116<sup>p53<sup>-/-</sup></sup>, compared to no cisplatin treatment ( $p=0.048$ , Figure 2C). In mock-transfected HCT116<sup>p53<sup>-/-</sup></sup> cellular viability was lower after the treatment with 20  $\mu$ M cisplatin compared to treatment with 10  $\mu$ M cisplatin ( $p=0.005$ , Figure 2C).  $\Delta$ Np73 $\beta$ -transfected HCT116<sup>p53<sup>-/-</sup></sup> cells were more viable than mock-transfected cells both with 0 and 10  $\mu$ M cisplatin at 72 h after transfection, however, the difference was not significant ( $p=0.076$  and  $0.34$  for 0 and 10  $\mu$ M, respectively, Figure 2D). In mock-transfected cells viability was decreased after the treatment with 20  $\mu$ M cisplatin as compared to the control ( $p=0.028$ , Figure 2D). A similar trend was found in the  $\Delta$ Np73 $\beta$ -transfected cells ( $p=0.09$ , Figure 2D).

HT29 cells were treated with the same concentrations of cisplatin. At 48 h after transfection, there was no difference between 0 and 10  $\mu$ M cisplatin, neither in  $\Delta$ Np73 $\beta$ -transfected cells ( $p=0.35$ ) nor in mock-transfected cells ( $p=0.76$ ). Cellular viability remained similar with treatment of 20  $\mu$ M cisplatin compared to treatment with 0 or 10  $\mu$ M, both in  $\Delta$ Np73 $\beta$ -transfected cells ( $p=0.10$  and  $0.84$  for 0 and 10  $\mu$ M, respectively) and in mock-transfected cells ( $p=0.47$  and  $0.85$  for 0 and 10  $\mu$ M, respectively, Figure 2E).  $\Delta$ Np73 $\beta$ -transfected HT29 cells were more viable than mock-transfected cells at the concentration of 20  $\mu$ M ( $p=0.001$ ). A similar trend was seen at 10  $\mu$ M ( $p=0.055$ ). At 72 h after transfection, cellular viability was decreased after treatment with 20  $\mu$ M cisplatin compared to no treatment, both in  $\Delta$ Np73 $\beta$ -

transfected cells ( $p=0.001$ ) and mock-transfected cells ( $p=0.045$  Figure 2F). There was a significant decrease in viability with 20  $\mu\text{M}$  cisplatin compared to 10  $\mu\text{M}$  in  $\Delta\text{Np73}\beta$ -transfected cells ( $p=0.002$ ) but not in mock-transfected cells ( $p=0.13$ ). Again, there was a trend that  $\Delta\text{Np73}\beta$ - transfected cells were more viable than the mock-transfected cells at the concentration of 10  $\mu\text{M}$  ( $p=0.074$ ) but not at 20  $\mu\text{M}$  ( $p=0.28$ ).

### Protein Expression

Expression of  $\Delta\text{Np73}$ , TAp73 and p53 proteins in  $\text{HCT116}^{\text{p53}+/+}$  and  $\text{HCT116}^{\text{p53}^-/-}$  cells was determined by Western blot at 48 and 72 h after the transfection (Figure 3). The expression of  $\Delta\text{Np73}$  was markedly increased after  $\Delta\text{Np73}\beta$ -transfection. Levels of the  $\Delta\text{Np73}\beta$  protein in both  $\text{HCT116}^{\text{p53}+/+}$  and  $\text{HCT116}^{\text{p53}^-/-}$  cells were decreased in a dose-dependent manner after cisplatin treatment. TAp73 expression was increased in both  $\Delta\text{Np73}\beta$ - and mock-transfected  $\text{HCT116}^{\text{p53}+/+}$  and  $\text{HCT116}^{\text{p53}^-/-}$  cells after the treatment with cisplatin. In  $\text{HCT116}^{\text{p53}+/+}$  p53 expression was increased in response to cisplatin. p53 expression was also increased in  $\Delta\text{Np73}\beta$  overexpressing cells, without the cisplatin treatment. The HT29 cells were transfected using the calcium phosphate transfection protocol as other transfection reagents were ineffective. An increase in the  $\Delta\text{Np73}$  protein levels could be observed after transfection (supplementary data).



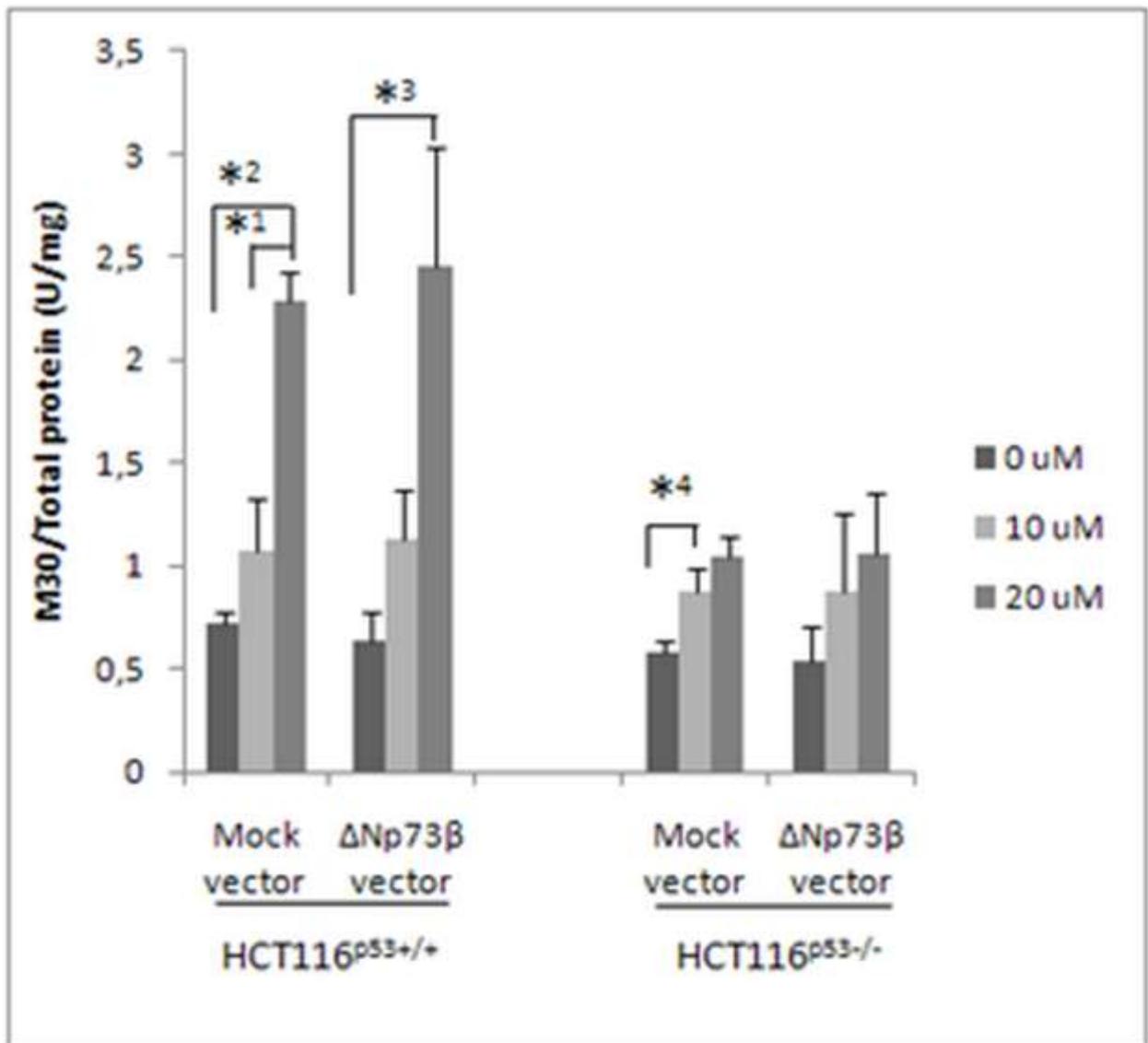
**Fig 3.** Protein expression examined by Western blot of  $\Delta$ Np73, TAp73 and p53 in cell lines HCT116<sup>p53+/+</sup> and HCT116<sup>p53-/-</sup> at 72 h after the transfection with  $\Delta$ Np73 $\beta$ - or mock- vector, treated with increasing concentrations of cisplatin (0-20  $\mu$ M).

### Apoptosis

Apoptosis was evaluated in HCT116 cells at 72 h after transfection using the M30-Apoptosense® ELISA kit (Peviva). The amount of apoptotic cells in  $\Delta$ Np73 $\beta$ -transfected HCT116<sup>p53+/+</sup> cells was increased 1.8 $\times$  at 10  $\mu$ M ( $p=0.062$ ) and significantly increased 3.9 $\times$  at 20  $\mu$ M ( $p=0.049$ ), after the treatment with cisplatin compared to the controls. Corresponding figures for mock-transfected HCT116<sup>p53+/+</sup> cells were 1.5 $\times$  at 10  $\mu$ M ( $p=0.33$ ) and 3.9 $\times$  at 20  $\mu$ M ( $p=0.004$ ). There was no difference between  $\Delta$ Np73 $\beta$ -transfected and mock-transfected HCT116<sup>p53+/+</sup> cells, either with or without treatment of cisplatin ( $p=0.68$ , 0.10 and 0.81 for 0, 10 and 20  $\mu$ M cisplatin, respectively) (Figure 4).

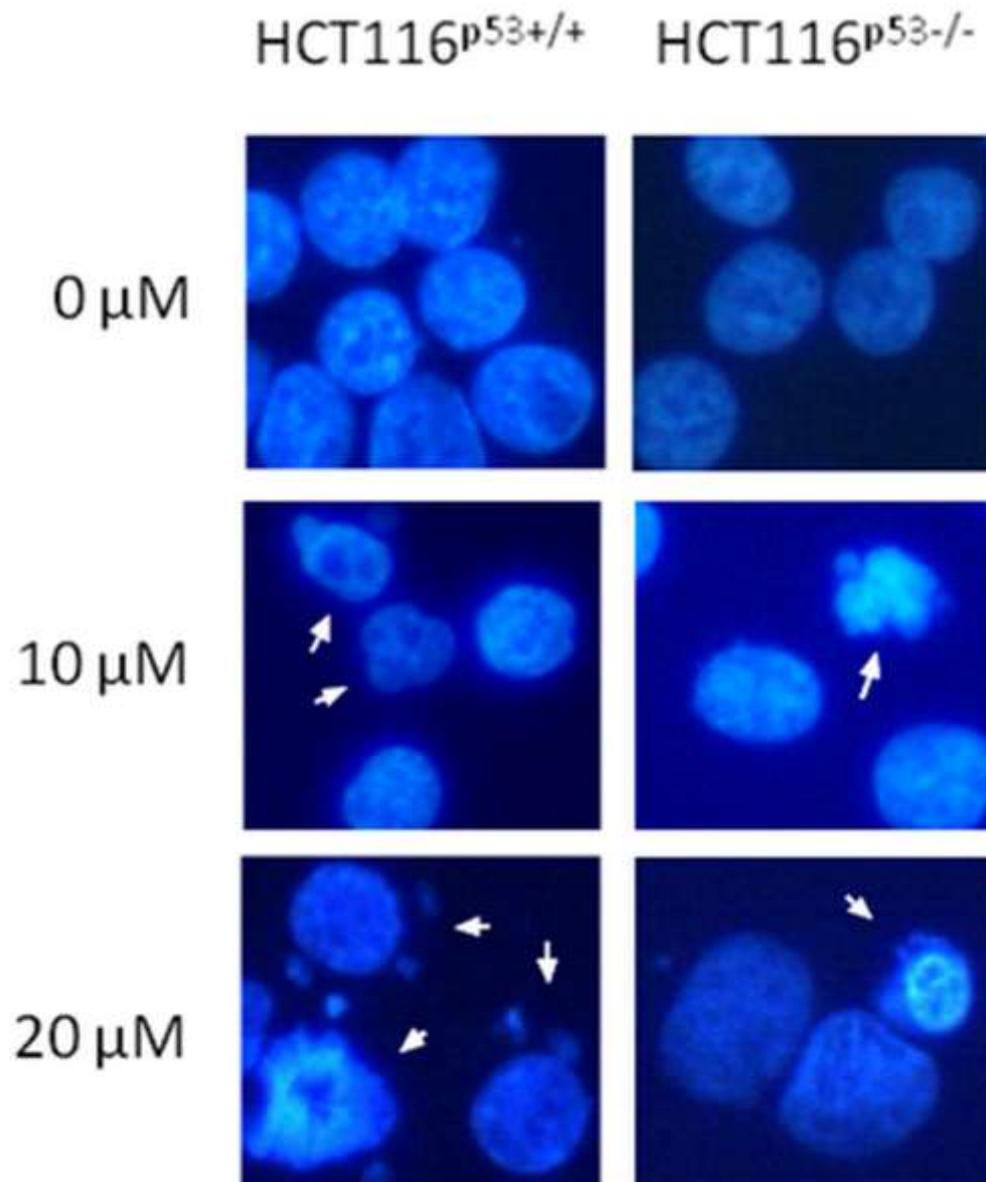
Apoptotic HCT116<sup>p53-/-</sup> cells increased after the cisplatin treatment, although not significantly, by 1.6 $\times$  at 10  $\mu$ M ( $p=0.16$ ) and 2.0 $\times$  at 20  $\mu$ M ( $p=0.059$ ) in  $\Delta$ Np73 $\beta$ -transfected cells, and by 1.5 $\times$  at 10  $\mu$ M ( $p=0.042$ ) and 1.8 $\times$  at 20  $\mu$ M ( $p=0.056$ ) in mock-transfected cells. No difference between  $\Delta$ Np73 $\beta$ - and mock transfected cells

was found, neither at 0, 10 or 20  $\mu\text{M}$  cisplatin ( $p=0.79, 0.38$  and  $0.92$ , respectively) (Figure 4).



**Fig 4.** Apoptosis at 72 h after the transfection with  $\Delta\text{Np73}\beta$ - or mock-vector in  $\text{HCT116}^{\text{p53}+/+}$  and  $\text{HCT116}^{\text{p53} -/-}$  cells measured by M30-Apoptosense® ELISA kit (Peviva), with and without cisplatin treatment. Values are normalized against total protein content of the samples, and presented as units (U) of M30 per mg of protein. The p-values from two-sided t-tests are \*<sup>1</sup>=0.004, \*<sup>2</sup>=0.048, \*<sup>3</sup>=0.049 and \*<sup>4</sup>=0.042.

DAPI staining was used to validate the results of the M30 assay. Apoptotic cells were distinguished from non-apoptotic cells by morphological hallmarks such as nuclear condensation and fragmentation. Representative pictures of DAPI stained cells are shown in Figure 5.



**Fig 5.** HCT116<sup>p53+/+</sup> and HCT116<sup>p53-/-</sup> cells undergo apoptosis after treatment with 0, 10 or 20 μM cisplatin for 48 h. The cells are stained with DAPI, apoptotic cells were observed (arrows).

## DISCUSSION

In this study, we investigated the effects of  $\Delta$ Np73 $\beta$  on colon cancer cells with respect of cytotoxicity of cisplatin. HCT116<sup>p53+/+</sup>, HCT116<sup>p53-/-</sup> and HT29 cells, were transfected with a  $\Delta$ Np73 $\beta$  expression vector and further treated with cisplatin, then colony formation potential, cellular viability, apoptosis, and protein expression were examined. We found that overexpression of  $\Delta$ Np73 $\beta$  significantly increased cellular viability in all the three cell lines. There were no significant differences between  $\Delta$ Np73 $\beta$ - and mock-transfected cells regarding apoptosis in HCT116<sup>p53+/+</sup> and HCT116<sup>p53-/-</sup> cell lines, indicating that the differences in cellular viability do not seem to be primarily due to increased apoptosis. A reduction in cell cycle arrest could possibly explain the difference were due to decreased cell cycle arrest rather than apoptosis.

Since  $\Delta$ Np73 $\beta$  was degraded in a dose dependent manner in response to the cisplatin, any resistance to cisplatin treatment conferred by  $\Delta$ Np73 $\beta$  should be minor at the concentrations used in the cellular viability and apoptosis assays. A relatively low concentration of cisplatin was used in the colony forming assay, and the survival fractions slightly increased in  $\Delta$ Np73 $\beta$ -transfected cells compared to mock-transfected cells, indicating resistance to cisplatin treatment.

It has been shown in sarcoma cell line SAOS-2 that  $\Delta$ Np73 is rapidly degraded in a proteasome dependent manner in response to DNA-damage [30].  $\Delta$ Np73 expression is induced by p53 and TAp73 [11], constituting a regulatory feedback mechanism. The degradation of  $\Delta$ Np73 may be a safety mechanism, allowing cell cycle arrest and apoptosis to occur properly in damaged cells without inhibition from the anti-apoptotic  $\Delta$ Np73. In contrast, cisplatin up-regulates  $\Delta$ Np73 in SH-

SY5Y neuroblastoma cells [17,31]. The signaling pathways leading to DNA damage-induced degradation of  $\Delta$ Np73 could be disturbed in some tumor types, providing a mechanism for resistance to various DNA-damaging treatments.

p53 contributes to cisplatin treatment by inducing cell cycle arrest and apoptosis [22], and we found the protein levels of p53 to be increased after the cisplatin treatment. Further, HCT116<sup>p53<sup>-/-</sup></sup> cells were less sensitive to the cisplatin treatment than HCT116<sup>p53<sup>+/+</sup></sup> cells, confirming the involvement of p53 in cisplatin treatment. We also found an increase in p53 protein in cells overexpressing  $\Delta$ Np73 $\beta$ , even without the cisplatin treatment. This could be due to blocking of the mdm2 promoter;  $\Delta$ Np73 $\beta$  has been previously shown to reduce the transcriptional activation of mdm2 [32]. Mdm2 promotes the ubiquitin-mediated degradation of p53 [33], and high levels of  $\Delta$ Np73 $\beta$  may, therefore, result in reduced degradation of p53.

In addition, we found that expression of TAp73 protein was increased after the cisplatin treatment. Inhibition of TAp73 has been shown to increase the resistance to the cisplatin treatment [24], suggesting that TAp73 is involved in cisplatin-induced cell cycle arrest and apoptosis. On the contrary, it has been reported that p73 is not induced in response to cisplatin in the HCT116 cell line as a result of deficient mismatch repair [25]. Cisplatin increases the half-life of p73 by activating the tyrosine kinase c-abl in mismatch-repair-proficient cells [25]. However, p73 has been stabilized by the transcription factor c-Jun in response to cisplatin, in cells defective in c-abl binding and phosphorylation [34], suggesting alternative pathways for p73 induction independent of c-abl and mismatch-repair signaling pathway.

Upregulation of  $\Delta Np73$  in tumors may disturb apoptotic signaling pathway by interfering with p53 and TAp73, and high expression of  $\Delta Np73$  has been correlated with a worse outcome of patients [35-36] and resistance to DNA-damaging drugs [37-38]. Recent studies have shown that overexpression of  $\Delta Np73$  protects cells from apoptosis, also when treated with cisplatin, by inhibiting p53 target genes such as p21 [30], while downregulation of  $\Delta Np73$  increases apoptosis [39]. However, the effect of the  $\Delta Np73\beta$  isoform remains indistinct. Unlike  $\Delta Np73\alpha$ ,  $\Delta Np73\beta$  possessed the capability of activating some p53-responsive genes, thereby inducing both cell cycle arrest and apoptosis [20]. Resulting from the use of alternative intron 3 promoter, the  $\Delta Np73$  isoforms contain 13 unique residues at the N-terminus shown to contain an activation domain [20]. The  $\Delta Np73\alpha$  and  $\beta$  isoforms are identical apart from the C-terminal where the  $\beta$ -isoform lacks exon 13 [1], suggesting that the C-terminal of  $\alpha$ -isoform represses transcription. Both  $\Delta Np73\alpha$  and  $\beta$  have been shown to inhibit the transcription of p53 responsive genes, although the  $\alpha$ -isoform is much more potent than the  $\beta$ -isoform which shows some intrinsic transactivation capacity [16]. Also in TAp73, the  $\alpha$ -isoform has lower transactivation ability than the  $\beta$ -isoform [40].

In conclusion, we demonstrate that cisplatin decreased clonogenic potential and cellular viability while increasing apoptosis. These effects were more pronounced in  $HCT116^{p53+/+}$  than  $HCT116^{p53-/-}$ , confirming the involvement of p53 in the cisplatin treatment. Both p53 and TAp73 were upregulated in cisplatin treated HCT116 cells. Overexpression of  $\Delta Np73\beta$  increased cellular viability in HT29 cells as well as both  $HCT116^{p53+/+}$  and  $HCT116^{p53-/-}$ . Resistance to cisplatin treatment increased slightly in  $\Delta Np73\beta$  overexpressing  $HCT116^{p53+/+}$  and

HCT116<sup>p53<sup>-/-</sup></sup> cells, indicating that both p53 and TAp73 were involved in cisplatin treatment.

**REFERENCES**

1. Kaghad M, Bonnet H, Yang A et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997;90:809-819.
2. Jost CA, Marin MC, Kaelin WG, Jr. p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature* 1997;389:191-194.
3. Yang A, Walker N, Bronson R et al. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 2000;404:99-103.
4. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323-331.
5. Stiewe T, Putzer BM. Role of p73 in malignancy: tumor suppressor or oncogene? *Cell Death Differ* 2002;9:237-245.
6. Sun XF. p73 overexpression is a prognostic factor in patients with colorectal adenocarcinoma. *Clin Cancer Res* 2002;8:165-170.
7. Zaika AI, Kovalev S, Marchenko ND, Moll UM. Overexpression of the wild type p73 gene in breast cancer tissues and cell lines. *Cancer Res* 1999;59:3257-3263.
8. Chen CL, Ip SM, Cheng D, Wong LC, Ngan HY. P73 gene expression in ovarian cancer tissues and cell lines. *Clin Cancer Res* 2000;6:3910-3915.
9. Tokuchi Y, Hashimoto T, Kobayashi Y et al. The expression of p73 is increased in lung cancer, independent of p53 gene alteration. *Br J Cancer* 1999;80:1623-1629.

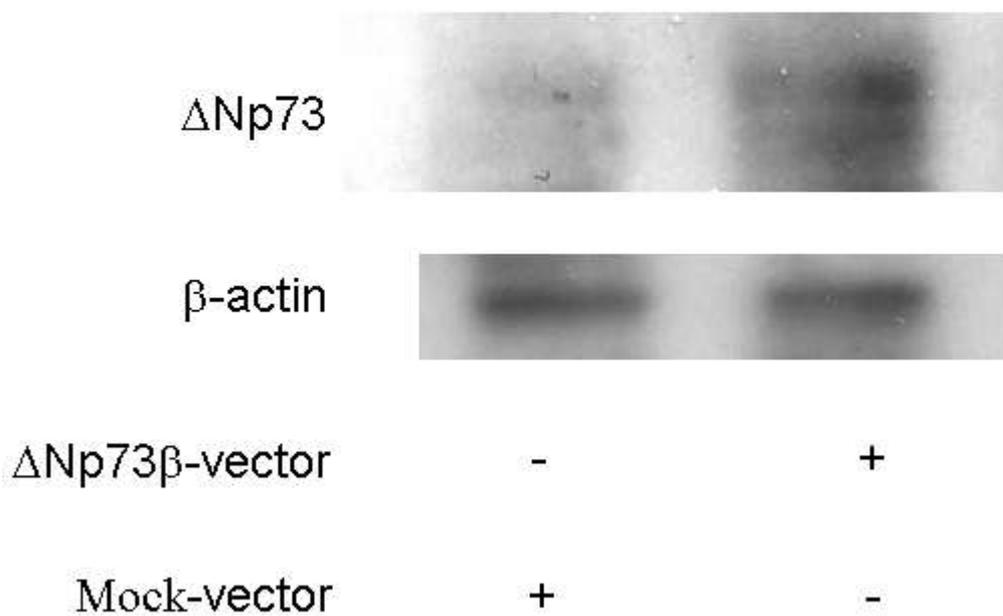
10. Tannapfel A, Wasner M, Krause K et al. Expression of p73 and its relation to histopathology and prognosis in hepatocellular carcinoma. *J Natl Cancer Inst* 1999;91:1154-1158.
11. Grob TJ, Novak U, Maisse C et al. Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell Death Differ* 2001;8:1213-1223.
12. De Laurenzi V, Costanzo A, Barcaroli D et al. Two new p73 splice variants, gamma and delta, with different transcriptional activity. *J Exp Med* 1998;188:1763-1768.
13. De Laurenzi VD, Catani MV, Terrinoni A et al. Additional complexity in p73: induction by mitogens in lymphoid cells and identification of two new splicing variants epsilon and zeta. *Cell Death Differ* 1999;6:389-390.
14. Ishimoto O, Kawahara C, Enjo K, Obinata M, Nukiwa T, Ikawa S. Possible oncogenic potential of DeltaNp73: a newly identified isoform of human p73. *Cancer Res* 2002;62:636-641.
15. Stiewe T, Zimmermann S, Frilling A, Esche H, Putzer BM. Transactivation-deficient DeltaTA-p73 acts as an oncogene. *Cancer Res* 2002;62:3598-3602.
16. Kartasheva NN, Contente A, Lenz-Stoppler C, Roth J, Dobbstein M. p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene* 2002;21:4715-4727.
17. Nakagawa T, Takahashi M, Ozaki T et al. Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter. *Mol Cell Biol* 2002;22:2575-2585.

18. 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;289:304-306.
19. Tomasini R, Tsuchihara K, Wilhelm M et al. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev* 2008;22:2677-2691.
20. Liu G, Nozell S, Xiao H, Chen X. DeltaNp73beta is active in transactivation and growth suppression. *Mol Cell Biol* 2004;24:487-501.
21. Marrazzo E, Marchini S, Tavecchio M et al. The expression of the DeltaNp73beta isoform of p73 leads to tetraploidy. *Eur J Cancer* 2009;45:443-453.
22. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 2003;22:7265-7279.
23. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108:153-164.
24. Irwin MS, Kondo K, Marin MC, Cheng LS, Hahn WC, Kaelin WG, Jr. Chemosensitivity linked to p73 function. *Cancer Cell* 2003;3:403-410.
25. Gong JG, Costanzo A, Yang HQ et al. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. *Nature* 1999;399:806-809.
26. Vayssade M, Haddada H, Faridoni-Laurens L et al. P73 functionally replaces p53 in Adriamycin-treated, p53-deficient breast cancer cells. *Int J Cancer* 2005;116:860-869.
27. Bunz F, Dutriaux A, Lengauer C et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 1998;282:1497-1501.

28. Fernández de Mattos S, Villalonga P, Clardy J, et al. FOXO3a mediates the cytotoxic effects of cisplatin in colon cancer cells. *Mol Cancer Ther* 2008;7:3237-3246.
29. Gambi N, Tramontano F, Quesada P. Poly(ADPR)polymerase inhibition and apoptosis induction in cDDP-treated human carcinoma cell lines. *Biochem Pharmacol* 2008;75:2356-2363.
30. Maise C, Munarriz E, Barcaroli D, Melino G, De Laurenzi V. DNA damage induces the rapid and selective degradation of the DeltaNp73 isoform, allowing apoptosis to occur. *Cell Death Differ* 2004;11:685-687.
31. Million K, Horvilleur E, Goldschneider D et al. Differential regulation of p73 variants in response to cisplatin treatment in SH-SY5Y neuroblastoma cells. *Int J Oncol* 2006;29:147-154.
32. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997;387:296-299.
33. Nakagawa T, Takahashi M, Ozaki T et al. Negative autoregulation of p73 and p53 by DeltaNp73 in regulating differentiation and survival of human neuroblastoma cells. *Cancer Lett* 2003;197:105-109.
34. Toh WH, Siddique MM, Boominathan L, Lin KW, Sabapathy K. c-Jun regulates the stability and activity of the p53 homologue, p73. *J Biol Chem* 2004;279:44713-44722.
35. Muller M, Schilling T, Sayan AE et al. TAp73/Delta Np73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. *Cell Death Differ* 2005;12:1564-1577.

36. Casciano I, Mazzocco K, Boni L et al. Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients. *Cell Death Differ* 2002;9:246-251.
37. Concin N, Hofstetter G, Berger A et al. Clinical relevance of dominant-negative p73 isoforms for responsiveness to chemotherapy and survival in ovarian cancer: evidence for a crucial p53-p73 cross-talk in vivo. *Clin Cancer Res* 2005;11:8372-8383.
38. Meier M, den Boer ML, Meijerink JP et al. Differential expression of p73 isoforms in relation to drug resistance in childhood T-lineage acute lymphoblastic leukaemia. *Leukemia* 2006;20:1377-1384.
39. Simoes-Wust AP, Sigrist B, Belyanskaya L, Hopkins Donaldson S, Stahel RA, Zangemeister-Wittke U. DeltaNp73 antisense activates PUMA and induces apoptosis in neuroblastoma cells. *J Neurooncol* 2005;72:29-34.
40. Dobbstein M, Wienzek S, König C, Roth J. Inactivation of the p53-homologue p73 by the mdm2-oncoprotein. *Oncogene* 1999;18:2101-2106.

## HT29



**Supplementary.** Western blot of  $\Delta Np73$  in HT29 colon cancer cells after the transfection with  $\Delta Np73\beta$ - or mock- vector.