



# Human $\Delta$ Np73 regulates a dominant negative feedback loop for TAp73 and p53

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## Abstract

Inactivation of the tumour suppressor p53 is the most common defect in cancer cells. p53 is a sequence specific transcription factor that is activated in response to various forms of genotoxic stress to induce cell cycle arrest and apoptosis. Induction of p53 is subjected to complex and strict control through several pathways, as it will often determine cellular fate. The p73 protein shares strong structural and functional similarities with p53 such as the potential to activate p53 responsive genes and the ability to induce apoptosis. In addition to alternative splicing at the carboxyl terminus which yields several p73 isoforms, a p73 variant lacking the N-terminal transactivation domain ( $\Delta$ Np73) was described in mice. In this study, we report the cloning and characterisation of the human  $\Delta$ Np73 isoforms, their regulation by p53 and their possible role in carcinogenesis. As in mice, human  $\Delta$ Np73 lacks the transactivation domain and starts with an alternative exon (exon 3'). Its expression is driven by a second promoter located in a genomic region upstream of this exon, supporting the idea of two independently regulated proteins, derived from the same gene. As anticipated,  $\Delta$ Np73 is capable of regulating TAp73 and p53 function since it is able to block their transactivation activity and their ability to induce apoptosis. Interestingly, expression of the  $\Delta$ Np73 is strongly up-regulated by the TA isoforms and by p53, thus creating a feedback loop that tightly regulates the function of TAp73 and more importantly of p53. The regulation of  $\Delta$ Np73 is exerted through a p53 responsive element located on the  $\Delta$ N promoter. Expression of  $\Delta$ Np73 not only regulates the function of p53 and TAp73 but also shuts off its own expression, once again finely regulating the whole system. Our data also suggest that increased expression of  $\Delta$ Np73, functionally inactivating p53, could be involved in tumorigenesis. An extensive analysis of

the expression pattern of  $\Delta$ Np73 in primary tumours would clarify this issue. *Cell Death and Differentiation* (2001) 8, 1213–1223.

**Keywords:** p73;  $\Delta$ Np73; p53; apoptosis; DNA damage

**Abbreviations:**  $\Delta$ N, amino-terminus deleted; TA, transactivation; DBD, DNA-binding domain; OD, oligomerisation domain; DMEM, Dulbecco's Modified essential Medium; PI, propidium iodide; GFP, green fluorescent protein

## Introduction

Physical or functional inactivation of the tumour suppressor p53 is the most common defect in cancer cells.<sup>1,2</sup> p53 is a sequence specific transcription factor that is activated in response to various forms of genotoxic stress to induce cell cycle arrest and apoptosis. p53 executes its function inducing genes responsible for cell cycle regulation like p21 or genes promoting apoptosis, e.g. PUMA, noxa and bax.<sup>3–8</sup> The steady state protein levels of p53 and thus its function are subjected to complex and strict control through several pathways in order to determine the fate of the cell.<sup>9</sup> For example, p53 induces MDM2 which in turn promotes the ubiquitin dependent degradation of p53, generating an important feedback loop that controls p53 action.<sup>9</sup>

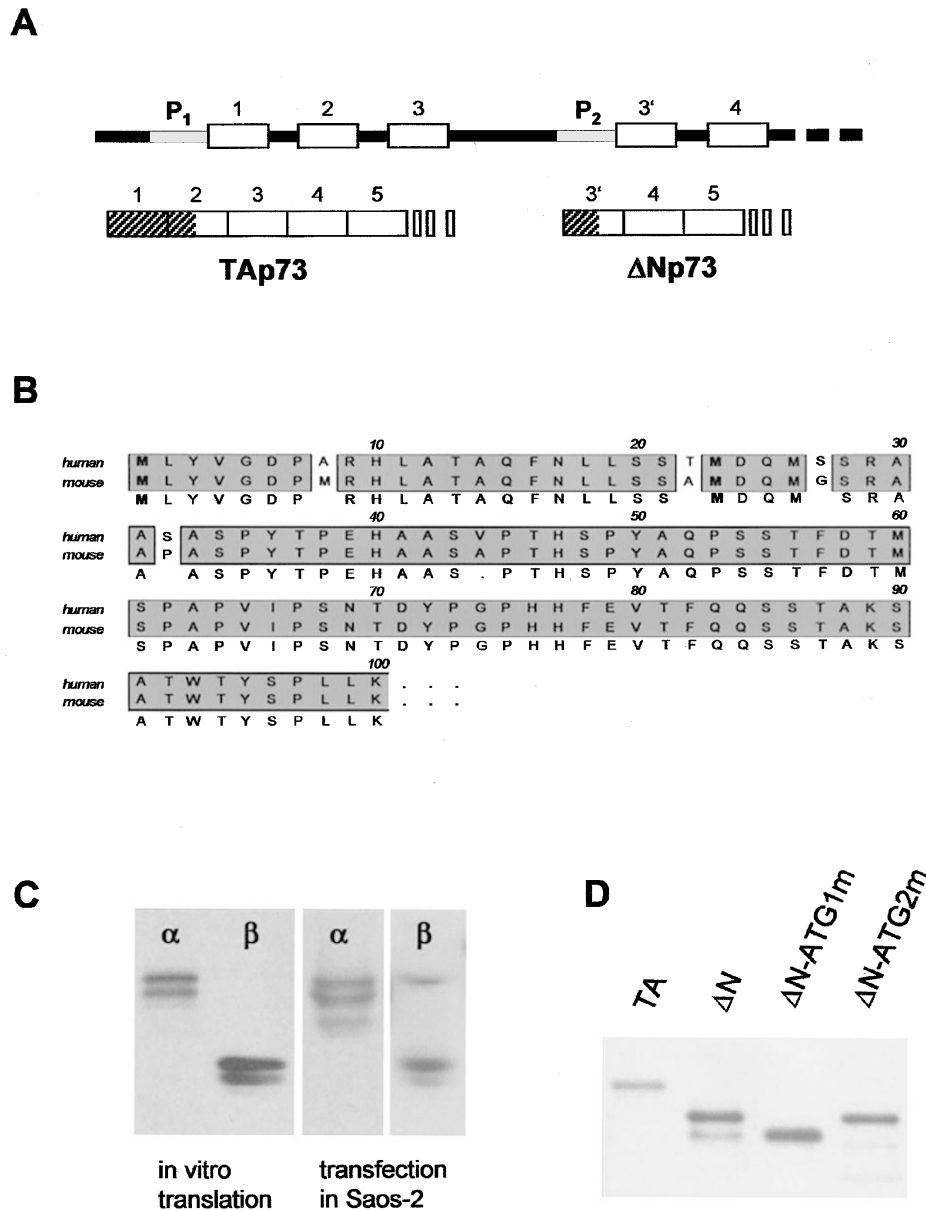
The p73 gene was discovered as the first homologue of the tumour suppressor p53.<sup>10</sup> The two proteins work as transcription factors and share significant structural similarity, being formed by three highly homologous domains, the N-terminal transactivation (TA) domain, a DNA-binding domain (DBD), and an oligomerisation domain (OD). These structural homologies lead the way for the search of functional similarities; indeed, p73 shows many p53-like features.<sup>11–16</sup>

Despite remarkable structural similarities of the genes, the knockout mice for p53, p63 and p73 display no obvious overlapping features.<sup>11</sup> In particular, p73<sup>-/-</sup> mice show abnormalities in fluid dynamics of the nervous and respiratory systems, defective neurogenesis and abnormal reproductive and social behaviour indicating a role for p73 in the development of the nervous and immune systems.<sup>17</sup> Conversely, several lines of evidence suggest its involvement in cancer. Exogenous expression of p73, similarly to p53, induces irreversible cell cycle and growth arrest and promotes apoptosis.<sup>10,18–25</sup> Although p73 is not transcriptionally regulated by DNA damage, p73 protein is stabilised by phosphorylation through the c-Abl tyrosine kinase pathway in response to DNA damage.<sup>26–28</sup> These data support the existence of a rescue pathway mediated by MLH1/c-Abl/p73 which triggers apoptosis following DNA damage, independent from p53. However, unlike p53, and despite its homology to the tumour suppressor gene p53,

p73 mutations are extremely rare in human cancers,<sup>13,29–31</sup> and in contrast to p53 knockouts, p73 knockout mice do not develop spontaneous tumours.<sup>17</sup> Still, several reports suggest that an altered expression of this gene rather than its mutation might be involved in cancer.<sup>13,16,17,32–41</sup>

In contrast to p53, p73 is expressed as several distinct forms differing either at the C- or at the N-terminus. Indeed, differential splicing of the 3' end of the gene leads to the

expression of several p73 C-terminal splice variants ( $\alpha$  to  $\zeta$ )<sup>10,18,42,43</sup> that differ *in vitro* in their potential to activate p53-responsive genes, such as p21<sup>Waf1/Cip1</sup> and BAX, thus showing different functional properties. In addition, in mice two variants with a different N-terminus exist, thought to derive from the usage of two different promoters one located upstream of exon 1 and one located upstream of exon 3.<sup>17</sup> Consequently, full length isoforms contain the



**Figure 1** ΔNp73 gene structure and amino acid sequence. (A) Schematic representation of the 5' end of the p73 gene giving rise to TA and ΔN variants. Distances are not proportional to genomic distances. White boxes represent exons (numbers are indicated above in arabic), dark grey shading represents 5' untranslated regions. The light grey shading indicates the two promoter regions: P<sub>1</sub> (coding for TAp73) and P<sub>2</sub> (coding for ΔNp73). (B) Alignment of mouse and human ΔN amino-termini, only four aminoacids differ in this region between the two sequences, one of which is encoded by exon 3'. Initial methionines are in bold. (C) Western blot of *in vitro* translated (left) and over-expressed (right) ΔNp73 $\alpha$  and ΔNp73 $\beta$  proteins, showing that two different forms derived from two different ATGs exist. Thirty  $\mu$ g of protein extract were separated and blotted and revealed with a anti p73 antibody, as indicated in the experimental section. (D) Western blot analysis of *in vitro* translated proteins showing that the double band observed in ΔN expressing cells is due to the use of different ATGs. (TA) TAp73 $\alpha$ ; (ΔN) ΔNp73 $\alpha$ ; (ΔN-ATG1m) ΔNp73 $\alpha$  carrying an a235c mutation removing the first ATG; (ΔN-ATG2m) ΔNp73 $\alpha$  carrying an a301c mutation removing the second ATG

transactivation domain (TA forms) and shorter or  $\Delta$ N isoforms lack this domain introducing additional complexity to the system. The  $\Delta$ Np73 variants do not activate transcription from p53-responsive promoters and inhibit the full length p73 variant (TAp73)<sup>17</sup> acting as dominant negatives. Ectopic expression of these variants in mice was shown to inhibit p53-induced apoptosis and to protect p73<sup>-/-</sup> neurons from death induced by nerve growth factor (NGF) withdrawal.<sup>44</sup> The dominant negative effect is thought to be mediated either by competition through its DBD and/or by hetero-oligomerisation and sequestration through its OD. The differential expression of transcriptionally active (TA) and inactive ( $\Delta$ N) p73 variants may determine p73 and p53 function within a particular cell type or in a particular phase of cell cycle or differentiation stage. The balance between the two forms might be finely regulated at the transcriptional level via alternative promoter usage. Alteration of the relative amounts of the two isoforms might be extremely important for its function (e.g. its involvement in development and possibly in carcinogenesis). Since the two forms have distinct (if not opposite) functions, it is important to identify them in humans, to clarify their normal expression pattern and functions and to clarify their differential regulation.

Here we describe the cloning and characterisation of the human  $\Delta$ Np73 variants, and of the second promoter region located in intron 3'. Our results show that p53 and TAp73 regulate  $\Delta$ Np73 protein levels by binding to the  $\Delta$ Np73 promoter and inducing its transcription, thus generating a negative feed-back loop that tightly regulates the activity of either TAp73 or p53.  $\Delta$ Np73 in turn blocks p53 and TAp73 action on its own promoter, further refining the regulation of the whole system. In addition our data suggest that an altered expression of the  $\Delta$ Np73 isoforms might be oncogenic by creating a functional inactivation of p53.

## Results

### Cloning of human $\Delta$ Np73 isoforms

The p73 gene shows a number of different splice variants clustered at the 3' end of the gene that result in a number of different C-termini. At least in mice, p73 also shows two different N-termini, one containing (TA forms) and one lacking ( $\Delta$ N forms) the transactivation domain.<sup>17,44</sup> The latter derives from the usage of a different ATG, located in an additional exon (exon 3'), driven by a second promoter (Figure 1A).

In order to clone the human homologues of the mice  $\Delta$ Np73 isoforms we performed a BLAST search in the genebank database using a sequence from mice exon 3' (y19235). This search allowed us to identify a genomic clone (AL136528) containing the entire human p73 gene. We were therefore able to design forward primers within human exon 3' and amplify the entire coding sequence of  $\Delta$ Np73  $\alpha$ ,  $\beta$  and  $\gamma$  [ $\Delta$ Np73 $\alpha$  (AY040827);  $\Delta$ Np73 $\beta$  (AY040828)  $\Delta$ Np73 $\gamma$  (AY040829)]. The human  $\Delta$ Np73 isoforms are highly homologous to the mouse  $\Delta$ Np73 isoforms (Figure 1B). Interestingly, the sequence of exon 3' contains two different in frame ATGs and translation can start with either one (Figure 1B). The existence of two

**Table 1** mRNA expression of TAp73 and Np73 determined by real-time quantitative RT-PCR

Normal tissues	TA/7s	$\Delta$ N/7s	TA/ $\Delta$ N
Adult skeletal muscle	$9.48 \times 10^{-7}$	$8.67 \times 10^{-8}$	10.9
Adult breast	$1.06 \times 10^{-5}$	$4.25 \times 10^{-6}$	2.5
Adult ovary	$8.18 \times 10^{-6}$	$6.29 \times 10^{-7}$	13.0
Adult kidney	$1.22 \times 10^{-6}$	$1.05 \times 10^{-7}$	11.6
Adult colon	$2.21 \times 10^{-6}$	$1.39 \times 10^{-7}$	15.9
Adult stomach	$1.77 \times 10^{-6}$	$4.13 \times 10^{-8}$	42.9
Adult liver	$4.35 \times 10^{-6}$	$2.61 \times 10^{-7}$	16.6
Adult lung	$8.76 \times 10^{-6}$	$6.01 \times 10^{-7}$	14.6
Fetal liver	$2.28 \times 10^{-6}$	$1.71 \times 10^{-7}$	13.3
Fetal lung	$5.00 \times 10^{-5}$	$4.42 \times 10^{-6}$	11.3
Fetal brain	$2.70 \times 10^{-6}$	$9.15 \times 10^{-7}$	2.9
<b>Cell lines</b>			
EPI	$1.63 \times 10^{-6}$	$3.47 \times 10^{-8}$	47.1
HepG2	$5.65 \times 10^{-5}$	$6.10 \times 10^{-7}$	92.5
LAN5	$5.93 \times 10^{-5}$	$2.26 \times 10^{-6}$	26.3
SK-N-BE	$1.44 \times 10^{-7}$	$1.68 \times 10^{-8}$	8.6
SH-Sy5y	$1.47 \times 10^{-5}$	$3.31 \times 10^{-7}$	44.5
SK-N-SH	$4.09 \times 10^{-5}$	$1.37 \times 10^{-6}$	30.0
SK-N-AS	$2.04 \times 10^{-8}$	ND	-
WI-38	$1.93 \times 10^{-7}$	$2.03 \times 10^{-8}$	9.5
HaCat	$2.49 \times 10^{-6}$	$1.05 \times 10^{-6}$	2.4
A2780	$3.35 \times 10^{-5}$	$8.10 \times 10^{-7}$	41.3
OVCAR3	$3.78 \times 10^{-5}$	$2.15 \times 10^{-6}$	17.6
Saos-2	$9.03 \times 10^{-7}$	$1.72 \times 10^{-8}$	52.4
Hela	$3.47 \times 10^{-7}$	$6.06 \times 10^{-8}$	5.7
MCF-7	$1.77 \times 10^{-5}$	$1.43 \times 10^{-7}$	123.8
Calu-1	$5.02 \times 10^{-6}$	$5.24 \times 10^{-8}$	95.8
A549	$6.77 \times 10^{-7}$	$2.87 \times 10^{-8}$	23.6
K562	$2.94 \times 10^{-5}$	$6.33 \times 10^{-7}$	46.4
HI60	$4.84 \times 10^{-7}$	$4.34 \times 10^{-8}$	11.2
JVM-2	$3.37 \times 10^{-4}$	$4.07 \times 10^{-6}$	82.8
Jurkat	$1.33 \times 10^{-9}$	$6.30 \times 10^{-9}$	0.2
Kasumi-1	ND	ND	-

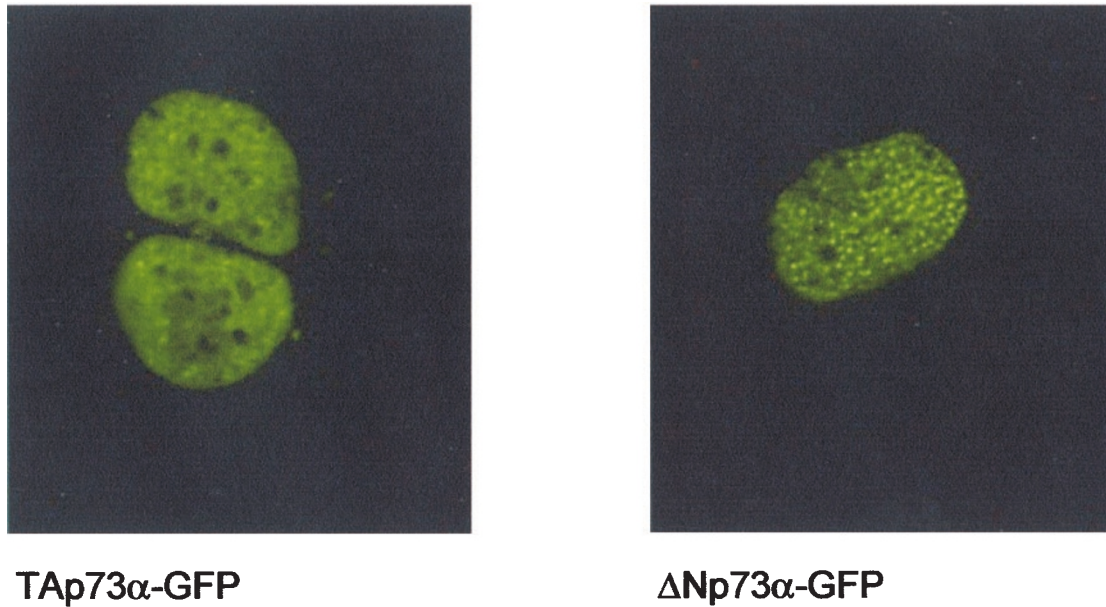
Values are expressed as a ratio between the number of transcripts measured for TAp73 or  $\Delta$ Np73 and 7s ribosomal RNA in 25 ng of total RNA. The same RNA sample is used for all three amplification reactions (TA,  $\Delta$ N and 7s) by real-time RT-PCR. The table reports the mean of the results obtained over three distinct measurements. The standard mean error was always within 10% of the measured value. ND indicates cases where no amplification of the gene was obtained

different translation start sites is confirmed by *in vitro* translation of a  $\Delta$ Np73 construct (Figure 1C) and by Western blot analysis of over-expressed (Figure 1C) and endogenous p73 (Figure 2B). In addition we confirmed the use of both ATGs by *in vitro* translating  $\Delta$ Np73 cDNAs in which either one of the two ATGs was mutated, showing that only one protein band was present (Figure 1D).

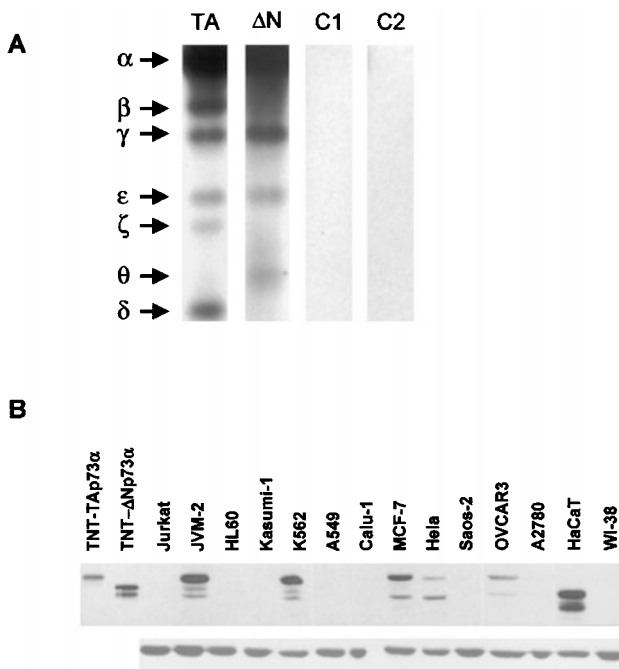
Fluorescence microscopy of cells transfected with TAp73 $\alpha$  and  $\Delta$ Np73 $\alpha$  show that both forms are localised in the nucleus (Figure 2).

### Expression pattern of human $\Delta$ Np73 isoforms

RT-PCR of the entire open reading frame of p73 using forward primers specific for either TA or  $\Delta$ N, followed by a nested PCR spanning exons from 8 to 14, shows that mRNA for all C-terminus variants exists as both TA and  $\Delta$ N variants (Figure 3A). Western blot analysis with an antibody directed against the C-terminus of p73 $\alpha$ , that recognises only the  $\alpha$  isoforms, shows that  $\Delta$ N and TAp73 proteins can be detected



**Figure 2** Fluorescence microscopy examination of Saos-2 cells transfected with TAp73 $\alpha$ -GFP and  $\Delta$ Np73 $\alpha$ -GFP plasmids showing that both forms are expressed in the nucleus. Cells were analysed at 490 nm with a CDD camera



**Figure 3** Expression of the different p73 isoforms. (A) RT-PCR demonstrating the existence of all different C-termini isoforms with both TA and  $\Delta$ N aminotermini. RT-PCR products of the open reading frames of both TA and  $\Delta$ N forms, obtained using specific forward primers, were re-amplified using nested primers spanning from exon 8 to exon 14, common to both p73 variants, then blotted and detected with a probe spanning from exon 8 to 10. Controls omitting the first amplification (C1) or omitting RNA in the amplification mix (C2) are shown. A representative experiment of three performed is shown. (B) Western blot of protein extracts from different cell lines, TNT-TAp73 $\alpha$  and TNT- $\Delta$ Np73 $\alpha$  indicate *in vitro* translated TA and  $\Delta$ N p73 $\alpha$  respectively. Bottom lane represents actin control. Thirty  $\mu$ g of proteins extract from each indicated cell line was separated electrophoretically, blotted and revealed with an anti-p73 antibody as described in Materials and Methods. A representative experiment of three performed is shown

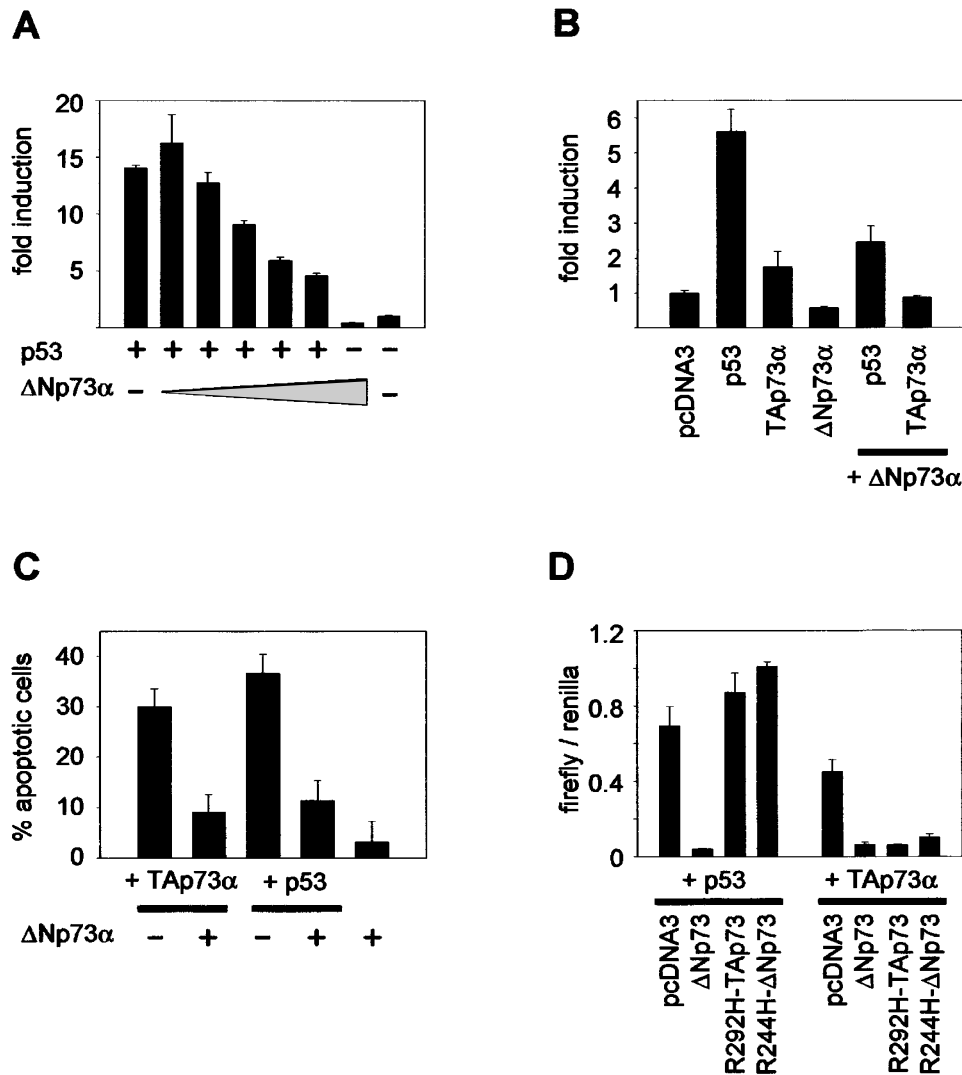
only in a small subset of different cell lines tested and that with the exception of HaCaT cells, the TA isoforms were the most represented (Figure 3B).

Since it has been previously reported by us and others<sup>10,33</sup> that in most tissues and cell lines p73 is expressed at very low levels, we developed a very sensitive quantitative real time RT-PCR to evaluate the expression levels of TA and  $\Delta$ N isoforms in different tissues and cell lines. Our results summarised in Table 1 show that differently from what was previously reported for mouse p73, in human tissues and cell lines TA isoforms are the most represented. Our results also suggest that foetal tissues express 10-fold more p73 (both TA and  $\Delta$ N) than the corresponding adult tissues, underlining its important role in development. Interestingly, breast and ovary show the highest expression levels in adult tissues. In all cases TA isoforms are more expressed than  $\Delta$ N in the same sample.

The expression levels of the  $\Delta$ N and TA isoforms detected by PCR do not always correspond to those measured by Western (this is particularly evident for HeLa and HaCaT cells), suggesting that  $\Delta$ N proteins might possibly have a longer half life than the corresponding TA forms.

### $\Delta$ Np73 isoforms function as dominant negatives

Mouse p73 and p63  $\Delta$ N isoforms have been shown to act as dominant negatives,<sup>11,17,44</sup> thus regulating the activity of the full length family members. In order to show that the human  $\Delta$ N isoforms we have cloned are also functioning as dominant negatives on p73 and on its homologue p53, we cloned the different  $\Delta$ N isoforms into a mammalian expression vector (pCDNA-3) under the control of the CMV promoter and performed cotransfection experiments.



**Figure 4** Characterisation of  $\Delta$ N isoforms function. (A) Luciferase assay with Saos-2 cells transfected with 900 ng of p21-luc, 20 ng of p53 and increasing concentrations (from 30 to 180 ng) of  $\Delta$ Np73 $\alpha$ . The histogram reports the results of three experiments performed. Results are expressed as fold induction over the reporter alone. (B) Luciferase assay with Saos-2 cells transfected with 1  $\mu$ g of p21-luc, and 40 ng of p53, or TAp73 $\alpha$  alone or in combination with 360 ng of  $\Delta$ Np73 $\alpha$ . The histogram reports the results of five experiments performed. Results are expressed as fold induction over the reporter alone. (C) Evaluation of hypodiploid apoptotic events after PI staining of Saos-2 cells transfected with 200 ng of p53, or TAp73 $\alpha$  alone or in combination with 200 ng of  $\Delta$ Np73 $\alpha$ . The histogram reports the results of five distinct experiments performed. (D) Luciferase assay with Saos-2 cells cotransfected with 800 ng of a p53 responsive promoter with 20 ng of either p53 or TAp73 $\alpha$  expressing plasmids together with 180 ng of either wild-type  $\Delta$ Np73 $\alpha$  ( $\Delta$ N) or DNA binding domain mutants of  $\Delta$ Np73 $\alpha$  (R244H- $\Delta$ Np73) or of TAp73 $\alpha$  (R292H-TAp73) or the empty vector (pcDNA3). The histograms reports the results of three experiments, expressed as fold induction over the reporter alone

As shown in Figure 4,  $\Delta$ Np73 is capable of blocking the ability of either p73 or p53 to transactivate the p21 promoter in a dose-dependent manner (Figure 4A,B). Experiments were also performed with plasmids expressing  $\Delta$ N isoforms starting with either of the two ATGs, and similar results were obtained, showing that both forms were able to block p53 and TAp73 induced transcription at comparable levels (data not shown).

We also investigated the ability of  $\Delta$ Np73 to interfere with a highly important cellular function of p53, namely the ability to induce apoptosis. In fact,  $\Delta$ Np73 is able to significantly reduce the apoptosis induced by over-expression of TAp73 or p53 when co-transfected into Saos-2 cells (Figure 4C).

The data reported very clearly indicate that  $\Delta$ Np73 is able to act as a natural dominant negative regulator of p73 and p53.

In order to rule out whether  $\Delta$ N isoforms block p53 and TAp73 by competing with the binding to DNA or by forming inactive complexes with p53 and TAp73 we produced mutants of the DNA binding domain of both TA and  $\Delta$ Np73 isoforms. We mutated the same arginine residue in the DNA binding domain into a histidine (R292H for TA; R244H for  $\Delta$ N). This mutation is known to determine loss of DNA binding activity of TAp73 and p53, transforming them into dominant negatives that inhibit the corresponding wild-type forms.<sup>21</sup> Our results show that while both R292H-TAp73

**A**

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-253 gccctcatgcctgggaacagaggtcgtttacggggtgagggcctggggcc
-202 ccccgagccttcccagggcaggcagcatctcgggaaggagcctgggtgggtt
-151 taattatggagccggcgtgaccggcgtcccgcctcccacagcagcctc
-100 ctgtgtcgggtccaacacatcaccgggcaagctgagccctgcccggactt
-49 ggatgaatactcatgagaaataaagggggtgggcccgggttttgtgttgg
3 ATTCAGCCAGTTGACAGAACTAAGGGAGATGGGAAAAGCGAAAATGCCAAC
54 AAACGGCCCGCATGTTCCCAGCATCCTCGGCTCTGCCTCACTAGCTGCG
105 GAGCCTCTCCCCTCGGTCCACGCTGCCGGGGCCACGACCGTGACCCCTT
156 CCCTCGGGCCGCCAGATCCATGCCTCGTCCACGGGACACCACTTCCTT
207 GCGGTGTGCAGACCCCCGGCCCTACC ATG CTG TAC GTC GGT
Met Leu Tyr Val Gly

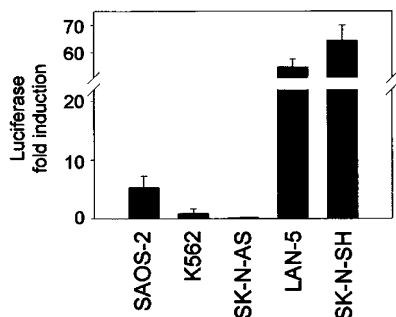
250 GAC CCC GCA CGG CAC CTC GCC ACG GCC CAG TTC AAT CTG
Asp Pro Ala Arg His Leu Ala Thr Ala Gln Phe Asn Leu

289 CTG AGC AGC ACC ATG GAC CAG ATG AGC AGC CGC GCG GCC
Leu Ser Ser Thr Met Asp Gln Met Ser Ser Arg Ala Ala

328 TCG GCC AGC CCC TAC ACC CCA GAG CAC GCC GCC AGC GTG
Ser Ala Ser Pro Tyr Thr Pro Glu His Ala Ala Ser Val

367 CCC ACC CAC TCG CCC TAC GCA CAA.....
Pro Thr His Ser Pro Tyr Ala Gln.....
    
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**B**



**Figure 5** ΔN promoter structure and characterisation. (A) Partial sequence of the ΔN promoter region, transcribed sequence is capitalised, the first transcribed nucleotide is numbered as +1. The box indicates the putative TATA-box. The two initiation codons are in bold. p53 consensus sequence is underlined. (B) Luciferase assay of cell lines expressing different amounts of ΔNp73 mRNA show comparable induction of the ΔN promoter: low expressing Saos-2, K562 (left) and high expressing LAN-5 and SK-N-SH (right) cells transfected with a vector containing a luciferase reporter gene (pGL3basic) under the control of a 2kb fragment of the ΔN promoter (construct A) are shown. The histogram reports the results of five experiments performed expressed as fold induction over cells transfected with the pGL3basic vector alone. Transfection of SK-N-AS cells that have undetectable levels of ΔNp73 are also shown and no induction of the promoter is observed

and R244H-ΔNp73 are still capable of inhibiting Tap73 transcription they both lose the ability to interfere with p53 activity (Figure 4D). This result strongly suggests that p53 inhibition is achieved through the competition for the binding to specific responsive elements while p73 is blocked also through a hetero-oligomerisation between the TA and the ΔN forms.

**Cloning and characterisation of ΔNp73 promoter**

To confirm that the transcription of ΔNp73 isoforms is driven by a different promoter located upstream of exon 3', we first determined the beginning of the messenger RNA by 5' race and RNA protection assay (data not shown) and then cloned a genomic fragment upstream of the transcription start site by PCR using primers designed on the genomic sequence previously described (AL136528).

**Table 2** Real-time RT-PCR experiments specific for Tap73 and ΔNp73. Transient transfection of p53, Tap73α or Tap73β in Saos-2 cells were performed by electroporation. p73 transcript numbers were normalised using 7s ribosomal RNA as a house keeping gene. Fold induction was calculated using the normalised transcript numbers

	Induction of ΔNp73 transcripts			
	Transcripts		Fold induction	
Transfected with:	TAp73/7s	ΔNp73/7s	TAp73	ΔNp73
pcDNA3	$3.45 \times 10^{-5}$	$6.19 \times 10^{-7}$	1.0	1.0
p53	$8.82 \times 10^{-4}$	$2.24 \times 10^{-3}$	25.6	3620.0
TAp73α		$1.35 \times 10^{-3}$		2177.8
TAp73β		$3.57 \times 10^{-3}$		5763.5

As shown in Figure 5A the messenger RNA contains 235 nucleotides of 5' untranslated RNA which is not interrupted by introns. A TATA box is in position -25.

We cloned an approximately 2 kb fragment (from nucleotide 43580 to nucleotide 45728 of sequence, AL136528) of the 5' flanking sequence into the pGL3-basic vector upstream of the luciferase gene (construct A), and tested its ability to drive transcription of the luciferase gene. This fragment drives the expression of the luciferase gene when transfected into cell lines expressing high levels of ΔN but not in those that are negative or low expressing. Figure 5B shows the experiments performed in five representative cell lines expressing different levels of ΔNp73.

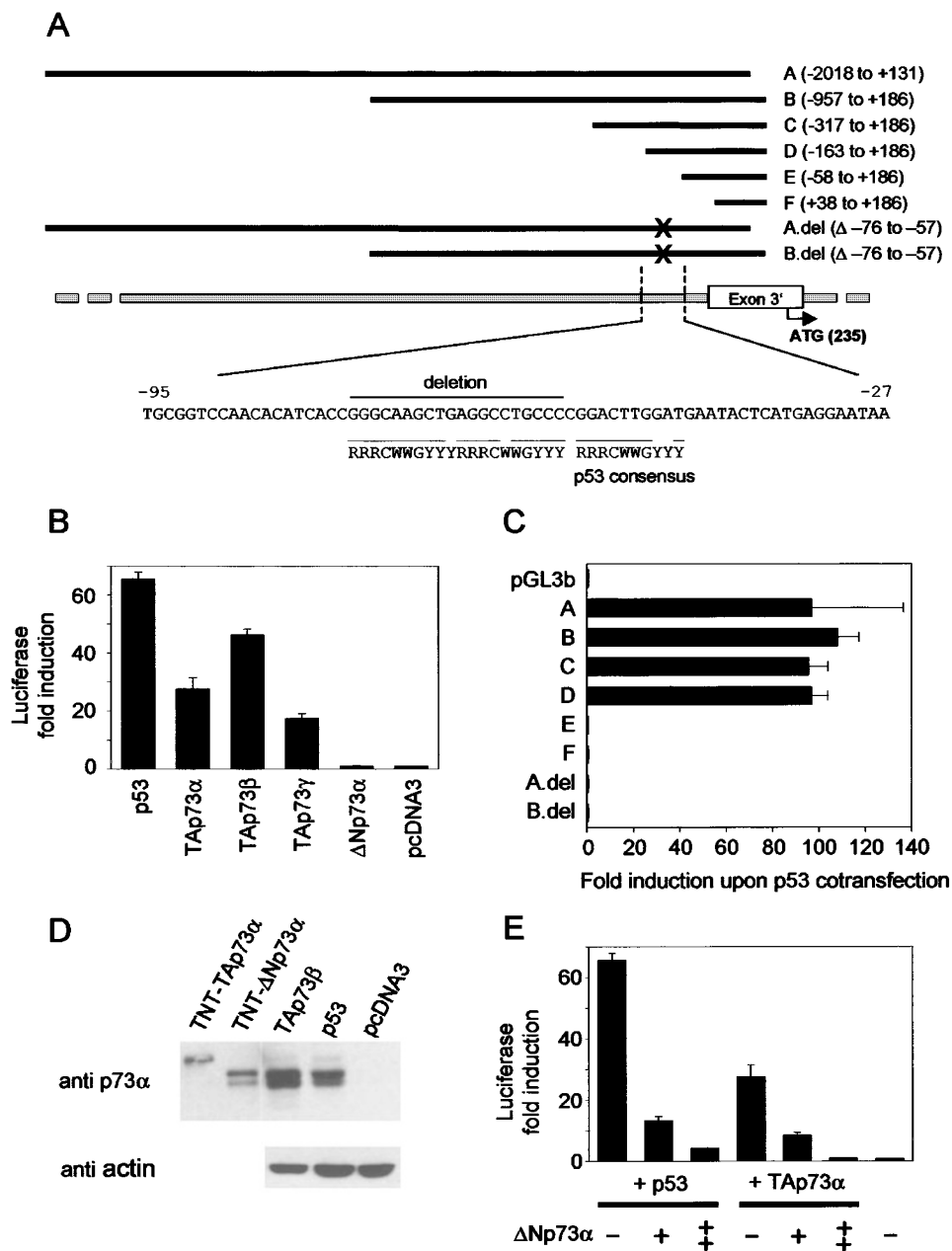
**p53 and Tap73 regulate ΔNp73 expression**

Sequence analysis of ΔNp73 promoter region revealed the presence of a single p53 responsive element, which consists of three half-binding sites, located at position -47/-78 from the cap-site (Figure 6A). This suggested that expression of the ΔNp73 isoforms could be regulated by p53 and by Tap73s.

In order to study whether p53 and Tap73s act directly on ΔNp73 promoter we performed luciferase assays co-transfecting the ΔNp73 promoter together with p53 and Tap73s. Figure 6B shows that ΔNp73 promoter is strongly induced by both p53 and Tap73 α, β and γ.

To confirm that p53 and Tap73 directly induce ΔNp73 acting on the p53 responsive element identified at position -47/-78, we generated progressive truncations of the ΔNp73 promoter and a deletion mutant lacking the p53 responsive element (Figure 6A). Luciferase constructs containing these truncated promoters were tested for the ability to respond to p53. Constructs containing more than 150 bases upstream of the TATA-box showed strong luciferase activity by p53 cotransfection (constructs A-D) while shorter constructs lacking the p53 responsive element were no longer inducible (constructs E and F) (Figure 6C). Similarly deletion of two of the p53 half-binding sites (constructs A.del and B.del) abrogated the induction by p53 completely demonstrating the existence of a single active p53 binding site (Figure 6C).

In order to confirm that p53 and Tap73s were able to regulate ΔNp73 we studied the expression levels of ΔNp73 after transfection of SAOS-2 cells with p53 or Tap73α and



**Figure 6** p53 induction of ΔNp73 promoter. **(A)** Schematic representation of genomic region upstream of exon 3' (open box) containing the ΔNp73 promoter. The sequence from bp-27 to bp-95 containing the p53 responsive element is shown. A to F progressive truncations of the ΔN promoter used for luciferase assays, the numbers in brackets indicate the nucleotides of origin and end of the fragments numbered from the cap site. A.del and B.del constructs contain a 20 base pair deletion. Deleted nucleotides are indicated. **(B)** Luciferase assay of Saos-2 cells co-transfected with ΔNp73 promoter construct (construct B) and p53 or TAp73  $\alpha$ ,  $\beta$ , and  $\gamma$  or the empty vector (pcDNA3). Values are expressed as fold induction over control transfected cells. Values are a mean of three experiments. **(C)** Analysis of deletion mutants of ΔNp73 promoter (A to F and A.del and B.del) co-transfected with a p53 expressing plasmid by luciferase assay. Values are expressed in fold over control cells, transfected with an empty vector (pcDNA3) and the corresponding reporter constructs (A-F, A.del, B.del). Values are a mean of three experiments. **(D)** Induction of ΔNp73 protein by p53. Western blot analysis of Saos-2 cells after transfection of p53 family members. An antibody specific for p73 $\alpha$  (detecting TA and ΔN forms) was used. Specific induction of ΔNp73 is shown by transfection of p53 and TAp73 $\beta$ . TAp73 $\alpha$  transfected cells are not shown because the over-expressed protein migrates very close to the ΔNp73 rendering detection impossible. *In vitro* transcribed and translated TAp73 $\alpha$  (TNT-TAp73 $\alpha$ ) and ΔNp73 $\alpha$  (TNT-ΔNp73 $\alpha$ ) proteins are shown. The two ΔNp73 bands result from the use of two different start codons. Western blot using an antibody detecting the HA-tag fused to the proteins was used as a transfection control. Equal loading is shown with an anti-actin antibody in the lower lane. **(E)** ΔNp73 blocks its own induction by p53 and TAp73. Luciferase assay of cells transfected with 800 ng of ΔNp73 promoter and 20 ng of plasmids expressing either p53 or TAp73 $\alpha$  expressing plasmids and increasing concentrations (from 50 to 180 ng) of ΔNp73 $\alpha$ . Results are expressed as fold over control cells transfected with ΔNp73 promoter and an empty vector (pcDNA3). Values are a mean of three experiments

$\beta$ . Transcript analysis by real time RT-PCR (Table 2) shows that p53 is capable of inducing both TA and ΔNp73 constructs, however while TA forms are induced 26-fold

ΔNp73 is induced up to 3620-fold. Similarly TAp73  $\alpha$  and  $\beta$  are capable of inducing ΔNp73,  $\beta$  having the strongest activity, up to 5763-fold. Figure 6D shows the protein levels

evaluation by Western blot analysis confirming the induction of ΔNp73 levels.

**ΔNp73 blocks its own induction by p53 and TAp73**

Since ΔNp73 is capable of counteracting the activity of p53 and p73 we investigated the possibility that ΔNp73 blocked its own induction. Co-transfection of p53 or TAp73 together with increasing concentrations of ΔNp73 and ΔN promoter construct shows that ΔNp73 is capable of blocking the action of p53 and TAp73 on its own promoter (Figure 6E). This creates an additional feedback loop that finely tunes the system.

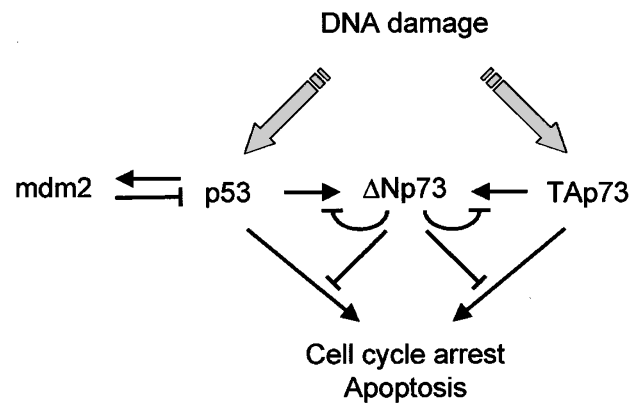
**Discussion**

The function of p73 has not been fully elucidated at molecular levels. Even though the data known so far are sufficient to indicate the importance of p73, its molecular regulations are just beginning to emerge. Not only are there physical and functional interactions within the members of the family (p53, p63, p73) and their isoforms in normal as well as in cancer-derived variants, but the existence of cross-reactive natural dominant negatives finely tune the cellular response.

Our results show that human ΔNp73 isoforms are expressed in different tissues and cell lines and that their expression is driven by a second promoter located in a genomic region upstream of exon 3'. At the functional level, not only do the two proteins have distinct actions, but ΔNp73 can act as a natural dominant negative regulator for both p53 and for TAp73 itself. In fact, ΔNp73 can block TAp73- and p53-dependent transcription and apoptosis. In contrast to what has been reported for mouse embryos, TA forms are always expressed 10–100-fold more than ΔN isoforms. In addition, most of the tumour cell lines tested showed an altered TA/ΔN ratio when compared to normal adult or foetal tissues, suggesting that the relative amounts of the two forms rather than the absolute amount is important for the normal cell function. This is understandable since ΔN isoforms exert a dominant negative effect on the TA forms. Therefore the final activity of the TA forms is tightly regulated by the amount of ΔN present in the cell. In addition, ΔN isoforms can interact with p53 thus regulating the function of this gene.

p53 is expressed in response to DNA damage and its induction results in cell cycle arrest and apoptosis, preventing multiplication of damaged cells. As for other killing genes, the levels and functions of p53 have to be carefully regulated in order to avoid wanton cell death. Intracellular p53 levels are therefore controlled by a balance of production and degradation. As a matter of fact, elevated p53 levels lead to the protein's own regulation by inducing the transcription of MDM2, that binds p53 and causes its ubiquitination and degradation in proteosomes.

Here we describe a novel way of controlling p53 function, without increasing its elimination. Our results show that p53 is capable of inducing ΔNp73, a protein that can block p53 action by competing for p53 DNA binding sites. p53 induces ΔNp73 by directly binding to its promoter. In addition ΔNp73



**Figure 7** Schematic representation of the p53 loop. DNA damaging agents induce p53 and p73 through distinct pathways. Levels and function of p53 and p73 are regulated by different pathways. MDM2 is induced by p53 controls its degradation. ΔNp73 is induced by both p53 and TAp73 and counteracts their action. ΔNp73 also controls its own induction by p53 and TAp73

counteracts p53 action on its own promoter creating an additional feedback loop that finely tunes the entire system (Figure 7). In addition, we show that a similar loop regulates TAp73 action, since ΔNp73 expression is also induced by TAp73, and ΔNp73 also regulates the function of the TA forms. The major implication of these results is that ΔNp73 plays an important role in regulating p53 function and that its over expression could play a role in tumorigenesis by generating a functional block of p53.

The complete absence of p73 could then be less damaging to most cells than the altered equilibrium of its isoforms, explaining the absence of increased number of tumours described in the p73-KO animals.

A very interesting observation arising from our data is the relatively high expression levels of p73 in cancer cell lines.<sup>45,46</sup> Indeed, it is worth noting that several cancers express a cancer-specific TA isoform of p73 with a specific deletion of exon 2 (Δ2-p73) that, because of the disruption of the TA domain, is functionally similar to ΔNp73.<sup>32,47</sup> It is possible that increased ΔN levels, and subsequent functional inactivation of p53, are involved in carcinogenesis. This possibility could be addressed by careful screening of a large number of primary tumour samples.

In conclusion we report here the cloning and characterisation of human ΔNp73 isoforms, the cloning and partial characterisation of the ΔN promoter, and the identification of a feedback loop that controls the function of p53 preventing unnecessary elimination of cells. Activation of the p53/TAp73-dependent death program would require the removal (ΔN downregulation) or the overcoming (p53 or TAp73 increased expression) of the ΔN block.

**Materials and Methods**

**Cell cultures and samples**

Cells were grown in Dulbecco's Modified essential Medium (DMEM) or RPMI-1640 supplemented with 10% v/v Foetal Bovine Serum, 1.2 g



bicarbonate per litre, 1% (v/v) non essential amino acids and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, at 37°C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub> in air. All chemicals and reagents were from Sigma (Buchs, Switzerland), unless specified. Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany) and from the American Type Culture Collection (Rockville, USA). The HaCaT cell line was a kind gift from N. Fusenig (Heidelberg, Germany). The panel of normal RNA was obtained from Stratagene (Amsterdam, The Netherlands).

### RNA extraction and reverse transcription

Total RNA was extracted using the RNeasy kit (Qiagen, Basel, Switzerland). During the extraction procedure, contaminating DNA was removed by DNase I: 600 ng RNA was treated with 10 U of DNase I (Roche, Basel, Switzerland) in a total volume of 10 μl containing 50 mM NaAc pH 5.0, 5 mM MgCl<sub>2</sub> and 25 U of RNase Inhibitor (Roche) for 15 min at 37°C, 5 min at 90°C and immediate placement on ice. Two hundred ng of total RNA was reverse transcribed in a 20 μl reaction volume, using 16 nM pd(N)6 random primers, 19.2 U of avian myeloblastosis virus reverse transcriptase, 50 U RNase inhibitor and 1 × AMV-RT-buffer (all reagents from Roche). The incubation was at room temperature for 10 min, followed by 42°C for 60 min and 95°C for 5 min. To check for contaminating genomic DNA, RNA samples were processed identically as for cDNA synthesis except that the reverse transcriptase was replaced by the same volume of water.

### Real-time quantitative RT-PCR

Real-time PCR was used for absolute quantitation of p73 N-terminal variants, 7S RNA was used for internal standard. Each PCR was carried out in a total volume of 25 μl containing cDNA reverse-transcribed from 25 ng and 0.375 ng total RNA for p73 and 7S respectively. Dual labelled (FAM/TAMRA) gene specific probes and TaqMan Universal PCR Master Mix (Applied BioSystems, Rotkreuz, Switzerland) were used for the PCR. ΔNp73 was amplified using 5'-GGAGATGGGAAAAGCGAAAAT-3' as the forward primer, 5'-CTCTCCCGCTCGGTCCAC-3' as the reverse primer (both 300 μM), and a ΔNp73 probe 5'-CAAACGGCCCGCATGTTCCC-3' (150 μM). For TAp73, the primers were forward 5'-GCACCACGTTT-GAGCACCTC-3', reverse 5'-TAATGAGGTGGTGGGCGGA-3' (both 300 μM), and the probe was 5'-TTCGACCTCCCCAGTCAAGCCG-3' (150 μM). For 7S RNA the primers and probe were: forward 5'-ACCACCAGTTGCCTAAGGA-3', reverse 5'-CACGGAGTTTT-GACCTGCT-3', probe 5'-TGAACCGGCCAGGTGCGAAAC-3' (300 μM each). All measurements were performed twice, and the arithmetic mean was used for further calculations.

For the determination of absolute transcript number analysis, cDNA was amplified from JVM-2 cells (TAp73 F 5'-ACGCAGC-GAAACCGGGGCCCG-3', R 5'-GCCGCGCGGCTGCTCATCTGG-3', ΔNp73 F 5'-CCCGGACTTGGATGAATACT-3', R 5'-GCCGCGCGGCTGCTCATCTGG-3') and from a 7S-plasmid with F 5'-GCTACTCGG-GAGGCTGAGAC-3', R 5'-AGGCGCGATCCCACTACTGA-3'). The amplicons were cloned into the pcDNA3.1/V5-His vector as described and the constructs were verified by sequencing. After digestion with *Nsi*I (Roche), a T7-dependent RNA synthesis was performed with the RiboMAX™ Large Scale RNA Production System (Promega, Wallisellen, Switzerland) according to the manufacturer's protocol. The synthesised RNA was extracted with a 5'-biotinylated oligo (5'-TTTCCACACCTAACTGACA-3') and the mRNA Isolation Kit (Roche) and quantified spectrophotometrically. Molecular concentra-

tions were calculated and random-primed cDNA synthesis was performed with the purified RNA adjusted to 200 ng with yeast RNA. A series of dilutions was prepared and measured by real-time quantitative RT-PCR as described above.

### Determination of C-terminal p73 mRNA splice variants

One hundred ng of cDNA obtained as described above from three different cell lines (JVM-2, HaCaT, and MCF-7) were amplified by PCR with forward primers specific for TAp73 and ΔNp73 and a reverse primer common to both variants: TAp73 F 5'-AAGATGGCCAGTC-CACCGCCACCTCCCCT-3' (exon 2); ΔNp73 F 5'-ATGCTG-TACGTGGTGACCC-3' (exon 3); common reverse primer R 5'-TCAGTGGATCTCGGCCTCC-3' (exon 14). The Expand High Fidelity PCR System enzyme was used as described above, but with an annealing temperature of 61°C. 0.1 μl of the first PCR product was used as a template for a nested PCR using a forward primer in exon 8 and a reverse primer in exon 14,<sup>35,45</sup> 15 cycles were used for TAp73 and 18 cycles for ΔNp73. The detection by blotting and hybridisation was performed as previously described.<sup>35,45</sup>

### Western blot analysis

Protein extraction was performed as previously described.<sup>45</sup> Thirty μg of total cellular protein was size-fractionated on an 8% SDS-polyacrylamide gel and blotted onto nitrocellulose (Protran; Schleicher and Schuell, Dassel, Germany). Equal loading and transfer efficiency were assessed by ponceau staining. p73 antibodies (AB7824, Chemikon, or a polyclonal p73 antibody kindly provided by D. Caput, Labège, France) were used for the detection as previously described.<sup>46</sup> As standards for the Western blots, ΔNp73α/β, and TAp73α/β (the latter two subcloned from pcDNA3-HA plasmids<sup>18</sup> were synthesised *in vitro* by TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocol. To assess for protein quality, 30 μg of protein was fractionated and blotted as above, and detected with a rabbit polyclonal antibody against actin (A2066; Sigma).

### Cloning of the ΔNp73 expression plasmids

PCR was carried out with 100 ng of cDNA from JVM-2 in a 50 μl reaction with Expand High Fidelity PCR System enzyme mix (Roche, Basel, Switzerland) according to manufacturer's protocol. Amplification consisted of 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 59°C for 30 s, and 72°C for 1.5 min with a cycle elongation of 5 s for cycles 11–35, and a final elongation at 72°C for 7 min. The primers were as follows: 5'-ATGCTG-TACGTGGTGACCC-3' and 5'-TCAGTGGATCTCGGCCTCC-3' which allowed the amplification of different C-terminal splice variants. The PCR product was cloned into the pcDNA3.1/V5-His Vector (TA Cloning Kit<sup>®</sup>, Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol and ΔNp73α, ΔNp73β, and the ΔNp73γ variants were sequenced completely in both directions. Point mutations on the DBD (TAp73-R292H and ΔNp73-R244H) were done using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with mutagenic primers (GTCCTTTGAGGGCCA-CATCTGCGCCTGTC sense and antisense) according to manufacturer's protocol. The same method was used for mutations of the start codons. For ΔNp73-ATG1m primers were GAATTGCCCT-TCTGCTGTACGTGGTGA sense and antisense, for ΔNp73-

ATG2m primers were CTGAGCAGCACCCCTGGACCAGAT sense and antisense.

Subcloning in pEGFP vector (Clontech) was performed by PCR. PCR products of the open reading frames of TAp73 and ΔNp73 were ligated by T/A cloning as described<sup>48</sup> using *Sma*I restriction enzyme (Roche) and the Rapid DNA Ligation Kit (Roche).

### Cloning of the ΔNp73 promoter region

Amplification of the 5' upstream region of ΔNp73 was performed using primers: with F1, 5'-GCTGGCCTTGGGAACGTT-3' and R1, 5'-GGCAGCGTGGACCGAGCGG-3' (construct A) designed on the genomic sequence of clone AL136528, with High Fidelity Taq (Life Technologies). Amplification consisted of 1 cycle at 94°C for 3 min, followed by 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min, and final elongation at 72°C for 7 min on a GeneAmp PCR 9600 (Perkin Elmer, Rothrist, Switzerland). The fragment was first cloned into PCR2.1 Invitrogen, then digested with *Xho*-I *Hind*III and cloned into pGL3basic vector (Promega).

Deletion constructs were generated by PCR. Different forward primers (Construct B: GTTGAAGGAAAGGGGAAAG; Construct C: ACACCATCTCTCCCCTTG; Construct D: CCCTGGTGGGTTAAT-TATGG; Construct E: CCCGGACTTGGATGAATACT; Construct F: AAGCGAAAATGCCAACAAAC) and a common reverse primer (GACGAGGCATGGATCTGG) were used. Amplification was carried out with Taq DNA polymerase (Roche) using an annealing temperature of 55°C and 35 cycles. Ligation into the pGL3basic vector was performed by T/A cloning as described<sup>48</sup> using *Sma*I restriction enzyme (Roche) and the Rapid DNA Ligation Kit (Roche).

Deletion of the p53 binding site on the A and B construct (A.del and B.del respectively) were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) with mutagenic primers (CAACACATCACCCGACTTGGATGAAT sense and antisense) according to manufacturer's protocol.

### Fluorescent microscopy

Saos2 cells were transfected with TAp73 $\alpha$ -GFP and ΔNp73 $\alpha$ -GFP expression vectors with Lipofectamine 2000 (Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. 36 h after transfection, cells were fixed in 4% paraformaldehyde. Slides were analyzed with a fluorescence microscope (excitation wavelength 490 nm) and images were acquired with a CCD camera (Biorad).

### Dual luciferase-assay

Saos-2 cells (10<sup>5</sup>) were plated and transfected with Lipofectamine 2000 (Life Technologies, Basel, Switzerland) according to the manufacturer's protocol. Indicated amounts of a reporter plasmid containing firefly luciferase under control of the p21<sup>WAF1/Cip1</sup> promoter<sup>33</sup> or ΔN promoter described below, together with p53, TAp73<sup>33</sup> and ΔNp73 expression plasmids were used for transfection. A total of 2 μg of plasmids were transfected using pcDNA3 (Invitrogen) to adjust for equal amounts. In all cases 10 ng of a renilla luciferase expression plasmid (pRL-CMV, Promega) was co-transfected to normalise for transfection efficiency. All transfections were done in triplicates and the Dual-Luciferase reporter assay system (Promega) was carried out after 24 h from transfection according to the manufacturer's protocol.

### Analysis of apoptosis

To estimate DNA fragmentation, Saos-2 cells were plated to approximately 50% confluency and transfected, using Lipofectamin

2000 reagent (Life Technologies) according to the manufacturer's protocol, with either TAp73 $\alpha$  or p53<sup>14,18</sup> in combination with pcDNA3 or ΔNp73 $\alpha$ , together with a GFP-spectrin expression vector<sup>49</sup> at a 1 to 5 ratio. Cells were collected at 800 × *g* for 10 min and fixed with 1 : 1 PBS and methanol-acetone (4 : 1 v/v) solution at -20°C. Hypodiploid events and cell cycle of GFP positive cells was evaluated by flow cytometry using a propidium iodide (PI) staining (40 mg/ml) in the presence of 13 kU/ml ribonuclease A (20 min incubation at 37°C) on a FACS-Calibur flow cytometer (Becton-Dickinson, CA, USA). Cells were excited at 488 nm using a 15 mW Argon laser, and the fluorescence was monitored at 578 nm at a rate of 150–300 events/s. Ten thousand events were evaluated using the Cell Quest Program (ibid). Electronic gating FSC-a/vs/FSC-h was used, when appropriate, to eliminate cell aggregates.

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