

*Laboratory Investigation* **$\Delta$ Np73 antisense activates PUMA and induces apoptosis in neuroblastoma cells**

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**Key words:** antisense, apoptosis, bax, neuroblastoma,  $\Delta$ Np73, PUMA

**Summary**

The p73 gene codes for various different protein isoforms. They include proteins expressed under the control of the P1 promoter that contain a transactivation domain and are similar in function to p53 (TAp73 isoforms), as well as proteins regulated by the P2 promoter that lack this domain and function as dominant negative inhibitors of TAp73 and p53 ( $\Delta$ Np73 isoforms). Whereas TAp73 functions as a tumor suppressor with pro-apoptotic function,  $\Delta$ Np73 is likely to prevent the induction of apoptosis in tumor cells and to participate in oncogenesis. Here we used a loss-of-function strategy to assess the role of  $\Delta$ Np73 in SH-SY5Y neuroblastoma cells. An antisense oligonucleotide designed to target  $\Delta$ Np73 mRNA, but not TAp73, was used to effectively downregulate this transcript.  $\Delta$ Np73 downregulation was accompanied by increased levels of the pro-apoptotic BH3 family member PUMA at the mRNA and protein level, and by conformational activation of BAX which translocated to mitochondria. These  $\Delta$ Np73 antisense-mediated alterations led to the induction of apoptosis as detected by decreased cell viability, augmented DNA fragmentation and increased caspase-3 activity in cell lysates. Our results demonstrate the cytoprotective role of  $\Delta$ Np73 in neuroblastoma and suggest its use as a target for molecular intervention therapy.

**Introduction**

p53 and p73 proteins have a relatively high sequence homology to each other and may act as transcription factors with common target sequences whose transcriptional activation can lead to cell cycle-arrest and apoptosis. p53 has a tumor-suppressor function as revealed by studies with knockout mice and based on data with primary tumors, which demonstrate its inactivation in more than 50% of all human cancers [1]. Comparable observations have not been reported for p73 [2,3], indicating that the role of p73 in neoplastic transformation is less well defined, likely due to the co-existence of distinct p73 isoforms with opposing functions, some acting as tumor suppressors and others as oncogenes.

In contrast to p53, the p73 gene codes for various mRNAs which arise according to alternative splicing and are under the control of at least two promoters [3]. One can distinguish between two major types of p73 isoforms, long and short, which differ in the amino-terminal sequence. The long isoforms are transcriptionally regulated by the P1 promoter and contain the transactivation domain encoded by exons 2 and 3 (TAp73), whereas the short isoforms arise upon activation of the P2 promoter and comprise, instead of the TA domain, a smaller sequence derived from an alternative 3'-exon ( $\Delta$ Np73). Within each of these two major types a variety of other proteins ( $\alpha$ - $\eta$ ) exist that arise from differential carboxy-terminal splicing. Whereas the TAp73-isoforms positively regulate several p53-responsive genes [3–5], the  $\Delta$ Np73-isoforms behave as dominant negative inhibitors of both the p53- and the TA-dependent activation of these genes [6–13].  $\Delta$ Np73 has been shown to

act as an oncogene [9] and to be up-regulated in human tumors relative to normal tissues [10].

p73 locates to chromosome 1p36, a locus prone to loss of heterozygosity in various tumor types, particularly in neuroblastoma [3] which is the most frequent solid tumor in children under 5 years of age [14]. To date, neuroblastoma is the only tumor type in which expression of  $\Delta$ Np73 has been clearly shown to be an adverse prognostic marker [15], and investigations into the prognostic value of the different p73 isoforms in other cancer types are underway. In the present study we used antisense oligonucleotides targeting a sequence present in  $\Delta$ Np73, but not in TAp73, to assess the role of these short p73 isoforms in SH-SY5Y neuroblastoma cells. Downregulation of  $\Delta$ Np73 led to an increase in the level of PUMA mRNA and protein, and to conformational activation of BAX. These alterations resulted in decreased viability by induction of apoptosis.

**Material and methods***Treatment of cells with oligonucleotides*

SH-SY5Y neuroblastoma cells were cultured in RPMI-1640 (Hyclone Europe, Ltd., Cramlington, UK), supplemented with 10% FCS (Hyclone Europe, Ltd.), 2 mM L-glutamine, 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin and 20  $\mu$ g/ml gentamicin, and maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Oligonucleotides with a full phosphorothioate backbone (Microsynth, Balgach, Switzerland) were delivered into cells in the form of complexes with Lipofectin

(Life Technologies, Glasgow, UK) as described in detail elsewhere [16]. The sequences were as follows: 7001, 5'-CCCATCTCCCTTAGTTCTGT-3'; sc-7001a, 5'-TA-ACCGTTTCTTCCTCGTCC-3'; sc7001b, 5'-GTTACCCTCCTTACTCGCTT-3'. A BLAST search of the NCBI database revealed no homology of these sequences to other known human genes.

#### *Real-time PCR*

Total RNA isolation, cDNA synthesis and real-time PCR were performed as previously described [17] with modifications. DNase was included during RNA isolation in order to eliminate any contaminating genomic DNA. The amplification of  $\Delta$ Np73 cDNA was performed using 5'-AAAAGCGAAAATGCCAACAA-3' as forward primer, 5'-GCAGGAGCCGAGGATGC-3' as reverse primer and 5'-CGGCCCGCATGTTCCCC-3' as Taqman probe (Applied Biosystems, Foster City, CA). These bind to the untranslated region of the 3' exon from the p73 gene which is characteristic and exclusive for all the  $\Delta$ Np73 isoforms, independent of their carboxy-terminal sequence. A BLAST search of the NCBI database revealed no homology of the primers and probe sequences to other known human genes. Amplification of rRNA, used as an internal standard, was performed in 100-times further diluted cDNA suspensions and the calibration curve method was used for relative quantification of  $\Delta$ Np73. For PUMA mRNA, Assay-on-Demand (Applied Biosystems) primers and probe were used and the data were quantified by the comparative  $C_T$  method. The mRNA level in untreated cells was taken as 100%, upon correction for the internal standard.

#### *Western blotting*

Western blot analysis was performed as previously described with modifications [17]. The blots were incubated during 2 h at room temperature (or overnight at 4 °C) with goat anti-human PUMA $\alpha$  (N-19) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).  $\alpha$ -tubulin detected with a mouse monoclonal antibody (TU-02, Santa Cruz Biotechnology) was used to assure comparable protein loading. To detect the primary antibody, blots were incubated with rabbit anti-goat or rabbit anti-mouse immunoglobulin peroxidase conjugates, respectively (Sigma, St. Louis, MO) for 1 h at room temperature.

#### *Immunocytochemistry*

Floating and adherent cells were removed from culture 6-well plates and resuspended in 100  $\mu$ l PBS. Upon subsequent cytospin centrifugation, the cells were fixed by adding 100  $\mu$ l of Cytofix/Cytoperm solution (BD Biosciences, San Diego, CA), washed with PermWash solution (BD Biosciences) and incubated with rabbit anti-human Bax (N-20) polyclonal antibody (Santa Cruz Biotechnology) for 30 min. After three washes

with PermWash solution, cells were incubated with goat anti-rabbit-FITC (Sigma) followed by 1  $\mu$ g/ml Hoechst 33342 (Sigma) in PBS. Finally, cells were washed twice with PBS, dipped in distilled water and mounted with Mowiol (Calbiochem, La Jolla, CA). The preparations were analysed under a DM IRB E Leica microscope (Leica, Wetzlar, Germany) equipped with a TCS4D confocal laser scanning system (Leica Lasertechnik, Heidelberg, Germany) including a UV-laser (Enterprise Argon ion laser, model 651, Coherent Inc., Santa Clara, CA, USA), a laser for visible excitation light (model 543 Argon-Krypton-laser, Omnicrome Inc. Chino, CA, USA) and SCANware software (Leica, Wetzlar, Germany). Images were processed using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA) or an Octane workstation (Solikon Graphics Inc., Mountain View, CA, USA) for the DIC pictures.

#### *Measurement of cell viability*

Cell viability was determined by use of a colorimetric assay based on the reduction of the tetrazolium salt MTT as described [17]. Cells were plated in 96-well plates (7500 cells/well), incubated overnight, treated in the absence or presence of various oligonucleotide concentrations during 6 h and, after renewal of the culture medium, further incubated for up to 72 h.

#### *Caspase-3 assay*

Caspase-3-like protease activity in cell lysates was analysed by use of the Caspase-3 Colorimetric Assay Kit (Alexis Corporation, Lausen, Switzerland) following manufacturer's recommendations. Cleavage of the labeled substrate DEVD-pNA was monitored at 405 nm using a SPECTRAMax 340 microplate reader. The caspase-3-like protease activity in lysates from apoptotic cells was calculated as fold increase of the absorbance signal obtained with lysates of untreated (viable) cells kept under identical conditions.

#### *Measurement of apoptosis by flow cytometry*

Cells were harvested 72 h after the start of oligonucleotide treatment (400 nM final concentration, 6 h transfection), washed with PBS containing 5 mM EDTA and fixed with 100% ice-cold ethanol. Upon treatment with phosphate-citrate buffer for 30 min to extract degraded DNA [18], the fixed cells were again washed with 5 mM EDTA in PBS and incubated with 5  $\mu$ g/ml RNase A for 30 min. Thereafter, propidium iodide was added to achieve a final concentration of 50  $\mu$ g/ml and the cells were subjected to flow cytometry using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). A total of 10,000 events were analysed and clusters as well as cell debris were eliminated by appropriate gating. The subpopulation of apoptotic cells with fluorescence signals lower than that of cells in the G1 phase (sub-G1 peak due to fragmented DNA) [19] was determined using the CellQuest software (Becton Dickinson).

## Results

### *Downregulation of $\Delta$ Np73 mRNA by antisense oligonucleotide 7001*

To study the role of  $\Delta$ Np73 isoforms in tumor cells, we screened for an antisense oligonucleotide to effectively and specifically downregulate these transcripts. Seven target sequences were validated, all of them located in the untranslated region of the 3' exon of the TP73 gene which is present in all  $\Delta$ Np73 but not in the TAp73 isoforms. All sequences were expected to be accessible to oligonucleotide annealing as predicted by the RNAdraw program [20]. Oligonucleotide 7001 targeting nucleotides 16–35 of the human  $\Delta$ Np73 mRNA (NCBI accession number AB055065,  $\alpha$  isoform) was the most effective in downregulating  $\Delta$ Np73 in various cancer cell types (data not shown). While oligonucleotide 7001 led to a more than 80% reduction of target expression, two scrambled sequence control oligonucleotides (sc7001a and sc7001b) did not inhibit gene expression (Figure 1). Downregulation of the  $\Delta$ Np73 transcript was observed as early as 2 h after addition of oligonucleotide 7001 (data not shown). The levels of the corresponding proteins in untreated cells were below the detection threshold of Western blotting using commercially available antibodies, which precluded the measurement of antisense effects on the protein level.

A recent technology in targeted gene silencing takes advantage of the process of RNA interference by use of short double-stranded RNA (siRNA, small interfering RNA) [21]. Our own results with siRNA targeting the site of 7001 hybridization in the  $\Delta$ Np73 mRNA revealed lower antisense activity compared to oligonucleotide 7001 (data not shown).

### *Transcriptional activation of PUMA upon downregulation of $\Delta$ Np73*

$\Delta$ Np73 may function as a dominant negative form of TAp73 and p53. Very recent work by other authors has shown that overexpression of  $\Delta$ Np73 is able to inhibit TAp73- and p53-induced apoptosis by preventing the transcriptional activation of the BH3-only protein PUMA (p53-upregulated modulator of apoptosis) [4]. We hypothesized that if endogenous  $\Delta$ Np73 would

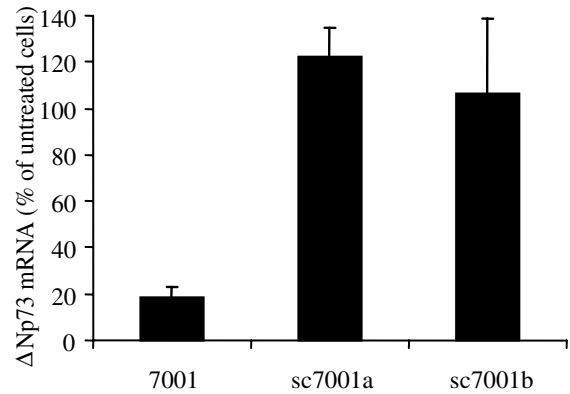


Figure 1. Antisense oligonucleotide 7001 downregulates  $\Delta$ Np73 mRNA in neuroblastoma cells. SH-SY5Y cells were incubated with 600 nM oligonucleotide 7001 or the corresponding scrambled sequence controls sc7001a and sc7001b, and harvested after a 20 h incubation. The relative levels of  $\Delta$ Np73 mRNA were quantified by real-time PCR. Data are shown as mean  $\pm$  SD of at least three independent experiments.

function in a similar way in SH-SY5Y cells, then its downregulation would result in transcriptional activation of PUMA. The level of PUMA mRNA increased with time during treatment with oligonucleotide 7001 (Figure 2A). Transcriptional activation of PUMA induced by antisense oligonucleotide 7001 (Figure 2) was accompanied by increased protein expression (Figure 3). Although at later time points treatment with the control oligonucleotides also slightly increased the expression of PUMA mRNA, this nonantisense effect was not observed on the functionally more relevant protein level (Figure 3). As shown in Figure 2B, NOXA, another BH-3 only protein, which is regulated by p53 on the transcriptional level [22], was not transcriptionally activated upon  $\Delta$ Np73 downregulation.

### *Silencing of $\Delta$ Np73 is associated with conformational activation of Bax*

PUMA may elicit conformational activation of the proapoptotic Bcl-2 family member Bax [4]. To examine this possibility under conditions when PUMA is transcriptionally activated by downregulation of  $\Delta$ Np73, we studied the conformation status of Bax using an

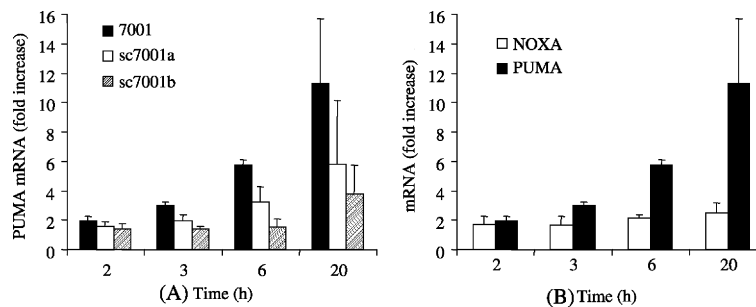


Figure 2. Antisense oligonucleotide 7001 increases the level of PUMA mRNA. (A) SH-SY5Y cells were incubated with 600 nM oligonucleotides 7001, sc7001a or sc7001b, and harvested after the indicated time periods. PUMA mRNA quantification was performed by real-time PCR. (B) SH-SY5Y cells were incubated with 600 nM oligonucleotide 7001 and harvested after the indicated time periods. PUMA and NOXA mRNA quantification was performed by real-time PCR. Data are shown as mean  $\pm$  SD of at least three independent experiments.



**Figure 3.** Antisense oligonucleotide 7001 increases the level of PUMA protein. SH-SY5Y cells were incubated with 300 nM oligonucleotides 7001, sc7001a or sc7001b, and harvested after the indicated time periods before PUMA protein level was quantitated by Western blotting.

N-terminus-specific antibody, which only has access to its epitope if the protein presents in an activated conformation. In untreated SH-SY5Y control cells, the immunofluorescence signal upon staining with the conformation-dependent anti-Bax antibody could not be distinguished from background (not shown). As shown in Figure A, treatment with oligonucleotide 7001 resulted in a clear fluorescence signal, indicating that the antibody's epitope was no longer occluded and that Bax had undergone a conformational change. The observed punctuate staining pattern suggests that activated Bax translocated to mitochondria. Moreover, cells positive for activated Bax were those in which condensed nuclei morphology was observed (Figure 4B), suggesting that activation of Bax initiated the apoptotic cascade. Bax activation occurred in the absence of a measurable increase in Bax protein level (not shown).

#### *SH-SY5Y cell viability is impaired upon treatment with $\Delta$ Np73 antisense*

To study the effect of  $\Delta$ Np73 downregulation on the survival of SH-SY5Y cells, different methods were used. As shown in Figure 5A, treatment with oligonucleotide 7001 specifically induced caspase-3-like activity that is indicative for the onset of apoptosis. Flow cytometry analysis was used to quantify the rate of apoptosis by measuring the sub-G1 population upon the various treatments. Figure 5B shows that downregulation of  $\Delta$ Np73 using oligonucleotide 7001 induced apoptosis in a significant fraction of the total cell population, which correlated with a concentration-dependent reduction of cell viability measured in colorimetric assays (Figure 5C). In all experiments, the unspecific toxicity of the control oligonucleotides sc7001a and sc7001b was sig-

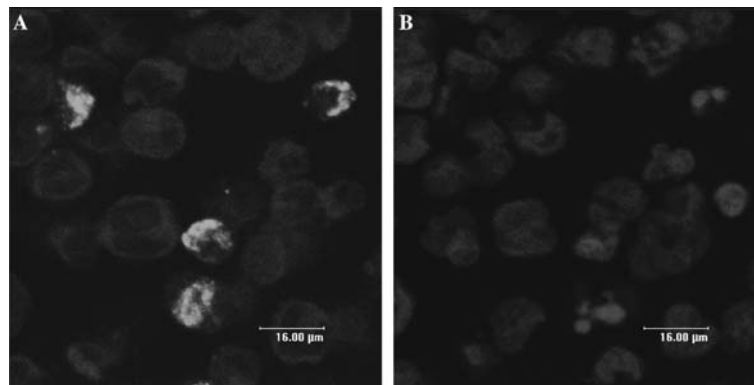
nificantly lower than the pro-apoptotic effect of the  $\Delta$ Np73-specific antisense oligonucleotide 7001.

#### **Discussion**

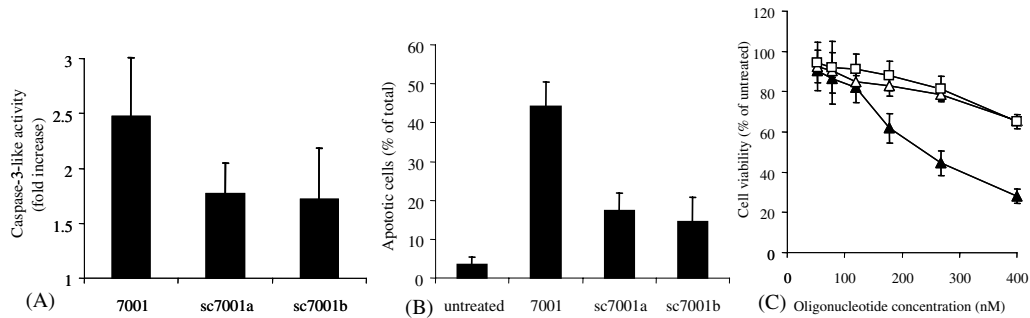
The co-existence of tumor suppressor and oncogene isoforms encoded by the p73 gene hampers the interpretation of data obtained in p73 knockout mice [2] or with antisense approaches targeting sequences common to both TA- and  $\Delta$ Np73 isoforms [11]. In the only study published so far with a  $\Delta$ Np73-specific antisense oligonucleotide, downregulation of the protein induced apoptosis in colon carcinoma cells, either upon expression of exogenous p53 or induction of DNA damage [10]. Our data reveal that 7001 is a highly effective  $\Delta$ Np73 antisense molecule and that silencing of these transcripts induces apoptosis in neuroblastoma cells.

Antisense 7001 is a first generation phosphorothioate oligonucleotide, the most commonly used type of antisense chemistry [23] and the one which could prove to be effective in clinical trials [24]. Nevertheless, phosphorothioate oligonucleotides are also known to exhibit sequence-independent nonantisense-related effects [25]. Since the quantification of unspecificity is of particular relevance in cell viability/apoptosis experiments, we included two scrambled sequence control oligonucleotides in our study. Independent of the method used (caspase-3-like activity, flow cytometry, cell viability assay), the effect of oligonucleotide 7001 on cell viability and apoptosis induction was significantly stronger than those of the control oligonucleotides, indicating that it primarily resulted from  $\Delta$ Np73 downregulation.

Several gain-of-function studies [7–13], including one performed with SH-SY5Y cells [6], demonstrated that



**Figure 4.** Antisense oligonucleotide 7001 induces conformational activation of Bax. SH-SY5Y cells were incubated with 300 nM oligonucleotide 7001 for 20 h. Staining of activated Bax (A) and cell nuclei (B) was performed as described under 'Materials and Methods'.



**Figure 5.**  $\Delta$ Np73 downregulation led to the induction of apoptosis in SH-SY5Y cells. (A)  $\Delta$ Np73 antisense treatment increased caspase-3 activity. Cells were incubated with 300 nM oligonucleotides 7001 or sc7001a or sc7001b for 6 h, and harvested 42 h later. Cell extracts were analysed in caspase-3 assays. (B)  $\Delta$ Np73 antisense treatment led to the appearance of apoptotic cells. Cells were treated with 400 nM antisense oligonucleotide 7001 or control oligonucleotides sc7001a or sc7001b for 6 h and harvested 72 h thereafter. The sub-G1 population was quantified using flow cytometry. (C)  $\Delta$ Np73 downregulation reduced cell viability. Cells were incubated with increasing concentrations of antisense oligonucleotide 7001 (solid triangles) or control sc7001a (solid squares) or sc7001b (open squares) for 20 h, and viability was measured in triplicate cultures 72 h thereafter. In all cases data are shown as mean  $\pm$  SD of at least three independent experiments.

$\Delta$ Np73 proteins are able to act on TAp73/p53 in a dominant-negative fashion by blocking their transactivation activity and ability to induce apoptosis. These studies were corroborated by investigations on the neuronal anomalies induced by cytomegalovirus which revealed that an accumulation of  $\Delta$ Np73 is involved in the apoptosis-blocking effect of this virus [12]. We believe that downregulating endogenous  $\Delta$ Np73 released its repressing effect on TAp73 and/or p53. The inhibition of p53/TAp73 could occur either via a physical interaction between  $\Delta$ Np73 and TAp73/p53, or through competition for the DNA-binding site of the p53/TAp73 targets [6]. SH-SY5Y cells express both TAp73 and wt-p53 [5,7], making a clear distinction between these two possible targets of  $\Delta$ Np73 difficult. Since p53 is functionally inactivated by sequestration into the cytosol in SH-SY5Y cells [5] and no activation could be observed upon  $\Delta$ Np73 antisense treatment using an ELISA-based assay (data not shown), we can exclude that our observations are due to a p53-mediated effect.

TAp73 and p53 are both able to induce apoptosis, though there are differences in the functional processes favored by the two proteins. Whereas p53 induces apoptosis by its ability to directly act on mitochondria [26] and through transcriptional activation of both PUMA and NOXA [27], TAp73-induced apoptosis seems to occur in the absence of a direct effect on mitochondria via an exclusively PUMA-mediated pathway [4]. The specific transcriptional activation of PUMA induced by  $\Delta$ Np73 antisense treatment was associated with a conformational change of Bax, a finding which is in agreement with previous data showing that overexpression of PUMA is able to activate Bax and promote its mitochondrial translocation [4]. Although it is known that PUMA, like other BH3-only proteins, requires Bax (or Bak) to induce apoptosis [22], the molecular mechanism of this interaction is not yet fully understood. Since the BH3-only proteins bind to a cleft formed by the BH1 and BH2 domains of anti-apoptotic Bcl-2 family members [27], it is conceivable that Bax-activation by PUMA requires additional proteins, i.e. Bcl-2 or Bcl-xL. The similarities between the TAp73-induced onset of cell death [4] and our data on

the activation of the apoptosis machinery by  $\Delta$ Np73 antisense are striking. Both processes seem to be dependent on the transcriptional activation of PUMA (but not NOXA) and involve a conformational (not transcriptional) activation of Bax. These similarities strongly suggest that by depleting the pool of endogenous  $\Delta$ Np73, its repressing effect on TAp73 could be diminished, resulting in restoration of the pro-apoptotic function of this protein.

Neuroblastoma is characterized by a broad clinical spectrum, including diseases with spontaneous regression and highly aggressive tumors [14], which points to the existence of a broad range of oncogenic determinants. Expression of  $\Delta$ Np73 correlates with poor overall and decreased disease-free survival in neuroblastoma patients independent of age, site of the primary tumor, stage and MYCN amplification [15]. Our data confirm the relevance of  $\Delta$ Np73 as a survival factor in neuroblastoma and shed new light on its biological function and implication in apoptosis resistance.

## Acknowledgements

We thank Dr. Anne Gret Bittermann (EMZ, University of Zurich) for assistance in fluorescence microscopy. This work was supported by the Stiftung für angewandte Krebsforschung Zürich.

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