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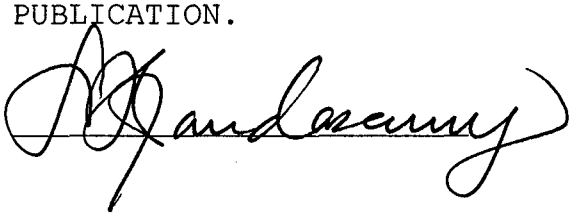

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Purpose & scope: The discovery of p73, a gene that in experimental conditions behaves like p53, requires us to determine what role it plays in breast cancer, whether a crosstalk exists between p73 and p53 actions and to delineate the differences and similarities between these two genes concerning their biological role and signaling pathways. Our understanding of p53's role in breast cancer has been made hazier again by the advent of p73's discovery. An additional challenge derives from the fact that the TP73 gene in principle can produce two diametruially opposed classes of protein products:full length forms and N-terminally truncated forms (that are missing the transactivation domain). Therefore, certain p73 isoforms could be dominant negative over p53 in heterotypic interactions. Conversely, it has already been shown that p73's transactivation and apoptotic function is inhibited by tumor-derived p53 mutants. This opens the possibility that the phenotype of mutant p53 tumor cells might in fact be due to an interference with normal p73 function. This scenario might explain why only 30 % of breast cancers have mutated their p53 gene. In this case, dominant negative p73 isoforms, when deregulated in breast cancer, could interfere with p53 and p73-mediated growth suppression. We had previously reported that about 40% of breast cancers overexpress p73, indicating its role in breast cancer tumorigenesis. A better insight into p73's function will add greatly to our understanding of its role in this disease. Major findings: A) We showed that certain relevant oncogenes signal to p73 *in vivo*. Endogenous full length (TA) p73 α and β proteins are upregulated and transcriptionally activated in p53-deficient tumor cells in response to deregulated oncogenes E2F1, c-MYC and E1A. Moreover, in the absence of p53, E2F1, c-MYC and E1A enlist p73 to induce apoptosis in tumor cells. B) Δ Functional studies indicate that ΔNp73 acts as a dominant negative inihibitor of both wild-type p53 and transactivation-component TA-p73.

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## Table of Contents

**Introduction:**

This grant was divided into two unrelated parts due to an approved change of scope for years 2-3. The first part deals with the risk of ATM heterozygosity for sporadic breast cancer, while the second deals with the role of the p53 homolog p73 in breast cancer.

**Body:**

**Part I: Risk for Sporadic Breast Cancer in Ataxia Telangiectasia Heterozygotes**

Scope: The scope was to assess whether heterozygosity for the ATM gene, due to a loss of function mutation in one of the 2 alleles and found in about 1% of the general population, confers a significant increase in breast cancer risk for women with sporadic breast cancer (without a family history of breast cancer). This is called the AT - carrier risk hypothesis for sporadic breast cancer.

The goals for the first 12 months were:

Aim I Genetic analysis of ATM in clinical samples.

IA) LOH mapping at 11q23.1 in sporadic breast carcinomas using intragenic and ATM flanking microsatellite markers (months 1-18).

Time Table

l LOH mapping at 11q23.1 in breast carcinoma/normal tissue matched pairs using intragenic and ATM flanking microsatellite markers (months 1-18)

l LOH mapping at 11q23.1 in DNA from normal controls using intragenic and ATM flanking microsatellite markers (months 1-18)

Reportable results in LOH mapping:
Using 6 polymorphic microsatellite markers in and around the ATM locus, we completed LOH analysis on 16 matched breast cancer/normal pairs with the following results:

| | |
|---|---|
| D11S2179 (intragenic ATM): | 4 of 16 (25%) |
| NS22 (intragenic ATM): | 3 of 16 (19%) |
| D11S1787 (centromeric): | 4 of 16 (25%) |
| D11S1778 (telomeric): | 6 of 16 (38%) |
| D11S1294 (telomeric): | 6 of 16 (38%) |
| D11S1818 (telomeric): | 4 of 16 (25%) |

Interpretation: Our results on frequencies of the ATM and flanking loci in breast cancer is similar to the ones reported in the literature.

In summary, our LOH results only confirmed the frequency data was already in the literature. Furthermore, the new mutational studies that appeared in the meantime did not show a significant mutational rate of the ATM gene. The latter is a strong but not absolute argument against a true suppressor role of the ATM gene in breast cancer. Rather than simply to continue LOH analysis on the originally planned 145 total cases, we decided to address the question from a different angle. We asked whether the expression status of ATM differed in breast cancers and breast cancer cell lines compared to normal breast tissue. If ATM has a suppressor role in breast cancer, a loss of wild type ATM expression rather than mutational inactivation could be expected.

With this rationale, we undertook a comprehensive ATM expression analysis using quantitative RT-PCR on 89 randomly selected breast cancer samples (from 3 different institutions), 7 breast cancer lines and 29 normal breast samples. Of these, 11 were matched normal/cancer pairs. Our working hypothesis was to find a decreased expression in cancer compared to normal breast tissue.

Results of ATM expression in breast cancer and normal breast tissues. Using a competitive semiquantitative RT-PCR approach, we determined relative ATM expression levels on 89 breast cancers and compared them to 29 normal breast samples. Eleven of these constituted matched tumor/cancer pairs. ATM and b2M transcripts were detectable in all breast tissues and the 7 breast cancer cell lines that we analyzed. While the expression of b2M was similar in all samples, ATM expression levels varied widely. Moreover, breast cancer tissues did not show a deficiency in ATM expression. In fact, cancers expressed mildly higher (1.5-fold) levels of ATM transcripts than normal breast tissues. However, due to the large variance in breast cancers and the relatively small difference between the geometric means of cancer versus normal tissue, the power to detect significant differences between the two groups was very low. The geometric mean of breast cancer was 0.484 +/- 2.5 standard deviation (Std.) compared to 0.329 +/- 0.30 Std. in normal breast tissue. In breast cancer, relative ATM expression ranged from 0.03 to 16.8 with a median of 0.57, and in normal breast it ranged from 0.093 to 1.31 with a median of 0.318. Examples of individual raw data are shown in Fig. 2. Repeat determinations from individual patients yielded reproducible results. Table II shows a subset of breast cancers and normal controls with their relative ATM expression levels, averaged from 2 independent measurements of the same sample. A mild tumor-associated increase in relative ATM transcript levels was also seen when the subgroup of matched pairs was analyzed separately. Seven of the 11 normal / cancer pairs showed a 1.2 to 2.3-fold increase in cancers compared to their adjacent normal tissue match, 3 cases were equal and only one case showed decreased (< 50%) ATM expression in the tumor. In line with the findings in primary cancers, breast cancer cell lines had even higher ATM expression with a geometric mean of 2.6 +/- 1.96 Std. and a range from 0.47 to 5.55.

We also performed a partial mutational analysis on two regions of the ATM gene (a middle region an dthe PI3 kinase region) on 8 cases of breast cancer with the highest ATM expression and found no mutations.

**Key Research Accomplishments:**

-Research Paper published (Kovalev S et al (2000) International Journal of Oncol 16: 825-831.

-Poster presentation at the Era of Hope Meeting, June 8-11, 2000 in Atlanta

**Reportable Outcome:**
1) This work was published: Kovalev S, Mateen A, Zaika AI, O'Hea BJ and UM Moll (2000) Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines. Int J Oncol 16: 825-831.

2) A repository of total RNA extracted from the 89 cases of breast cancer and 29 cases of normal breast tissue has been made an dwill be availabe for future molecular studies.

3) Based on the experience and training received from the work supported by this award, Dr. Sergey Kovalev obtained a faculty position in his native country at the University of Yekaterinburg, Russia. There, he is working in the field of molecular diagnosis of malignancies, including breast cancer.

**Conclusions:**
Although the ATM locus falls within a region of frequent LOH in breast and other human cancers, we did not find a reduction in ATM mRNA expression levels in our cohort of 89 sporadic breast cancers. Based on the available mutations data (mostly truncations leading to unstable protein), such a reduction would be expected at least in some cases if the ATM gene would play a causal role in breast cancer. Our study did not find evidence in support of the hypothesis that ATM is a tumor
suppressor gene causally involved in sporadic breast cancer. Our study agrees withseveral new studies in the literature which appeared since the proprosal was originally submitted. For reasons of higher relevance and pay-off, I requested a change of scope to the p73 project.

**References:** See reprint.
**Appendices:** Kovalev S, Mateen A, Zaika AI, O'Hea BJ and **UM Moll** (2000) Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines. *Int J Oncol* **16**: 825-831.

Part II: **In vivo role of the p73 gene in breast cancer**

The p73 gene is a structural and, in overexpression systems, functional p53 homolog. Ectopic p73 expression can activate a broad subset of p53-responsive genes, induce apoptosis, cell cycle arrest and growth suppression. Nevertheless, p73's role in tumorigenesis is unclear. Current genetic data exclude that p73 is a Knudson-type tumor suppressor. However, endogenous p73 is induced and activated for apoptosis by c-abl in response to cisplatin-induced DNA damage, suggesting some similarity to p53 in regulating checkpoint control pathways. Defining the upstream pathways that signal to p73 will be crucial for understanding its biological role.

In this study, we asked whether oncogenes can induce and activate endogenous p73. We show that p73 b, and to a lesser extent p73 a proteins are upregulated in p53-deficient H1299 cells in response to a panel of the overexpressed oncogenes E2F1, cMyc and E1A. The oncogene-mediated p73 accumulation is stronger than the p73 response after cisplatin, which is restricted to p73 a and occurrs only in one of two cell lines tested. E2F1, cMyc and E1A-

mediated p73 upregulation leads to the activation of p73 transcription function, as shown by the induction of the endogenous p73 target proteins p21 and HDM2, and by p73-responsive reporter activity which is inhibited by a dominant negative mutant of p73 (p73DN). Moreover, oncogene-mediated activation of endogenous p73 induces apoptosis in Saos2 cells, which is largely abrogated by p73DN, indicating p73 dependence. In contrast, in stable H1299 clones that overexpress cMyc, p73 protein accumulates but largely fails to activate a reporter and to induce endogenous response genes. The latter indicates that in p53-deficient tumor cells with activated oncogenic pathways, clonal outgrowth favors loss of p73 function. Taken together, this data shows that oncogenes can signal to p73 *in vivo*. Moreover, our data provide a mechanism for the fact that a broad spectrum of human tumors, with their frequent deregulation of oncogenes such as E2F1 and myc, overexpress p73 compared to their normal tissue of origin.

**Statement of Work: Task 1A.** To identify if downregulation of p73 with an antisense strategy alters growth rate of breast cancer cells months 1-9). This section using antisense downregulation was not done because of technical difficulties. We were unable to identify antisense oligonucleotides that effectively downregulated endogenous p73 mRNA.

**Task 1B.** Determine if cellular oncogenes induce p73 protein(s) .

**Results**

**Endogenous 73 a and b proteins are induced in response to cellular and viral oncogenes E2F1, cMyc and E1A.** The majority of functional and regulatory p73 studies to date used ectopically expressed p73 proteins. To reliably detect endogenous p73 proteins, we used 3 different p73 specific antibodies. They comprised the p73 b-specific monoclonal GC15, a p73 a-specific polyclonal raised against a C-terminal peptide (poly p73 a) and a pan p73 polyclonal raised against an N-terminal peptide (poly p73 N). Using transfected p73 a and b as positive controls (Fig 1, lanes 2 and 7), the antibodies recognize endogenous p73 a and b in several tumor cell lines including the p53-deficient human H1299 line (Fig 1). H1299 cells express a basal level of p73 a and b (see lanes 1 and 9). Simian COS cells express the highest level of both isoforms (lanes 4, 5 and 8) in contrast to SK-N-AS cells which express no detectable p73 b (lane 6) and only minute amounts of p73 a (lane 3), consistent with previous reports (19).

Next we tested whether viral and cellular oncogenes, which are major upstream signals for p53 activation, are also physiologically relevant upstream signals for endogenous p73 induction. H1299 cells were transiently transfected with various oncogene-expressing plasmids and expression was verified by immunoblotting with their respective antibodies (Fig 2A). As seen in Fig 2B and C, both p73 a and b proteins were markedly induced after expression of E2F1, cMyc or adenoviral E1A when compared to empty vector (see vimentin for equal loading). Since the transfection efficiency, as judged by cotransfected GFP, varied between 50 - 70%, a slight variation in fold induction from experiment to experiment was observed. A representative experiment is shown in Figs 2B and C, where p73 a and p73 b induction ranged from 1.9 - 3.7-fold compared to cells transfected with empty vector. P73 b reproducibly was induced to a greater degree than p73 a (2.3 - 3.7-fold versus 1.9 - 2.0-fold in this experiment).

Cisplatin (CDDP, 25 mM exposure for 24 h) was recently found to induce endogenous p73 in the human colon carcinoma cell line HCT 116-3(6) and in mouse embryo fibroblasts

(MEF) (37). The latter report did not specify which p73 isoform was induced (37). Cisplatin-induced p73 accumulation was due to posttranslational induction rather than an increase in p73 mRNA, analogous to the mechanism of p53 induction by DNA damage and oncogenic stress (37). When we treated H1299 and the human diploid fibroblast line MRC5 with cisplatin under the same conditions (CDDP, 25 mM for 24 h), p73 was responsive, albeit only to a moderate degree and in a cell type specific manner. Fig 3 shows that MRC5 cells showed a 1.4-fold induction of p73 a but not p73 b, while H1299 cells failed to respond completely. In contrast, p53 was induced 3.6-fold in MRC5 cells by this treatment. Taken together, we conclude that oncogenes induce p73 more potently than cisplatin and that they induce both isoforms.

**Oncogene-mediated p73 induction leads to activation of p73 response genes.** P73 shares many response genes with p53 *in vivo*. This has been shown in several cell systems using transient or inducible p73 expression (1, 8, 9). For example, p73 induces p21 and HDM2 mRNAs by Northern blot analysis, although somewhat less efficiently than p53 (9). We therefore tested whether the oncogene-mediated induction of endogenous p73 also translates into transcriptional activation by p73. When p53-deficient H1299 cells were transiently transfected with expression plasmids for E2F1, cMyc and E1A, endogenous p73 protein was upregulated (Fig 4A, top panel, compare with empty vector) and this upregulation was accompanied by the induction of the p73 response gene products p21 and HDM2 (Fig 4A, middle panels). These two response genes are direct *in vivo* targets of p73 a and b, as shown by ectopic p73 inducibly expressed in p53-deficient EJ and H1299 cells (9, 36). Oncogene expression was confirmed by immunoblots (data not shown). E2F1 reproducibly caused a stronger transactivation of the p21 and HDM2 genes than cMyc and E1A. A representative example is shown in Fig 4A with a 4.0-fold induction of p21 and a 1.7-fold induction of HDM2. We reason that in p53-deficient H1299 cells p73 substitutes for p53. In p53 expressing cells, transactivation of mdm2 in response to a broad spectrum of overexpressed oncogenes including the panel used here has been shown to be indirect and strictly dependent on p53 (for review see Ref. 47). Transactivation of p21 by cMyc and E1A is also p53-dependent, although E2F1, in addition to activating the p21 promoter through p53, can transactivate p21 directly (48). Taken together, this data strongly suggests that with the partial exception of E2F1, the induction of HDM2 and p21 after oncogenes is mediated through p73 in these p53-defocoent cells.

The oncogene-mediated activation of the p73 transcription function was further supported in luciferase reporter assays of H1299 cells using the p73 responsive PG13-Luc reporter. As shown in Fig 4B, E2F1 was the strongest activator of p73 while the other oncogenes showed lower but significant activity compared to vector controls. P73 b exhibited 80% of the activity of p53 (on a molar basis) in transactivating the PG13-Luc reporter (data not shown). To confirm that the oncogene-induced PG13 reporter activity is mediated through p73, we tested the effect of a co-expressed dominant negative mutant of p73 (Fig 4C). P73DN encodes aa 345-636 of human p73 a and acts as a dominant negative mutant by inhibiting p73 a-dependent transactivation (Fig 4C) but does not interfere with p53-dependent transactivation. This mutant also binds to p73 a and b *in vitro* and *in vivo* but not to p53 (M Irwin and WG Kaelin, to be published elsewhere). When co-expressed with E2F1, E1A and cMyc, the p73DN mutant suppressed reporter activity by 44% (E2F1), 58% (E1A) and 35% cMyc. A very similar suppressive effect was seen with p73DNs (aa 313-404) (data not shown). Taken together, we

conclude that oncogenes induce and activate endogenous p73 for transactivation of effector genes.

**Oncogene-mediated activation of endogenous p73 induces apoptosis in p53-deficient tumor cells.** The activation of the transcription function of p73 by oncogenes also suggested that these upstream signals might mediate the activation of the apoptotic function of p73. To test this prediction, we performed apoptosis assays on transiently transfected Saos-2 cells with or without co-expressed p73DN using the in situ TUNEL assay. As seen in Fig 5, expression of E2F1, cMyc and E1A in p53-deficient Saos-2 cells induces apoptosis that resembles the one seen with transfecting p73 b directly. Importantly, the apoptotic avtivity of each oncogene was greatly suppressed when co-expressed with p73DN (84 % for E2F1, 96% for cMyc and 72% for E1A). Taken together, this data shows that oncogene-mediated apoptosis in p53-deficient tumor cells depends on p73 function.

**Stable H1299 clones overexpressing cMyc have upregulated p73 protein levels but favor selection for loss of p73 function.** Our previous results on transiently transfected H1299 and Saos-2 cells indicates that, in the absence of p53, overexpressed oncogenes are able to activate the transcriptional and apoptotic activity of p73. From this data one might predict that clonal outgrowth of p53-deficient tumor cells with stable overexpression of oncogenes selects for loss of p73 transactivation function. To test this hypothesis, vector control and cMyc transfected H1299 cells (1x107 cells seeded) were selected in G418 for 3 weeks. Surviving foci were then ring cloned and expanded into sublines. Of the only 22 surviving cMyc foci, 7 were randomly picked and successfully established. As shown in Fig 6A, all clones overexpressed cMyc, albeit to various degrees compared to vector control. Cell extracts were then probed for p73 protein levels (Fig 6B, top 2 panels). As already seen with transient cMyc transfections, p73 a and b were found to be markedly induced in all subclones (Fig 6B; fold inductions compared to vector alone are indicated). However, in 6 of the 7 subclones upregulated p73 protein failed to induce p21 protein above baseline as present in the vector control (Fig 6B, third panel). Moreover, 3 of the 7 clones failed to induce significant levels of HDM2. Although clones 3 and 5 did show increased HDM2 levels, they are likely to be unrelated to p73 since these clones did not induce p21 concordantly. Only clone 7 exhibited concordant p21 and HDM2 induction. Interestingly, this was the only one among the seven clones with extremely slow growth, while all others grew at a much faster and relatively similar rate. It is therefore possible that p73 function in clone 7 is retained but exerts a strong negative effect on the cell cycle.

The lack of p73 transcriptional activity in these cMyc-expressing stable subclones was further confirmed in luciferase reporter assays. As shown in Fig 6C, transiently transfected p73 b gave a strong response. In contrast, our panel of cMyc clones exhibited no significant transactivation activity with reporter levels comparable to the empty vector clone, with the possible exception of clone 7. This clone showed a marginal p73 reporter activity consistent with its effects on endogenous target genes (see Fig 6B) and its extremely slow growth. Of note, by Western blot analysis with polyclonal anti-p73 a and monoclonal GC15 antibody we did not detect any aberrant p73-related polypeptides or a form consistent with delta Np73 in these cMyc clones. This suggests that the accumulated p73 proteins might harbor missense or small deletion mutations, and mutational analysis of these clones is underway. Taken together, our results show that clonal outgrowth tends to select for loss of p73 function. Conversely, cells with

constitutive activation of functional p73 appear to be largely unable to successfully establish stable clones. We conclude that in p53-deficient tumor cells with activated oncogenic pathways, p73 appears to exert an important suppressor function *in vivo*.

**Key Research Accomplishments:**
-Research Paper published (Zaika et al *J Biol. Chemistry* **276**:11310-11316, 2001.

- August 2000 Cancer Genetics &Tumor Suppressor Genes, Cold Spring Harbor Symposium, NY "Oncogenes induce and activate endogenous p73 protein"

**Reportable Outcome:** Zaika AI, Irwin M, Sansome S, **UM Moll** (2001) Oncogenes Induce and Activate Endogenous p73 Protein. *J Biol. Chemistry* **276**:11310-11316.

**Conclusions:** This data shows that oncogenes can signal to p73 *in vivo*. Moreover, our data provide a mechanism for the fact that a broad spectrum of human tumors, with their frequent deregulation of oncogenes such as E2F1 and myc, overexpress p73 compared to their normal tissue of origin.

**References:** See reprint
**Appendix:** Reprint of published paper.

**Statement of Work :Task 2.** Combinatorial assays to determine if dominant negative interactions among p73 isoforms and between p73 and p53 can be demonstrated months 12-24).

**Introduction**
The p53 family member p73 has significant homology to the p53 tumor suppressor. Human full length p73 (TA-p73) shares 63% amino acid identity with the DNA-binding region of TP53 including conservation of all DNA contact residues, as well as 38% and 29% identity with the tetramerization domain and transactivation domain, respectively. Ectopically overexpressed TA-p73a and b (two C-terminal splice variants) largely mimic p53 activities including the induction of apoptosis, cell cycle arrest and the transactivation of an overlapping set of target genes. Moreover, deregulation of oncogenes E2F1, cMyc and E1A induces apoptosis in tumour cells in a p53-independent manner by transcriptionally inducing and activating endogenous TA-p73 proteins. Furthermore, endogenous TA-p73 is activated to mediate apoptosis by a restricted spectrum of DNA damage such as cisplatin, taxol and g-irradiation via a pathway that depends on the non-receptor tyrosine kinase c-abl. Thus, TA-p73 might function synergistically with p53 in a tumour surveillance pathway. However, despite this homology, data from human tumours and p73-deficient mice argue against a classical Knudson-type tumour suppressor role for the TP73 gene. TP73-deficient mice lack a spontaneous tumour phenotype and inactivating mutations in human tumours are extremely rare (over 900 tumours analyzed to date). Moreover, while all normal human tissues studied express very low levels of p73, multiple primary tumour types and tumour cell lines overexpress p73, including cancers of the breast, lung, esophagus, stomach, colon, bladder, ovary, liver, bile ducts, ependymal lining, myelogenous leukemia and neuroblastoma. To date, most studies identifying p73 overexpression in primary human tumours have examined total levels of p73 with a few exceptions that specifically measured TA-p73 or Ex2Del p73. Importantly, in mouse, an

N-terminally truncated DNp73, generated from an alternative promoter in Intron 3, plays an essential anti-apoptotic role during p53-driven developmental neuronal death *in vivo* by acting as a dominant negative inhibitor of p53. We therefore sought the human counterpart of DNp73 and examined its potential role in cancer.

**The human TP73 gene can produce DNp73**

Mouse DNp73 differs from TA-p73 by a novel Exon 3', which replaces the first 3 Exons, and is spliced in frame to Exon 4 of the TP73 gene[11]. By sequence alignment of a human genomic BAC clone containing TP73 (GenBank Accession Nr. AL 136528), we identified a region with 77% identity to the 5'UTR of mouse DNp73 mRNA (Genbank Accession Nr. Y 19235). This allowed us to predict the human Exon 3' and design isoform-specific primers for human DNp73. Full length DNp73a cDNA, spanning Exons 3'-14 and including 220 bases of 5'UTR and 103 bases of 3'UTR, was cloned by RT-PCR from total RNA of human placenta and MDA 231 breast cancer cells and sequence confirmed (Fig. 1a). Human Exon 3' consists of 13 unique amino acids with almost complete identity to mouse Exon 3' (12 of 13 residues are identical) (Fig. 1a). Human Intron 3 contains the predicted TATA box 30 nt upstream of the transcriptional start site, which is located 7.6 kb downstream of Exon 3.

**Analysis of DNp73 and TA-p73 expression in human tumours**

Unique cDNA primers were designed for the specific amplification of DNp73 from tissues by semiquantitative RT-PCR (Fig. 1b). We then determined the expression levels of DNp73 in 52 breast cancers and compared them to 8 unrelated normal breast tissues (Fig. 2a). All but one normal breast tissues showed either non-detectable or very low levels of DNp73. In contrast, 16 of 52 breast cancers (31%) expressed DNp73 levels that were between 6 and 44 - fold higher than the normal tissue average (indicated by red line in Fig. 2a). Since we previously showed that breast cancers also overexpress TA-p73[14], we next used isoform-specific semiquantitative RT-PCR to measure DNp73 and TA-p73 simultaneously. Among these 16 cancers with a 6 to 44-fold increase of DNp73, 12 cancers showed much higher upregulation of DNp73 than of TA-p73 (data not shown).

We next analyzed a spectrum of tumours that were matched with the patients' normal tissues of origin (Table 1; examples in Fig. 2b). Of 16 matched cancer pairs (ovarian, breast, cervix, kidney and colon cancer) and 1 large benign ovarian tumour (serous cystadenoma), DNp73 was specifically upregulated 3 to 78-fold in 10 tumours (63%) (Table 1, second column), while TA-p73 was upregulated 3 to 155-fold in 7 tumours (41%) (third column), compared to their respective normal tissues of origin. Importantly, when upregulation of DNp73 and TA-p73 in a given tumour is analyzed more closely, in 7 of the 10 tumours (70%) DNp73 is upregulated disproportionately to a far greater degree than TA-p73 upregulation (fourth column). These include 4 ovarian tumours, 2 breast cancers and 1 cervical cancer. Their excessive rise in DNp73 compared to their rise in TA-p73 ranges from 5 to 16-fold. Four cancers within this group exhibited exclusive upregulation of DNp73 (tumours Nr. 5, 6, 8 and 10). Only 2 tumours exhibited an inverse ratio with an excessive TA-p73 rise compared to their DNp73 rise (tumours Nr. 3 and 7). One additional tumour (Nr. 9) with concomitant upregulation of both isoforms could not be quantitated because the corresponding normal tissue levels were undetectably low (ND). Furthermore, among the remaining 7 tumours that did not upregulate DNp73, 2 of 4 tumours that we analyzed (Nr. 11 and 17) showed tumour-specific upregulation of Ex2Del p73

instead of DNp73. Both of those tumours also failed to upregulate TA-p73. Ex2Del p73 is a dominant negative isoform of p73 lacking the transactivation domain, which is generated from the TA promoter by splicing out Exon 2. It has been previously shown to be upregulated in some ovarian cancers and breast cancer cell lines[1,15,17]. Thus, a total of 9 tumours in our series (53%) exhibit either exclusive or excessive upregulation of dominant negative p73. Moreover, of the 14 tumours available for p53 mutational analysis by immunocytochemistry, 10 tumours had undetectable p53 levels, suggesting wild-type status, while 4 tumours showed nuclear overexpression, suggesting p53 mutation. This estimated mutational rate (29%) is in good agreement with the reported rates of p53 mutations in these tumor types (about 30 %)[18]. Thus, it appears that in a total of 8 analyzable tumours with disproportional upregulation of a dominant negative p73 isoform (7 tumours with DNp73 and 1 tumour with Ex2Del), 7 tumours likely harbored a wild type p53 genotype, while only 1 tumour exhibited a concomitant p53 mutation (fifth column).

**DNp73 is an Efficient Dominant Negative Inhibitor of wild-type p53 and TA-p73 function**
To test the hypothesis that human DNp73 is a dominant negative inhibitor of human wild-type p53 and TA-p73, we first performed reporter assays with expression plasmids for wild-type p53, TA-p73a and b and a p53/TA-p73-responsive Luciferase reporter in the presence or absence of DNp73a in p53 *null* H1299 cells and SaOs2 cells (Fig. 3 and data not shown). DNp73a exhibited a dose-dependent, complete suppression of the transcriptional activity of wild-type p53 and TA-p73a and b (Fig. 3a and data not shown). Moreover, a molar ratio of 1:1 between DNp73a and wild-type p53 potently inhibited p53 activity, yielding a reduction by 92% (Fig. 3b). In comparison, a 1:1 molar ratio reduced TA-p73b activity by 62%, although a 3-fold molar excess of DNp73a completely blocked TA-p73b activity (97% reduction) (Fig. 3b). The latter suggests that DNp73a is a stronger inhibitor of wild-type p53 than of TA-p73b. Furthermore, DNp73a also efficiently suppresses endogenous target gene products of wild-type p53 and TA-p73 (Fig. 3c). In HeLa and H1299 cells, transfection of wild-type p53 or TA-p73 induces endogenous HDM2, 14-3-3s and p21Waf1 compared to basal levels seen with empty vector. However, the concomitant expression of DNp73a strongly suppresses each of these response gene products (Fig. 3c, compare lanes 2 and 3 and lanes 4 and 5).

Moreover, DNp73a is a strong inhibitor of apoptosis induced by wild-type p53 and TA-p73 (Fig. 4a). HeLa and SaOs2 cells undergo wild-type p53- and TA-p73 dependent cell death as assessed by Annexin V staining and TUNEL assay. This apoptotic activity is completely abolished by co-expression of DNp73a (Fig. 4a and data not shown). The inhibitory action of DNp73a is dependent on the presence of transcription-competent wtp53 and TA-p73, since DNp73a alone cannot affect apoptosis. Furthermore, in agreement with the above results, DNp73a is an inhibitor of colony suppression mediated by wild-type p53 and TA-p73 (Fig. 4b and Table 2). Reintroduction of wild type-p53 and TA-p73 suppresses growth of SaOs2 cells[2,19] and this suppression is thought to be largely due to apoptosis[20]. In keeping with these results, transfection of wild-type p53 strongly suppresses macroscopic colony formation of SaOs2 cells compared to many visible colonies with vector backbone alone (4 foci for wild-type p53 versus 1778 foci for vector control). In contrast, co-expression of DNp73a together with wild-type p53 at a 1:1 molar ratio counteracts this effect, leading to a 12.5-fold increase in the number of colonies from 4 to 51. Likewise, TA-p73a, although not quite as potent as wild-type p53, suppresses colony formation (82 foci)[2], but co-expression of DNp73a together with TA-p73a

again antagonizes this effect and increases the number of macroscopic colonies by 8.1-fold to 669 foci. The higher rescue ability of DNp73a with respect to wild-type p53 is reminiscent of its stronger inhibition of wild-type p53-mediated transactivation compared to TA-p73 -mediated transactivation (Fig. 3b). Entirely consistent with this finding were data from a subsequent p53 expression analysis of surviving colonies. A complete loss of p53 protein expression was found in 2 of 2 randomly picked and expanded colonies that were derived from plates transfected with wild-type p53 alone (Fig. 4c). This is in agreement with the fact that wild-type p53 expression is incompatible with the outgrowth of colonies in such an assay and the rare colonies that do grow escape because they have lost wild-type p53 expression[21]. In contrast, all 3 randomly picked colonies from plates cotransfected with wild-type p53 and DNp73a had detectable levels of p53 protein (Fig. 4c), indicating that DNp73a neutralizes the growth suppressive effect of wild-type p53, thereby removing the selection pressure to delete the wild-type p53 plasmid. Thus, DNp73a is able to counteract p53 and TA-p73 – induced colony suppression in transformed human cells.

**DNp73 inhibits wild-type p53 and TA-p73 function by heterocomplex formation**

One explanation for this dominant negative effect is a direct physical interaction between DNp73 and either wild-type p53 or TA-p73 proteins, analogous to the dominant negative mode of action of mutant p53 proteins towards wild-type p53. To test this hypothesis directly, lysates prepared from p53 ***null*** SaOs2 cells cotransfected with wild-type p53 and DNp73a were immunoprecipitated with monoclonal antibody, ER15, which recognizes DNp73a. Immunoblot analysis with an antibody specific for p53 (CM1) revealed a complex of the 2 proteins (Fig. 5a, left lane). As a control, no such complex was seen in SaOs2 cells transfected with DNp73a alone and immunoprecipitated with ER15 (Fig. 5a, center lane), indicating the specificity of the detection. Of note, TA-p73 isoforms are unable to form a protein complex with wild type p53[16,22-24], excluding the possibility that the observed p53 band was co-immunoprecipitated via the endogenous TA-p73 protein of SaOs2 cells. Moreover, a similar complex was seen in wild-type p53 expressing human U2OS cells after transfection with DNp73a alone. Fig. 5b shows a specific complex between endogenous wild-type p53 and ectopic DNp73a that was immunoprecipitated by ER15 (left lane). No such complex is seen when an irrelevant monoclonal antibody against green fluorescent protein (GFP) is used (right lane). The same specific complex can again be immunoprecipitated from U2OS cells using a monoclonal antibody specific for p53 (421) and immunoblotted with a polyclonal antibody specific for DNp73 that does not crossreact with any TA-p73 proteins (Fig. 5c, left lane). Again, no such complex is found with preimmune mouse IgG (center lane).

**Conclusion**

The p53 family member p73 has significant homology to p53, but tumour-associated upregulation of p73 and genetic data from human tumours and p73-deficient mice rule out a classical Knudson-type tumour suppressor role. We report that the human TP73 gene gives rise to an N-terminally truncated isoform, DNp73, which is derived from an alternative promoter. DNp73 is frequently overexpressed in a variety of primary human cancers with upregulated p73. Functional studies indicate that DNp73a, which lacks the transactivation domain of full length p73 (TA-p73), acts as a dominant negative inihibitor of both wild-type p53 and transactivation-competent TA-p73. Moreover, DNp73a counteracts apoptosis and tumour cell growth suppression induced by wild type p53 and TA-p73. The underlying mechanism of inhibition is heterocomplex formation between DNp73a and e.g. wild type p53. Thus, DNp73 mediates a novel inactivation mechanism of wild-type p53 and TA-p73 via a dominant-negative family network. Increased expression of DNp73 appears to bestow oncogenic activity upon the TP73 gene, a trait that is selected for in human cancers.

**Key Research Accomplishments:**

The p53 family member p73 has significant homology to p53, but tumour-associated upregulation of p73 and genetic data from human tumours and p73-deficient mice rule out a classical Knudson-type tumour suppressor role. We report that the human TP73 gene gives rise to an N-terminally truncated isoform, DNp73, which is derived from an alternative promoter. DNp73 is frequently overexpressed in a variety of primary human cancers with upregulated p73. Functional studies indicate that DNp73a, which lacks the transactivation domain of full length p73 (TA-p73), acts as a dominant negative inihibitor of both wild-type p53 and transactivation-competent TA-p73. Moreover, DNp73a counteracts apoptosis and tumour cell growth suppression induced by wild type p53 and TA-p73. The underlying mechanism of inhibition is heterocomplex formation between DNp73a and e.g. wild type p53. Thus, DNp73 mediates a novel inactivation mechanism of wild-type p53 and TA-p73 via a dominant-negative family network. Increased expression of DNp73 appears to bestow oncogenic activity upon the TP73 gene, a trait that is selected for in human cancers.

**Reportable Outcome:**

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- Nov 2001 Programmed Cell Death Meeting, Cold Spring Harbor Symposium, NY

**References:** see reprint.
**Appendix:** Submitted Manuscript

In addition, I was invited to write a comprehensive Review Article on the p53 gene family:
U. M. Moll , S. Erster and A. Zaika (2001). p53, p63 and p73 - solos, alliances and feuds among family members.
This article is in press in Biochimica and Biophysica Acta Reviews on Cancer .

**Appendix:** manuscript in press.

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# Lack of defective expression of the ATM gene in sporadic breast cancer tissues and cell lines

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**Abstract.** Homozygous mutations of the gene mutated in ataxia telangiectasia (ATM) causes the AT syndrome, a pleiotropic phenotype that includes an increased risk of cancer. Most of the known mutations at the ATM gene lead to truncations which are usually associated with instability of mRNA and protein. A decrease or loss of ATM protein expression is associated with specific lymphoid malignancies in AT and non-AT patients. ATM is located within a region in chromosome 11q22-23 that is frequently undergoing loss of heterozygosity in sporadic breast cancer. Epidemiological studies estimated a 4-fold increase in breast cancer risk in heterozygous women. However, direct mutational analysis failed to clearly support a role for mutant ATM alleles in breast carcinogenesis. If ATM does have a suppressor role in this tissue, one would expect deficient ATM expression. We therefore tested the hypothesis that the expression of the ATM gene is reduced in sporadic breast cancer. We determined ATM transcript levels using competitive RT-PCR on 89 randomly selected sporadic breast cancer samples and 29 normal breast tissues. Of these, 11 were matched normal/cancer pairs. We also evaluated 7 breast cancer cell lines. Deficiency in ATM expression was not observed. Of the 11 matched pairs, 7 tumors expressed mildly higher levels, 3 tumors expressed the same amount and only 1 tumor expressed <50% of the normal match. In addition, 3 cancers with tumor-associated LOH of the ATM gene expressed higher mRNA levels in the tumors than in their normal tissue matches, suggesting that no correlation exists between tumors with LOH and decreased ATM expression. In summary, our results do not support a suppressor role for ATM in the development of sporadic breast cancer.

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*Abbreviations:* LOH, loss of heterozygosity; T-PLL, T-cell prolymphocytic leukemia; B-CLL, B-cell chronic lymphocytic leukemia; ß2M, ß2-microglobulin

*Key words:* ATM gene, expression, loss of heterozygosity, mutation, breast cancer

## Introduction

The characterization of BRCA1 and BRCA2 gene mutations as high risk factors in familial breast cancer served as paradigm for the search for other more common but less penetrant genes as potential risk factors for sporadic breast cancer. The ATM gene is such a candidate, since AT homozygous patients have, among many other symptoms, an increased frequency of lymphoid malignancies and their cells exhibit excessive radiosensitivity. AT heterozygote carriers occur at a prevalence of 0.5-1% in the general population but are clinically free of AT symptoms. However, their cells show an intermediate radiosensitivity *in vitro* as well as a defective control of the mitotic spindle checkpoint after X-rays, although a defect in apoptosis is controversial (3,4). Before the ATM gene was cloned in 1995, 4 epidemiological studies had estimated a relative risk of breast cancer in AT heterozygotes of 3.9 (5-8). LOH analysis of sporadic breast cancer on chromosome 11q23 shows up to 40% frequency of loss of heterozygosity spanning an approximately 6 Mb region that includes the ATM locus (9,10). This leaves it unclear whether the true deletional target is ATM and/or another unknown suppressor gene(s) thought to reside in this region (9,11). Consistent with this data, LOH of ATM using the intragenic marker D11S2179 and the distal marker D11S1818 are associated with poorer survival (11). However, since the ATM gene has been cloned, direct mutational analysis of mainly constitutive DNA but also tumor DNA on cumulatively over 500 breast cancer patients from non-AT families, failed to clearly support the hypothesis that a mutant ATM allele plays a role in breast cancer risk/development and that ATM is a *bona fide* suppressor gene in this tissue (12-15). This includes a failure to detect increased germline mutations in the ATM gene of women with early onset (<40 years) sporadic breast cancer (12) and of women from breast and gastric cancer families, which appear to be the most frequent malignancies seen in AT carriers (13,14). Also, in the few breast cancers analyzed to date which harbored a mutant ATM allele somatically or constitutionally, no selection pressure appears to exist against the retained wild-type allele in the tumor (14,15). ATM mutations and constitutional ATM heterozygosity also failed to correlate with complications after radiotherapy in breast cancer patients with an adverse reaction (16-18). Clearly, these studies show that diagnostic or occupational exposure to ionizing radiation is not enough to increase the relative risk of breast cancer significantly (19).

The overall picture that emerges to date from LOH and mutational studies on over 1,200 breast cancers indicates that the connection between AT heterozygosity and breast cancer remains unclear. The difficulty in proving a connection, however, suggests that AT heterozygosity is not a significant genetic determinant in unselected sporadic breast cancer (12-15,19,20). Furthermore, the studies suggest that among women with heterozygous ATM mutations there is: i) at best a slightly increased risk for those from families with AT syndromes (20,21) or from select families with breast cancer, leukemias and lymphomas (13,14,21).

Although direct mutational studies in breast cancer did not show a significant mutational rate of the ATM gene, the data, albeit strong, is not an absolute argument against a genuine suppressor role of the ATM gene in sporadic breast cancer. Haploinsufficiency, particularly in light of a significant LOH status at and around the ATM locus, or epigenetic modes of downregulating the expression could in theory be alternate mechanisms. Importantly, a decrease or loss of ATM protein expression due to mutational inactivation is associated with specific lymphoid malignancies in AT and non-AT patients. ATM expression is decreased in the rare T-cell prolymphocytic leukemia (T-PLL) that occurs in young AT patients (22) or in older patients due to somatic loss of both alleles (23) and in an aggressive subgroup of B-cell chronic lymphocytic leukemia (B-CLL) with LOH at the ATM locus (24,25). We therefore tested the hypothesis that the expression of the ATM gene is also reduced in breast cancers and breast cancer cell lines compared to normal breast tissue. If ATM does indeed have a suppressor role in breast cancer, lost or decreased wild-type ATM expression might reflect or substitute for mutational inactivation. So far, one expression analysis on breast cancer has been reported (39 cases), and this study found a reduction in the mean ATM transcript level in carcinomas vs normal breast tissues (26). However, the basis for the reduced expression was unclear since LOH analysis did not include the ATM gene itself and direct analysis of the PI3 kinase region of ATM failed to detect mutations (26). With this background, we undertook a comprehensive ATM expression analysis using competitive RT-PCR on 89 randomly selected sporadic breast cancer samples and 29 normal breast tissues. Of these, 11 were matched normal/cancer pairs. We also included 7 breast cancer cell lines. In this cohort, primary breast cancers and breast cancer lines did not express reduced levels of ATM transcripts compared to normal breast tissue.

## Material and methods

*Tissues and cell lines.* Malignant tissues were obtained from 39 women at University Hospital at SUNY Stony Brook undergoing surgery for breast cancer and from 57 additional breast cancer patients through the Cooperative Human Tissue Network, Western Division (Case Western Reserve University, Cleveland OH). All cancers had pathologically confirmed diagnosis. Eighty-three cancers were invasive ductal carcinomas, 9 were ductal carcinoma *in situ* and 4 were invasive lobular carcinomas. Our series also comprised 36 normal breast tissues, 18 of which were matched pairs of cancer and adjacent normal tissue from the same patient while 18 were from unrelated reduction mammoplasties. Of the matched pairs, 11 were used for expression analysis and 16 were used for LOH analysis. Freshly harvested tissues were immediately snap frozen in liquid nitrogen and stored at -80°C until needed. Human breast cancer cell lines MDA 468, MDA 361, MDA 231, MDA 435, MCF-7, T47D and SK-BR-3 were grown in 10% FCS containing DMEM at 5% $CO_2$.

*RNA and DNA extraction.* Snap frozen tissue was homogenized under liquid nitrogen in 2 ml of RNA STAT-60 (Tel-Test, Inc. Friendswood TX). After adding chloroform, total RNA was precipitated in isopropanol, washed twice in 75% ethanol and dried. Concentrations of reconstituted RNA were measured in triplicate by UV spectrophotometry and adjusted to 1 µg/ml. To obtain corresponding DNA, DNA reverse extraction from the same samples was performed using DNA STAT-60 (Tel-Test, Inc.).

*Competitive RT-PCR.* A competitive RT-PCR method was used to determine the amount of ATM transcripts in individual samples as described (26,27). Briefly, a predetermined constant amount of a mutagenized (deleted) homologous competitor RNA was added to 250 ng of individual tumor RNA prior to the RT/PCR reaction (single tube format, Titan Kit, Boehringer/Roche). To make competitor RNA, template cDNA product was generated by a first PCR reaction using forward primer (ATMf) 5'-TGTCATTACGTAGCTTCTCC and reverse primer (ATMr) 5'-GCTGAGTAATACGCAAA TCC (nucleotide positions 4646-4665 and 4924-4905, GenBank #U33841). The reaction was performed using a standard PCR protocol. Amplicon I was subsequently mutagenized by a second PCR reaction that introduced a 5' deletion using the forward competitor primer 5'-TGTCATTACGTA GCTTCTCCacttactgtaaggatgctctag (position 4646-4665/ 4708-4729) and the ATMr primer. For generating RNA, amplicon II was cloned into the pPCR-Script Amp SK(+) cloning vector (Stratagene) followed by *in vitro* transcription (Stratagene RNA transcription kit). Competitor concentration was determined by spectrophotometry. To determine the proper amount of competitor addition, serial dilutions (10 fg to 100 pg) were added to 250 ng of pooled sample RNA and subjected to RT-PCR using primers ATMf and ATMr. Optimal calibration was defined at equal signal intensity between sample and competitor and was chosen for all subsequent individual tumor measurements. To standardize for RT efficiency, expression levels of the housekeeping gene ß2 microglobulin (ß2M) was determined analogously in a separate reaction including generation of a competitor and its calibration (ß2Mf primer 5'-TGTCTTTCAGCAAGGACTGG, ß2Mr primer 5'-GATGCTGCTTACATGTCTCG and ß2M competitor primer 5'-TGTCTTTCAGCAAGGACTGGaaa gatgagtatgcctgccgt). Amplicons were separated on a 6% denaturing acrylamide gel and quantitated by PhosphoImage analysis (model 445 SI, Molecular Dynamics). ATM expression levels were calculated using the competitive algorithm (ATM patient/ATM $_{competitor}$/ß2M $_{patient}$/ß2M $_{competitor}$) and in some cases also the simple algorithm (ATM $_{patient}$/ß2M patient). For statistical analysis, groups were analyzed using the Stastistix program.

Table I. Relative ATM expression in breast cancer tissues.

| Breast cancer case # | Relative ATM expression | Breast cancer case # | Relative ATM expression |
|---|---|---|---|
| BC1 | 0.165 | BC47 | 0.06 |
| BC2 | 0.25 | BC48 | 0.11 |
| BC3 | 0.24 | BC49 | 0.25 |
| BC4 | 1.63 | BC50 | 0.48 |
| BC5 | 1.08 | BC51 | 0.65 |
| BC6 | 2.3 | BC52 | 0.37 |
| BC7 | 0.77 | BC53 | 0.26 |
| BC8 | 0.61 | BC54 | 0.35 |
| BC9 | 1.29 | BC55 | 0.05 |
| BC10 | 0.63 | BC56 | 0.07 |
| BC11 | 0.89 | BC57 | 0.09 |
| BC12 | 2.11 | BC58 | 0.08 |
| BC13 | 1.38 | BC59 | 0.2 |
| BC14 | 0.44 | BC60 | 0.19 |
| BC15 | 0.75 | BC61 | 0.1 |
| BC16 | 0.8 | BC62 | 0.08 |
| BC17 | 0.68 | BC63 | 0.03 |
| BC18 | 5.3 | BC64 | 0.04 |
| BC19 | 0.53 | BC65 | 1.06 |
| BC20 | 0.36 | BC66 | 4.35 |
| BC21 | 0.71 | BC67 | 1.12 |
| BC22 | 1.44 | BC68 | 0.5 |
| BC23 | 0.96 | BC69 | 0.41 |
| BC24 | 0.64 | BC70 | 0.08 |
| BC25 | 1.39 | BC71 | 0.26 |
| BC26 | 1.76 | BC72 | 0.594 |
| BC27 | 1.46 | BC73 | 0.186 |
| BC28 | 0.49 | BC74 | 0.096 |
| BC29 | 1.15 | BC75 | 0.393 |
| BC30 | 6.37 | BC76 | 0.195 |
| BC31 | 0.76 | BC77 | 7.67 |
| BC32 | 0.73 | BC78 | 1.99 |
| BC33 | 0.478 | BC79 | 2.78 |
| BC34 | 0.57 | BC80 | 7.34 |
| BC35 | 0.87 | BC81 | 2.97 |
| BC36 | 1.1 | BC82 | 5.84 |
| BC37 | 2.36 | BC83 | 6.56 |
| BC38 | 0.052 | BC84 | 16.79 |
| BC39 | 0.07 | BC85 | 0.479 |
| BC40 | 0.06 | BC86 | 0.065 |
| BC41 | 0.483 | BC87 | 0.61 |
| BC42 | 0.12 | BC88 | 0.05 |
| BC43 | 0.09 | BC89 | 0.76 |
| BC44 | 0.46 | | |
| BC45 | 0.07 | geoMean | 0.484 |
| BC46 | 0.09 | SD | 2.5 |

Table I. Continued.[a]

| Normal breast tissue | Relative ATM expression | Normal breast tissue | Relative ATM expression |
|---|---|---|---|
| Normal 1 | 0.16 | Normal 17 | 0.628 |
| Normal 2 | 0.215 | Normal 18 | 0.176 |
| Normal 3 | 0.275 | N1 (of BC1) | 1.31 |
| Normal 4 | 0.51 | N61 (of BC61) | 0.093 |
| Normal 5 | 0.42 | N62 (of BC62) | 0.333 |
| Normal 6 | 0.36 | N63 (of BC63) | 0.2 |
| Normal 7 | 0.1 | N70 (of BC70) | 0.11 |
| Normal 8 | 0.31 | N71 (of BC71) | 0.24 |
| Normal 9 | 0.87 | N72 (of BC72) | 0.292 |
| Normal 10 | 1.1 | N73 (of BC73) | 0.261 |
| Normal 11 | 0.56 | N74 (of BC74) | 0.318 |
| Normal 12 | 0.43 | N75 (of BC75) | 0.258 |
| Normal 13 | 0.39 | N76 (of BC76) | 0.191 |
| Normal 14 | 0.83 | | |
| Normal 15 | 0.48 | geoMean | 0.329 |
| Normal 16 | 0.462 | SD | 0.3 |

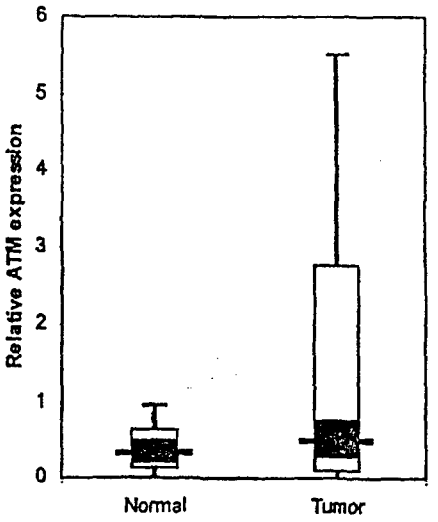
| Breast cancer lines | Relative ATM expression | Breast cancer lines | Relative ATM expression |
|---|---|---|---|
| T47D | 5.55 | MDA468 | 0.47 |
| MDA435 | 5.8 | MDA361 | 1.91 |
| MDA231 | 4.05 | | |
| SkBr-3 | 2.14 | geoMean | 2.6 |
| MCF7 | 3.21 | SD | 1.96 |

[a]Relative ATM expression in normal breast tissues and breast cancer cell lines.

*LOH analysis.* The microsatellite markers D11S2179 and NS22 (both intragenic for ATM), D11S1787 (centromeric), D11S1778 (telomeric), D11S1294 (telomeric) and D11S1818 (telomeric) (28,29) were amplified using a standard PCR protocol and $^{32}$P-labeled primers. Amplicons were analyzed on 6% acrylamide gels followed by PhosphoImage analysis.

*Partial mutational analysis.* For mutational analysis, 2 regions of the ATM gene were amplified from the RT-PCR reactions of 8 tumors with the highest ATM expression (BC30, BC66, BC77, BC79, BC80, BC82, BC83, BC84). The first region was identical to the one used for competitive RT-PCR (nucleotide positions 4646-4905 of GenBank #U33841), while the second was in the PI3 kinase domain (nucleotide positions 7980-8310). Products were purified, sequenced in both directions with the

A

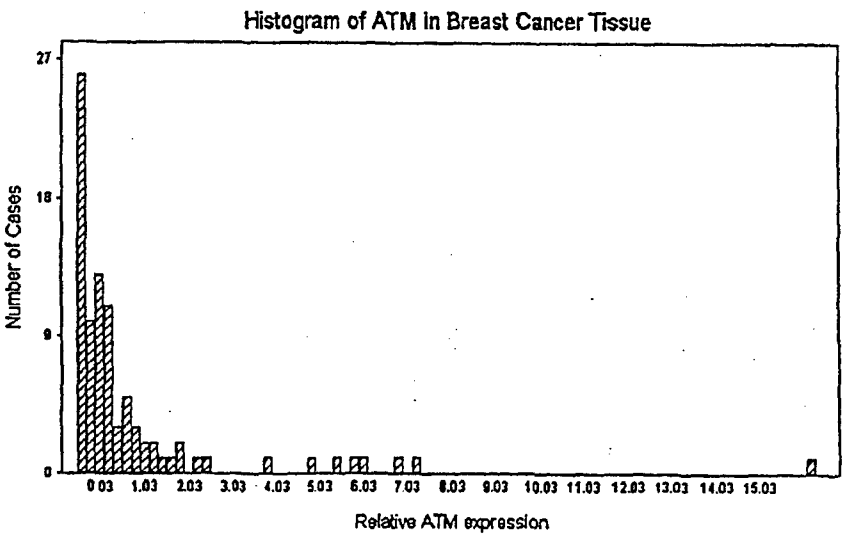



ABI Dye terminator cycle sequencing kit (Perkin Elmer) and analyzed on the ABI sequencer model 377.

## Results

*ATM expression in breast cancer and normal breast.* Using a competitive semiquantitative RT-PCR approach, we determined relative ATM expression levels on 89 breast cancers and compared them to 29 normal breast samples (Table I). Eleven of these constituted matched tumor/cancer pairs. ATM and ß2M transcripts were detectable in all breast tissues and the 7 breast cancer cell lines analyzed. While the expression of ß2M was similar in all samples, ATM expression levels varied widely. Moreover, breast cancer tissues did not show a

B



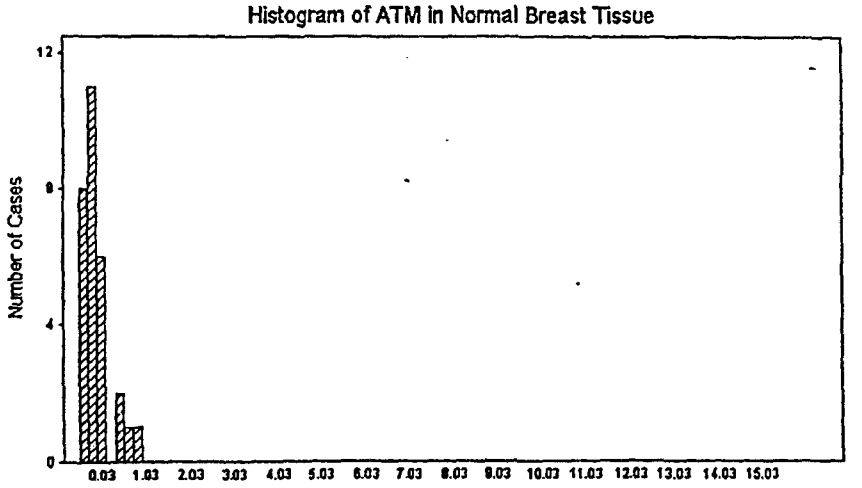




Figure. 1. A, Box plot of relative ATM expression levels in 89 breast carcinomas and 29 normal breast tissues as detected by RT-PCR. ATM expression varies widely in malignant tissue and is 1.5-fold higher on average than in normal tissue. The geometric mean of breast cancer is 0.484±2.5 SD compared to 0.329±0.30 SD in normal breast tissue (p=0.0005). Relative ATM expression in breast cancer range from 0.03 to 16.8 and in normal breast from 0.093 to 1.31. The geometric mean (black bar) with 95% confidence limits (line) and 75% of values (white box) are indicated for both groups. The shaded area represents the 95% confidence interval for the true mean. Values are normalized using the algorithm $ATM_{patient}/ATM_{competitor}/ß2M_{patient}/ß2M_{competitor}$. Breast cancer cell lines have the highest ATM levels with a geometric mean of 2.60±1.96 SD and a range from 0.47 to 5.55. B, The same data plotted as a histogram. Step size for the x-axis is 0.2.

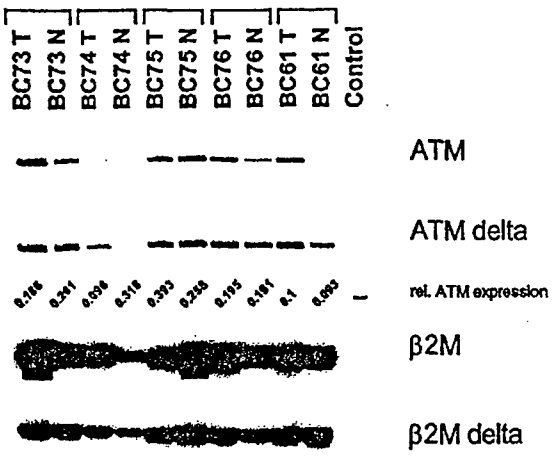



Figure 2. Some examples of raw RT-PCR data for ATM and ß2M mRNA expression. Matched tumor/normal pairs are shown. Numerical values are the relative ATM expression levels after normalization by competitive algorithm. BC74N shows a faint band on the original gel.

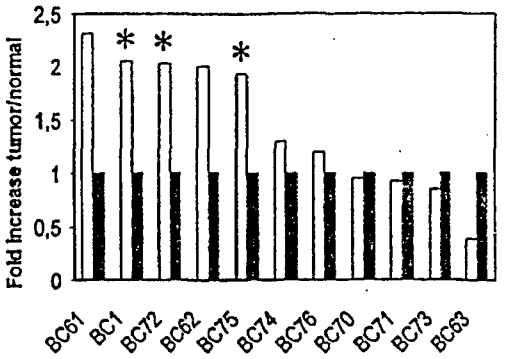



Figure 3. ATM expression levels in 11 matched tumor/normal tissue pairs. Values are normalized using the simple algorithm. White bars tumor, black bars normal. Stars indicate tumors with LOH of the ATM gene. Raw data of some cases are shown in Fig. 2. The overall result was the same when the group was normalized using the competitive algorithm.

Table II. Reproducibility of ATM expression measurements.

| Breast cancer case # | Measurement #1 | Measurement #2 | Average relative ATM expression |
|---|---|---|---|
| BC1 | 0.15 | 0.18 | 0.165 |
| BC12 | 2.07 | 2.15 | 2.11 |
| BC16 | 0.7 | 0.81 | 0.8 |
| BC30 | 6.25 | 6.48 | 6.37 |
| BC33 | 0.465 | 0.49 | 0.478 |
| BC38 | 0.048 | 0.056 | 0.052 |
| BC45 | 0.05 | 0.09 | 0.07 |
| BC49 | 0.23 | 0.26 | 0.25 |
| BC55 | 0.05 | 0.05 | 0.05 |
| BC66 | 4.3 | 4.4 | 4.35 |
| Normal 17 | 0.14 | 0.18 | 0.16 |
| Normal 1 | 0.62 | 0.636 | 0.628 |

deficiency in ATM expression. Table I contains the complete set of normalized expression data. Fig. 1A shows it graphed as a box plot and Fig. 1B shows the same data plotted as a histogram. In fact, cancers expressed mildly higher (1.5-fold) levels of ATM transcripts than normal breast tissues. However, due to the large variance in breast cancers and the relatively small difference between the geometric means of cancer versus normal tissue, the power to detect significant differences between the two groups was very low. The geometric mean of breast cancer was 0.484±2.5 standard deviation (SD) compared to 0.329±0.30 SD in normal breast tissue (Fig. 1A). In breast cancer, relative ATM expression ranged from 0.03 to 16.8 with a median of 0.57, and in normal breast it ranged from 0.093 to 1.31 with a median of 0.318. Examples of individual raw data are shown in Fig. 2. Repeat determinations from individual patients yielded reproducible results. Table II shows a subset of breast cancers and normal controls with their relative ATM expression levels, averaged from 2 independent measurements of the same sample. A mild tumor-associated increase in relative ATM transcript levels was also seen when the subgroup of matched pairs was analyzed separately. Seven of the 11 normal/cancer pairs showed a 1.2 to 2.3-fold increase in cancers compared to their adjacent normal tissue match, 3 cases were equal and only one case showed decreased (<50%) ATM expression in the tumor (Fig. 3). In line with the findings in primary cancers, breast cancer cell lines had even higher ATM expression with a geometric mean of 2.6±1.96 SD and a range from 0.47 to 5.55.

*LOH analysis of 11q22-23.* Sixteen matched pairs were analyzed for 6 markers at and around the ATM locus at chromosome 11q22-23. Of these, 2 were intragenic (D11S2179 and NS22), 1 was centromeric (D11S1787) and 3 were telomeric (D11S1778, D11S1294 and D11S1818, in increasing distance) to the ATM gene. Loss of heterozygosity for the ATM markers D11S2179 and NS22 was found in 31% and 25% of the informative cases, respectively. LOH for the extragenic markers was 20% for D11S1778, 20% for D11S1294, 20% for D11S1818 and 33% for D11S1787. Examples are shown in Fig. 4. These frequencies are consistent with those reported in the literature (30,31). Of note is that BC1, BC72 and BC75, all cases with tumor-associated LOH of the ATM gene, expressed higher levels of transcripts in the tumors than in their normal tissue matches (see Fig. 3). This indicates that no correlation exists between tumors with LOH for ATM and decreased expression of the gene product. Since breast tumors with LOH for ATM can in fact overexpress the gene, it renders the possibility of haploinsufficiency less likely. One case with a decreased ATM expression in the tumor could not be assessed for ATM LOH as it was non-informative.

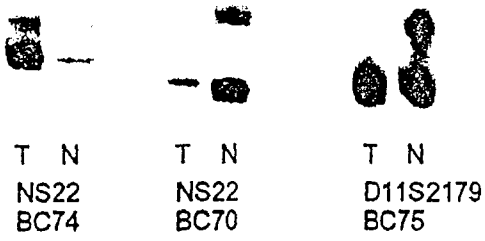



Figure 4. Examples of cases with LOH or retention of heterozygosity within the ATM gene using the indicated markers. N, constitutional DNA; T, tumor DNA.

*Partial mutational analysis of the ATM gene.* No mutations were found in the 8 tumors with the highest relative ATM expression.

## Discussion

In this study, we carried out a comparative analysis of ATM expression in 89 unselected sporadic breast cancers and 29 normal breast tissues. A subgroup of 11 cases comprised matched normal/cancer pairs. The study also included 7 breast cancer cell lines. In contrast to our working hypothesis, we found that cancer tissues did not show a deficiency in ATM expression. In fact, breast cancers expressed 1.5-fold higher levels on average compared to normal breast tissue. Moreover, of the 11 matched cases, 7 cases showed mild tumor-associated increases in ATM expression ranging from 1.2 to 2.3-fold compared to adjacent normal breast tissue. This tumor-associated increase is also reflected in a broad spectrum of breast cancer cell lines (T47D, MDA435, MDA231, MDA468, MDA 361, SkBr-3 and MCF7), which exhibit the highest relative levels of ATM expression compared to normal breast tissue (8-fold). Taken together, our results show that breast cancers and cell lines express somewhat higher ATM levels than normal breast tissues. This effect is independent of previous genotoxic exposure since none of the matched pair patients had received neoadjuvant treatment before surgery. It remains to be shown that the increased ATM levels reflect wild-type transcripts with increased biologic activity. However, this is likely to be the case since our partial mutational analysis was negative and known ATM mutations like those seen in AT patients are mostly truncation and frameshift mutations spread throughout the entire gene. Missense mutations are less common. The amplicon used in this study lies in the middle of the ATM open reading frame (nucleotide position 4646-4905 of 9385 nucleotides). Therefore, it appears unlikely that the majority of our over-expressing samples harbored an unrecognized truncation mutation downstream of the probed region. ATM has been thought of as a constitutive protein and very little is known about the regulation of its expression. ATM protein levels remain unchanged during the cell cycle and after DNA damage by γ-IR (2). However, as seen by the variability and extent of increased expression of some cases in this study, significant regulation seems to occur at the transcriptional level in breast cancer. Interestingly, the proliferative myoepithelial cells in sclerosing adenosis, a benign proliferative disease of breast ducts, exhibit an upregulation of ATM protein, while myoepithelial cells of normal breast have low ATM levels (31). Therefore, a speculative but reasonable interpretation of our results is that the proliferative program of tumor cells elicits a mild upregulation of the ATM gene in breast cancer.

Our ATM measurements for the breast cancer group is in agreement with the ATM levels and variance determined by Waha *et al*, who compared breast carcinomas from 39 patients to normal breast tissues from 4 unmatched control individuals (27). However, the two studies differ in size and values for their control groups (29 controls in this study, of which 11 were matched). Since Waha *et al* found a high ATM expression in their 4 unmatched controls (geometric mean of 5.6), the authors concluded that breast cancers exhibit a reduced ATM level. Therefore, the reason for the different conclusions is unlikely to be technical but could be due to a limited control sample size in the study of Waha *et al*. Of note is that both studies employed the same RT-PCR method including primers and competitors. Taken together, our study does not support the conclusion that decreased ATM expression is specifically associated with neoplastic potential and a reliable marker of breast cancer.

ATM is a member of the phosphatidylinositol-3 kinase family, a conserved family of very large proteins required for a DNA damage-sensitive checkpoint pathway in yeast, *Drosophila* and mammalian cells. Homology is conferred through the carboxy-terminal phosphatidylinositol 3-kinase (PI3-K) domain with 60% homology among family members. A second, cysteine-rich region of lesser homology (50% among family members) is present immediately upstream. ATM is a so-called 'stress kinase' and a DNA strand-break sensor that is involved in the γ-IR induced DNA damage response (33,34), meiotic recombination (35,36) and telomere length monitoring (37). P53 and the non-receptor tyrosine kinase c-abl are important downstream signaling targets for ATM (33,34,38). Moreover, ATM plays a role in p53-independent S and G2/M checkpoints. While ATM exhibits a suppressor role with loss of function mutations in specific sporadic and familial lymphoid malignancies (T-PLL and B-CLL), no convincing evidence for such a role could be shown in sporadic breast cancer despite considerable effort. Consistent with this data, our study shows a lack of defective ATM expression in this disease and therefore does not support a role for ATM in sporadic breast cancer risk or development.

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# Oncogenes Induce and Activate Endogenous p73 Protein*

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**The identification of upstream pathways that signal to *TP73* is crucial for understanding the biological role of this gene. Since some evidence suggests that *TP73* might play a role in tumorigenesis, we asked whether oncogenes can induce and activate endogenous *TP73*. Here, we show that endogenous p73 α and β proteins are up-regulated in p53-deficient tumor cells in response to overexpressed E2F1, c-Myc, and E1A. E2F1, c-Myc, and E1A-mediated p73 up-regulation leads to activation of the p73 transcription function, as shown by p73-responsive reporter activity and by induction of known endogenous p73 target gene products such as p21 and HDM2. Importantly, E2F1-, c-Myc-, and E1A-mediated activation of endogenous p73 induces apoptosis in SaOs-2 cells. Conversely, inactivation of p73 by a dominant negative p73 inhibitor (p73DD), but not by a mutant p73DD, inhibits oncogene-induced apoptosis. These data show that oncogenes can signal to *TP73 in vivo*. Moreover, in the absence of p53, oncogenes may enlist p73 to induce apoptosis in tumor cells.**

*TP53* is a crucial tumor suppressor for preventing the malignant transformation of cells. Surprisingly, despite *TP53*'s central role in carcinogenesis, no related genes were known for 20 years. In 1997, two novel family members were identified and termed *TP73* and *TP63* (1–4). p73 shares 63% identity with the DNA-binding region of p53 including the conservation of all DNA contact residues, 38% identity with the tetramerization domain, and 29% identity with the transactivation domain. In contrast to *TP53*, human *TP73* produces six C-terminal splice variants (p73 α–Φ) (1, 5, 6). For example, *TP73* α encodes all 14 exons, while *TP73* β lacks exon 13. In addition, mouse *TP73* has an alternative promoter in intron 3, which encodes a p73 protein that lacks the transactivation domain (ΔN p73) and acts as a dominant negative suppressor of p73 α (7). When ectopically overexpressed in cell culture, p73 α and β closely mimic p53 activities. Ectopic p73 β, and to a lesser extent p73 α, transactivate many p53-responsive promoters, although relative efficiency differences on a given promoter are observed (8–10). Like p53, p73 forms a complex with p300/CBP, which mediates transcription by p73 (11). Ectopic p73 also promotes apoptosis irrespective of the p53 status (1), and overexpression of p73 α, β, and δ suppresses focus formation, while p73 γ does not (5, 8). The suppressor activities of isoforms ε and Φ have not been determined.

Despite this experimental evidence, the role of *TP73* in tumorigenesis is as yet unclear. Current genetic data have ruled out that *TP73* is a Knudson-type tumor suppressor. Although *TP73* maps to chromosome 1p36.3, which undergoes frequent loss of heterozygosity in breast cancer, neuroblastoma, and several other cancers (12), mutations in the *TP73* gene are extremely rare in human tumors. Initially, imprinting of the *TP73* locus was thought to be an explanation to satisfy the two-hit hypothesis in tumors with loss of heterozygosity but no mutations. However, imprinting is highly variable from patient to patient and tissue to tissue (6, 13–15). In fact, in lung, esophageal, gastric, and renal carcinoma, the second *TP73* allele is specifically activated in the tumor compared with the normal tissue of origin (loss of imprinting) (16–19). Furthermore, p73-deficient mice lack a spontaneous tumor phenotype but have neurological and immunological defects (7).

Both differences and similarities to p53 are found with respect to p73 inactivation by viral oncoproteins. SV40 T antigen, adenovirus E1B 55-kDa protein, and HPV E6, which all target and inactivate p53 during host cell transformation, do not target the p73 protein physically or functionally (20–22). Indeed, ectopic p73, but not p53, induces apoptosis in E6-transformed cells, highlighting *TP73*'s potential for gene therapy in HPV-mediated cancers (23). However, the adenovirus E4orf6 oncoprotein specifically represses p73 but not p53 transactivation in some experimental systems (22, 24), indicating that adenoviral transformation targets both p53 and p73 via different viral products. Stable transfectants of a human rhabdoid tumor cell line expressing E1A or adenovirus 5 large E1B showed increased p73 levels, although functional activation of the protein was not tested (24). The Tax protein of the human T-cell leukemia virus type 1 represses the transactivation function of p73 α and β via a p300-dependent mechanism (25). Moreover, analogous to p53, Mdm2 suppresses p73 transactivation function via a negative feedback loop (26–28). However, in contrast to p53, cellular Mdm2 and the HPV E6 protein do not mediate degradation of exogenous p73 (26, 27, 29), suggesting that the regulation of p73 degradation might be distinct from the one regulating p53.

In all normal human tissues studied, p73 is expressed at very low levels (13, 30). In contrast, multiple primary tumor types and tumor cell lines overexpress p73. Work by us and others showed that the most common cancer-specific alteration is an overexpression of p73 rather than a loss. To date, p73 overexpression has been found in tumors of breast, neuroblastoma, lung, esophagus, stomach, colon, bladder, ovary, ependymoma, hepatocellular carcinoma, and myeloid leukemia (CML blast crisis and acute myeloid leukemia) (6, 13, 14, 17–19, 31–36). For example, using quantitative reverse transcription-polymerase chain reaction, we found overexpression (5–25-fold) of *TP73* mRNA in 38% of 77 invasive breast cancers and in 5 of 7 breast cancer cell lines (13–73-fold) but not in normal breast (6). Likewise, we found that *TP73* mRNA is overexpressed in a

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subset of neuroblastoma and related embryonal tumors (8–80-fold) and in 12 of 14 neuroblastoma cell lines (8–90-fold) (13). The cause of p73 overexpression is unknown.

Unlike p53 protein, which becomes stabilized and activated in response to a very broad spectrum of cellular stresses, little is known about the upstream signals that induce a p73 response. p73 is not activated by UV, actinomycin D, doxorubicin, and mitomycin C (1, 37), all of which stabilize and activate p53. However, recently it has been shown that endogenous p73 is activated for apoptosis in response to cisplatin and γ-ionizing irradiation in a pathway that depends on the nonreceptor tyrosine kinase c-Abl (38–40). This DNA-damage-dependent upregulation of p73 by c-Abl may be partly responsible for p53-independent apoptosis. What is already evident is that *TP73*, at least qualitatively, utilizes the same or very similar effector pathways as *TP53*. Complete identification of all upstream signals of *TP73* that operate physiologically will be very important in elucidating its normal function and role, if any, in tumorigenesis. We therefore asked whether deregulated oncogenes, which are a preeminent signal for triggering p53-dependent transactivation and apoptosis, also induce and activate p73 function. We report that overexpression of cellular and viral oncogenes do up-regulate endogenous p73 proteins and activate their transactivation function. Moreover, E2F1-, c-Myc-, and E1A-mediated activation of endogenous p73 induces apoptosis in p53-deficient tumor cells. Disruption of p73 function by a dominant negative p73 inhibitor (p73DD), but not by a mutant version thereof, inhibited oncogene-induced apoptosis. These data show that oncogenes can signal to *TP73 in vivo*.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Reagents*—The human lung carcinoma line H1299 and the human osteosarcoma line SaOs-2 each carry a homozygous deletion for *TP53* and were used for transfection. Other cell lines were SK-N-AS (human neuroblastoma), MRC5 (human diploid fibroblasts), and COS (monkey kidney cells). All cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Cisplatin was purchased from Sigma.

*Plasmids*—The following mammalian expression plasmids were used: pE1A, expressing E1A 12 S protein driven by the natural adenovirus E1A enhancer/promoter (41); pCMXM45 E2F1, expressing human E2F1 (42); pmc-Myc, expressing mouse c-Myc protein (gift from M. Cole, Princeton University); pC53-C1N3 expressing human wild type p53; and pcDNA3-p73 α and pcDNA3-p73 β expressing HA[1]-tagged human p73 α and β (5).

pcDNA3-p73DD and pcDNA3-mtp73DD were gifts of Dr. William Kaelin and are described in detail in Ref. 43. Briefly, pcDNA3-p73DD expresses T7-tagged amino acids 327–636 of human p73 α and acts as a dominant negative p73 *in vivo*. The corresponding loss-of-function mutant named mtp73DD, which contains a L371P point mutation, is inactive as inhibitor. Green fluorescent protein expression plasmid (CLONTECH) was cotransfected in transient transfections to verify relative transfection efficiency. The p53/p73-responsive reporter construct PG13-Luc and its mutant counterpart MG15-Luc (gift of B. Vogelstein) were used for luciferase assays. p73 β exhibited 80% of the activity of p53 (on a molar basis) in transactivating the PG13-Luc reporter and no activity with the MG15-Luc reporter (data not shown).

*Antibodies and Immunoblots*—Cell lysates were prepared as described (45), subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nylon membranes. Immunoblots were visualized by ECL (Pierce). Antibodies to p73 were the monoclonal GC15 (AB-3 from Oncogene Science; recognizes amino acids 380–499 of human p73 β), the polyclonal p73N (raised in rabbit against the N-terminal peptide FHLEGMTTSVMAQF), and the polyclonal p73 α (raised in rabbit against a C-terminal α-specific peptide; gift of K. Vousden). Oncogene expression was confirmed with antibodies against E1A (clone 13S-5; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), E2F-1 (clones C20 and KH95; Santa Cruz Biotechnology), and c-Myc (Ab-3; Oncogene Science). Antibodies against p53 (clone DO-1), HDM2 (clone IF2), and p21 (Ab-1) were from Oncogene Science. For normalization of protein loading, blots were reprobed with antibodies specific for vimentin (BioGenex).

*Transfections*—H1299 cells were plated in 60-mm dishes and grown overnight to 80% confluence. For transient transfections, 2 μg of expression plasmid or empty vector was cotransfected with 200 ng of green fluorescent protein-encoding plasmid using the LipofectAMINE Plus reagent (Life Technologies, Inc.). Cells were collected after 24 h. Stable transformants were seeded into P100 plates ($1 \times 10^7$ cells) and selected for 21 days in medium containing 0.5 mg/ml G418 (Life Technologies), ring-cloned, and expanded into single cell clones. For the SaOs-2 experiments (see Fig. 5*D*), transformants were pooled after 3 weeks of G418 selection and transiently transfected with c-Myc prior to parallel TUNEL assays and immunofluorescence staining 24 h later. For luciferase assays, cells were seeded into 24-well plates and transfected with an expression vector or empty vector (400 ng) together with the p53-responsive PG13-Luc from firefly (80 ng) and pRL-TK *Renilla* luciferase cDNA (8 ng). To test the effect of the dominant negative inhibitor and its mutant, each well was transfected with 100 ng of expression vector plus 300 ng of empty vector, with 100 ng of expression vector plus 300 ng of p73DD, or with 100 ng of expression vector plus 300 ng of mtp73DD, together with the reporter constructs as above. Luciferase activity was measured after 24 h by the dual luciferase reporter assay (Promega), and transfection efficiency was standardized against *Renilla* luciferase.

For apoptosis, SaOs-2 cells were seeded in duplicates into eight-well chambers 48 h prior to transfection. At about 70% confluence, cells in duplicate wells were transfected with expression plasmid (150 ng) plus empty vector (350 ng) or expression plasmid (150 ng) plus p73DD (350 ng) or expression plasmid (150 ng) plus mtp73DD (350 ng). After 24 h, cells were fixed and processed in parallel for the TUNEL assay (Roche Molecular Biochemicals) and for immunofluorescence using the appropriate antibodies against p73, E2F1, c-Myc, or E1A. Expression was reproducibly about 30%, similar among all constructs and evenly distributed throughout the wells. For each construct, TUNEL-positive cells (494 fields at 40×) and transfected cells (30 fields at 40×; around 200 cells) from duplicate chambers were counted, and the percentage of apoptosis of transfected cells was determined after correction for background with vector alone (500 ng per well). Experiments were performed 3–6 times, depending on the construct.

## RESULTS

*Endogenous p73 α and β Proteins Are Induced in Response to E2F1, c-Myc, and E1A*—The great majority of functional and regulatory p73 studies to date have used ectopically expressed p73 proteins. To reliably detect endogenous p73 proteins, we used three different p73-specific antibodies. They comprised a p73 β-specific monoclonal (GC15), a p73 α-specific polyclonal raised against a C-terminal peptide (poly-p73α) and a pan-p73 polyclonal raised against an N-terminal peptide (poly-p73N). The antibodies detected endogenous full-length p73 α and β in several tumor cell lines including the p53-deficient human H1299 line (Fig. 1). H1299 cells express a basal level of p73 α (*lane 1*) and β (*lane 9*). Simian COS cells express the highest level of p73 α (*lane 4*) and p73 β (*lanes 5* and *8*). In contrast, SK-N-AS cells express no detectable p73 α (*lane 3*) or p73 β (*lane 6*), consistent with previous reports (20).

Next we tested whether viral and cellular oncogenes, which are major upstream signals for *TP53* activation, are also physiologically relevant for triggering the induction of endogenous TP73. To this end, H1299 cells were transiently transfected with various oncogene-encoding plasmids. Their expression was verified by immunoblotting with the respective antibodies (Fig. 2*A*). Both p73 α and β proteins were markedly induced after expression of E2F1, c-Myc, and adenoviral E1A when compared with empty vector. A representative experiment is shown in Fig. 2, *B* and *C*. By molecular weight standards and reactivity with the polyclonal p73N antibody, only full-length proteins were observed, with p73 α migrating at about 83 kDa and p73 β at about 75 kDa. The equal loading of immunoblots was confirmed by reblotting the membranes with an antibody

[1] The abbreviations used are: HA, hemagglutinin; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

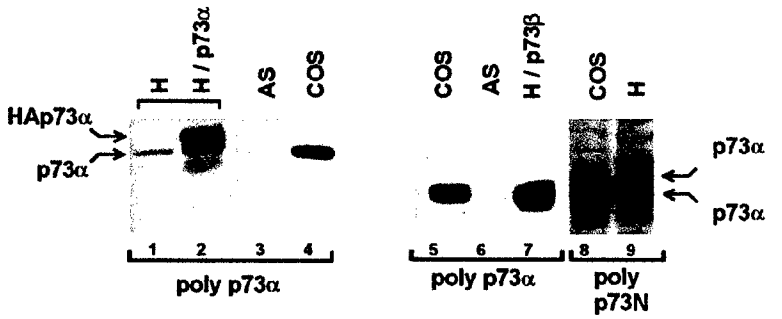


FIG. 1. **Endogenous p73 α and β proteins are detectable by various p73-specific antibodies.** Immunoblots of cell lysates from p53-deficient H1299 cells (*H*), neuroblastoma SK-N-AS cells (*AS*), and SV40 T antigen-transformed simian COS cells are shown. Polyclonal p73N was raised against an N-terminal peptide, polyclonal p73 α against a C-terminal α-specific peptide, and GC15 against amino acids 380–499 of human p73 β. Transiently transfected HA-tagged p73 α and β serve as positive control. HA-tagged exogenous p73 α shows a retarded migration (*lane 2*), while HA-tagged exogenous p73 β does not (*lane 7*). Each lane contains 30 μg of total cell extract. Detection was by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

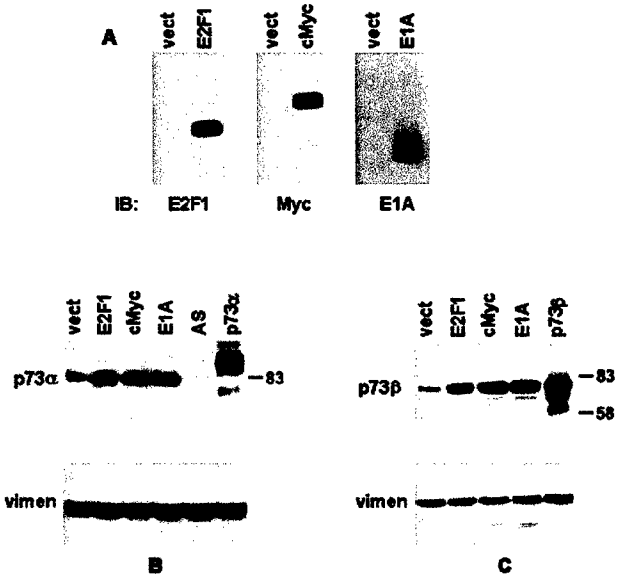


FIG. 2. **p73 α and β proteins are induced in response to cellular and viral oncogenes.** *A*, immunoblots of H1299 cells after transient transfection with empty vector or expression vectors for E2F1, c-Myc and E1A. Blots were developed with the indicated antibodies. Immunoblots (*IB*) are shown of H1299 cells probed with polyclonal p73 α antibody (*B*) or with p73 β antibody (GC15) (*C*) after transient transfection with empty vector (*vect*) or expression vectors for E2F1, c-Myc, and E1A. HA-tagged p73 α and p73 β transfected into H1299 cells serve as positive controls, respectively. Exogenous p73 α shows a retarded migration. SK-N-AS cells (*AS*) are used as negative control. Membranes were reblotted for vimentin to ensure equal loading.

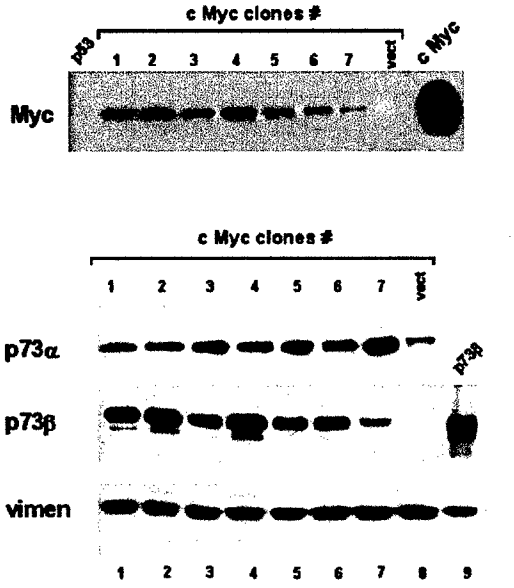


FIG. 3. **Stable H1299 clones overexpressing c-Myc recapitulate the overexpression of p73 proteins.** *Upper panel*, Myc immunoblot of all seven H1299 subclones overexpressing c-Myc and an empty vector clone. The first and last lanes show H1299 cells transiently transfected with p53 and c-Myc, respectively. Thirty μg of total protein per lane were loaded. *Lower panels*, immunoblots of the seven subclones and the empty vector clone were probed for p73 α and β. *Control lane 9* shows H1299 cells transiently transfected with p73 β expression plasmids. The p73 β blot was reprobed for vimentin to ensure equal loading (30 μg/lane).

specific for vimentin. Since transfection efficiency in these transient assays ranged between 30 and 40% (judged by coexpressed green fluorescent protein), the actual degree of induction is higher than the one detected here by immunoblots, since this assay is subject to dilution by untransfected cells. Significant induction of endogenous *TP73* was also seen after oncogene transfection in SaOs-2 cells (see Fig. 5*B*).

*Stable H1299 Clones Overexpressing c-Myc Recapitulate the Up-regulation of p73 Proteins*—Since transient overexpression of oncogenes induces the accumulation of p73 α and β (Fig. 2), we next asked if stable oncogene overexpression similarly would lead to long term up-regulation of p73. To this end, vector control and c-Myc-transfected H1299 cells were selected in G418 for 3 weeks. Of the surviving c-Myc foci, seven were randomly picked, ring-cloned, and successfully expanded into stable sublines. As shown in Fig. 3, all seven clones overexpressed c-Myc, albeit to various degrees compared with vector control. Clones 1, 2, and 4 showed the highest c-Myc expression. Cell extracts were then probed for p73 protein levels. As already seen with transient c-Myc transfections, p73 α and β were found to be induced above base line in all seven subclones (Fig. 3). p73 β induction appeared proportional to the level of c-Myc expression in the individual clones, with clones 1, 2, and 4 showing the highest p73 β accumulation. Interestingly, for reasons that remain to be elucidated, p73 α reproducibly behaved inversely to β (*i.e.* whenever β was high, α was low, and *vice versa*). Taken together, this result indicates that stable deregulation of the c-Myc oncogene recapitulates the p73 overexpression already seen with transient deregulation of c-Myc.

*Oncogene-mediated Up-regulation of Endogenous p73 Protein Leads to p73 Transcriptional Activation*—We then tested whether the oncogene-mediated up-regulation of endogenous p73 translates into activation of p73 transcriptional function.

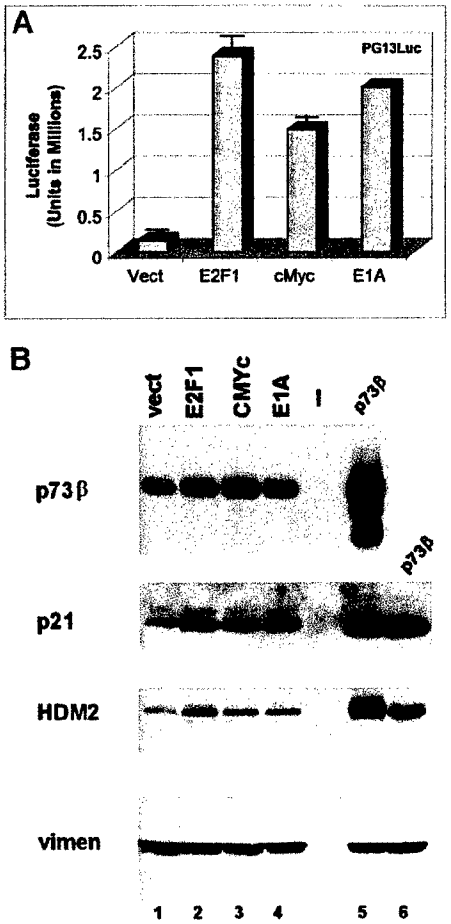


FIG. 4. **Oncogene-mediated induction of endogenous *TP73* leads to functional activation of p73.** *A*, oncogene-mediated activation of the p73-responsive reporter PG13-Luc. H1299 cells were transiently transfected with empty vector or the indicated expression plasmids (400 ng each) together with 80 ng of PG13-Luc and 8 ng of *Renilla* luciferase. Luciferase activity was measured 24 h later and standardized for *Renilla* activity. E2F1 exhibited a 16.5-fold, c-Myc a 10.3-fold, and E1A a 13.9-fold induction compared with vector controls. Results are the average ± S.D. of three independent experiments. *B*, H1299 cells were transiently transfected with empty vector (*vect*) or the same amount of the indicated expression plasmids (2 μg). Total cell extracts were immunoblotted for p73 β, Waf1p21, and HDM2. Membranes were reblotted for vimentin to ensure equal loading (30 μg/lane). H1299 cells directly transfected with 2 μg of HA-p73 α and β expression plasmids, respectively, are shown as positive controls (*lanes 5* and *6*). Four independent experiments gave similar results.

To this end, we carried out luciferase reporter assays in transiently transfected H1299 cells using the p53/p73-responsive PG13-Luc reporter. As shown in Fig. 4*A*, all three oncogenes were able to activate p73 reporter activity. E2F1 exhibited a 16.5-fold, c-Myc a 10.3-fold, and E1A a 13.9-fold induction of the p73-responsive reporter compared with vector controls. These data indicate that oncogenes induce the transcriptional activation of endogenous p73.

*Oncogene-mediated Up-regulation of Endogenous p73 Leads to Activation of TP73 Response Genes—TP73* shares many response genes with *TP53 in vivo*. This has been shown in several cell systems using transient or inducible expression of ectopic p73 (1, 8, 9, 38). To further support our previous results, we tested whether oncogene-mediated accumulation of endogenous p73 leads to the induction of *TP73* target gene products. When p53-deficient H1299 cells were transiently transfected with expression plasmids for E2F1, c-Myc, and E1A, endogenous p73 β protein was again up-regulated (Fig. 4*B*, *top panel*, *lanes 2–4*, compare with empty vector in *lane 1*). Oncogene expression was confirmed by immunoblots (data not shown). The p73 up-regulation was accompanied by the induction of the *TP73* response gene products Waf1p21 and HDM2 (Fig. 4*B*, *middle panels*, *lanes 2–4*; compare with empty vector in *lane 1*). *Lanes 5* and *6* are positive controls after direct transfection of p73 α and β expression plasmids. Although the induction is modest, it could be due to the fact that we are relying on endogenous rather than ectopic p73. Furthermore, since transfection efficiency was only between 30 and 40%, the actual induction is probably higher than the one detected here by immunoblots, due to the dilutional effect by the untransfected majority of cells. E2F1 reproducibly caused a stronger transactivation of the p21 and HDM2 genes than c-Myc and E1A. Previous studies have shown that Waf1p21 and HDM2 are direct *in vivo* targets of ectopic p73, as demonstrated by detecting their products in response to inducibly expressed p73 α and β in EJ (mutant p53) and p53-deficient H1299 cells (9, 38). In p53-expressing cells, transactivation of HDM2 in response to a broad spectrum of overexpressed oncogenes including the panel used here has been shown to be indirect and strictly dependent on p53 (for a review, see Ref. 47). Transactivation of p21 by c-Myc and E1A is also p53-dependent, although E2F1, in addition to activating the p21 promoter through p53, can transactivate p21 directly (47). Overall, these data strongly suggest that with the partial exception of E2F1, the induction of p21 and HDM2 in response to oncogenes is mediated through p73 in H1299 cells. Together with the reporter assays, these results are consistent with the idea that in the absence of p53, oncogene-induced endogenous p73 is capable of activating its target genes.

*Oncogene-mediated Activation of Endogenous p73 Induces Apoptosis in p53-deficient Tumor Cells; Conversely, Inactivation of p73 Inhibits Oncogene-induced Apoptosis*—The activation of the p73 transcription function by oncogenes suggested that these upstream signals might also induce the activation of the apoptotic function of p73. To test this prediction, we performed apoptosis assays on transiently transfected SaOs-2 cells using the *in situ* TUNEL assay.

To confirm that the oncogene-induced apoptotic activity is mediated through *TP73*, we tested the effect of a coexpressed dominant negative inhibitor of p73 (p73DD) (43). p73DD is modeled after the dominant negative p53 inhibitor (p53DD) (48) and encodes amino acids 327–636 of human p73 α. p73 DD acts as a specific inhibitor of p73 α and β-dependent transactivation (Fig. 5*A*) but not of p53-dependent transactivation (data not shown). Moreover, p73DD binds to p73 α and β proteins *in vitro* and *in vivo* but not to p53 protein (43). When coexpressed with p73 β, p73DD suppressed PG13-Luc reporter activity by 98%, making it an efficient specific inhibitor (Fig. 5*A*). In contrast, an inactive point mutant called mtp73DD, carrying a L371P exchange, does not bind p73 α and β and does not block p73 α- and β-dependent transactivation (43). mtp73DD was completely incapable of suppressing the reporter activity of p73 β (only 4% inhibition compared with p73 β alone) (Fig. 5*A*). Together, these results indicate the specificity of these two reagents.

Prior to doing apoptosis assays, we needed to demonstrate that oncogenes induce endogenous p73 in p53-deficient SaOs-2. As seen in Fig. 5*B*, expression of E2F1, c-Myc, and E1A in these cells markedly induced p73 levels, analogous to what we already saw in H1299 cells (see Fig. 2). Importantly, the induced p73 protein was functionally active (Fig. 5*C*). After transient transfection, all three oncogenes induced apoptosis in SaOs-2 cells, which resembled the one seen after transfecting p73 β directly (*light grey columns*). Moreover, the apoptotic activity of

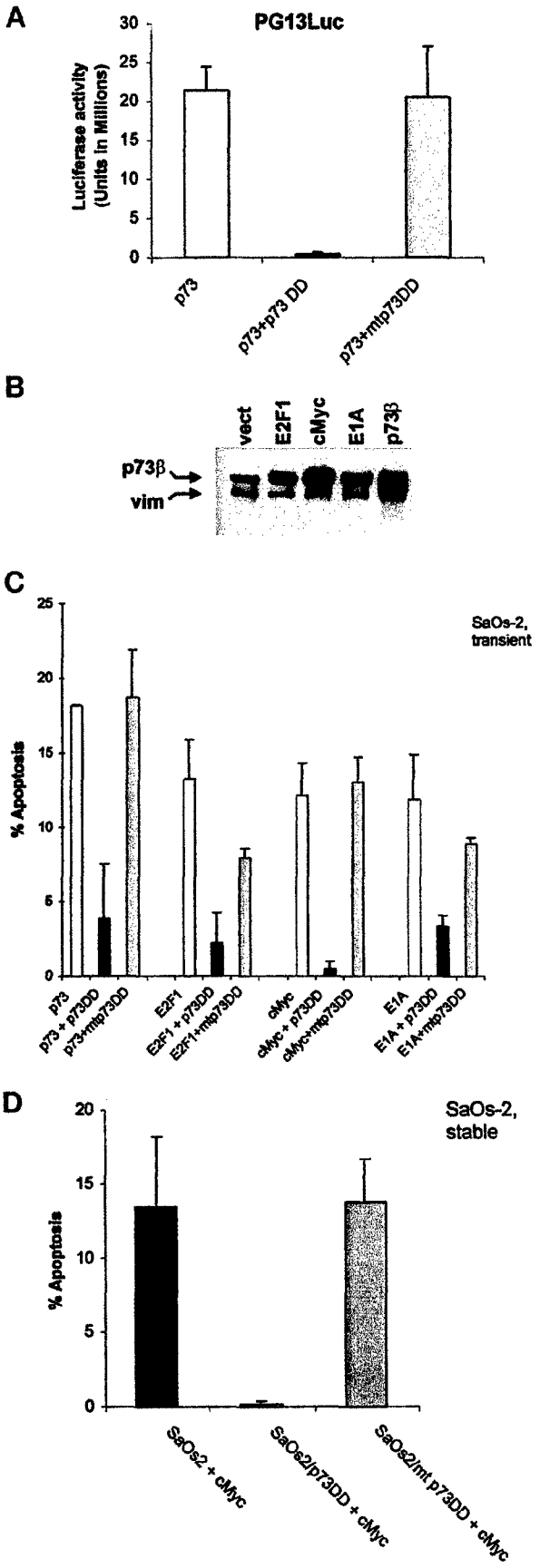


FIG. 5. **Oncogene-mediated activation of endogenous p73 induces apoptosis in p53-deficient tumor cells. Inactivation of p73 inhibits oncogene-induced apoptosis.** *A*, inhibition of p73 β-dependent transactivation by the dominant negative inhibitor p73DD but not by the inactive mtp73DD (L371P) mutant. H1299 cells were transiently transfected with either p73 β (100 ng) plus empty vector (300 ng), p73 β (100 ng) plus p73DD inhibitor (300 ng), or p73 β (100 ng) plus mtp73DD mutant (300 ng), together with 80 ng of PG13-Luc and 8 ng of *Renilla* luciferase. Luciferase activity was measured 24 h later and standardized for *Renilla* activity. Results are the average ± S.D. of three independent experiments. *B*, immunoblot of SaOs-2 cells after transient transfection with empty vector or expression vectors for E2F1, c-Myc, and E1A. The blot was developed with a mixture of GC15 and vimentin antibodies. *C*, SaOs-2 cells in duplicate wells were transfected with either expression plasmid (150 ng) plus empty vector (350 ng), expression plasmid (150 ng) plus p73DD inhibitor (350 ng), or expression plasmid (150 ng) plus mtp73DD mutant (350 ng). After 24 h, cells were processed in parallel for TUNEL and for immunofluorescence to determine expression. The percentage of apoptosis of transfected cells is shown after correction for background with vector alone (500 ng/well). The results represent the average ± S.D. of three independent experiments. *D*, SaOs-2 cells were transfected with empty vector or expression vectors for p73DD or mtp73DD. Cells were selected for 3 weeks in G418 (550 μg/ml) prior to transfection with c-Myc or empty vector. Cells were seeded into duplicate eight-well chamber slides. After 24 h, cells were processed in parallel for TUNEL and for immunofluorescence to determine expression. The percentage of apoptosis of transfected cells is shown after correction for background with vector alone (500 ng/well).

each oncogene was greatly suppressed or abrogated when oncogenes were coexpressed with the dominant negative inhibitor p73DD, with 84% suppression for E2F1, 96% for c-Myc, and 72% for E1A (*black columns*) (Fig. 5*C*). The suppression of oncogene-mediated apoptosis by a p73-specific inhibitor strongly suggests that E2F1, c-Myc, and E1A mediate their apoptotic effects through p73. This conclusion is further confirmed by the lack of significant suppression when p73DD is exchanged for the functionally inactive inhibitor mtp73DD (*dark grey columns*) (Fig. 5*C*). Furthermore, the dominant negative p73DD inhibitor also prevented apoptosis when it was stably expressed in SaOs-2 cells, while its mutant counterpart failed to prevent apoptosis (Fig. 5*D*). Also, E2F1- and c-Myc-induced apoptosis was found to be partially mediated through p73 in transiently transfected HeLa cells (data not shown; E1A not tested). Taken together, these data show that E2F1-, c-Myc-, and E1A-mediated apoptosis in p53-deficient tumor cells largely depends on p73.

## DISCUSSION

Our results show that during short term exposure, overexpression of three different oncogenes induce the endogenous *TP73* gene and activate it for transcriptional and apoptotic function in p53-deficient cells. We demonstrate that disruption of p73 function inhibits oncogene-induced apoptosis in p53-deficient human tumor cells. Collectively, our findings identify an important novel afferent signaling pathway to p73 that appears to operate *in vivo*. This conclusion is in agreement with evidence from several other experimental systems. For example, Myc-induced apoptosis in $p53^{-/-}$ mouse embryo fibroblasts is only attenuated but not abrogated compared with wild type p53 cells, supporting the idea that part of the apoptotic response is p53-independent (49). Based on our results, this p53-independent apoptosis may be due, at least in part, to p73. The demonstration that endogenous p73 is induced and activated by oncogenes is in line with the known p73 activation by cisplatin and γ-ionizing irradiation (38–40). Together, these data suggest that *TP73* can be a component of a tumor surveillance pathway, which in the absence of p53 might respond to different types of incoming signals in a p53-compensatory fashion.

While this work was ongoing, W. G. Kaelin's group obtained similar data on the relationship of E2F1 and p73 (43). They also found that p73 mediates E2F1-induced apoptosis in SaOs-2 cells and $p53^{-/-}$ mouse embryo fibroblasts. Moreover,

they identified the mechanism of this interaction to be directly transcriptional; *i.e.* E2F1 induces transcription of full-length p73 $\alpha$ and $\beta$ via E2F1 binding sites in the P1 promoter of *TP73* (43). A functional interaction between E2F1 and p73 was also recently shown in T-cell receptor activation-induced cell death (44). A direct transcriptional mechanism of *TP73* induction by E2F1 is in complete agreement with two predicted E2F1 binding sites at positions −284 and −1862 of the *TP73* promoter (50). Moreover, the c-Myc-mediated up-regulation of *TP73* that we observed most likely also operates via E2F (51–53). E2F1 (as well as E2F2 and E2F3a) is induced by c-Myc via a group of E-box elements in its promoter conferring positive Myc responsiveness. The *TP73* promoter itself has not been reported to contain Myc binding sites. In cells with functional Rb such as in H1299 cells, E1A-mediated up-regulation of *TP73* might again be mediated through E2F1, albeit indirectly via inactivation of Rb by E1A (54). SaOs-2 cells, which were also used in our study, are deficient for Rb, indicating an additional Rb-independent mechanism of *TP73* induction by E1A. Taken together, our paper independently confirms the *E2F1-TP73* signaling pathway and extends it to additional oncogenes, thus broadening the concept of *TP73* activation by oncogenes.

In contrast to transcriptional activation of *TP73* by oncogenes, cisplatin activates endogenous p73 by a posttranscriptional mechanism (38). Cisplatin increases protein stability from 45 min to 2 h without altering *TP73* transcript levels. Protein stability of endogenous and exogenous p73 $\alpha$ has been shown to be regulated in part through proteasome-dependent degradation, since cells accumulate p73 $\alpha$ after treatment with proteasome inhibitors (27, 29, 55). Furthermore, $\gamma$-ionizing irradiation activates p73 through c-Abl-mediated tyrosine phosphorylation without protein stabilization (39, 40). Taken together, it appears that the regulation of p73 activity occurs both on a transcriptional and posttranscriptional level and might depend on the specific activating stimulus.

We also find that stable deregulation of c-Myc in H1299 subclones recapitulates the p73 overexpression seen with transient deregulation of c-Myc. One might ask how stable clones can be generated, given that in transiently transfected tumor cells deregulated c-Myc was able to activate the transcriptional and apoptotic activity of p73. One possibility is that clonal outgrowth of cells with stable overexpression of oncogenes selects for loss of p73 transactivation function. In keeping with this idea, a preliminary analysis on three clones with the highest p73 $\beta$ overexpression suggested that this might be the case. In contrast to transient Myc transfection, Myc clones 1, 2, and 4 (Fig. 3) failed to show evidence of p21 and HDM2 induction compared with vector. The same clones also failed to show transactivation activity in a p73-responsive reporter assay.[2] Moreover, in a previous study on five human breast cancer cell lines with p73 overexpression (four of the five lines were mutant for p53), we found no correlation with p21 mRNA and protein levels (6). While it is tempting to speculate, it is important to point out that more extensive analyses need to be done before definitive statements about the functional consequence of constitutive p73 overexpression in tumor cells can be made. Nevertheless, the sustained p73 overexpression in stable Myc clones is highly reminiscent of the fact that multiple primary human tumor types and tumor cell lines overexpress p73. This includes tumors of breast, neuroblastoma, esophagus, stomach, lung, colon, bladder, ovary, ependymoma, hepatocellular carcinoma, and myeloid leukemia (6, 13, 14, 17–19, 31–36).

Most human tumors harbor deregulated oncogenes, including the Myc gene (56). In particular, many human tumors have suffered a deregulation of the E2F1 activity through mutations that inactivate the Rb pathway (57–59), thus derepressing E2F1-responsive genes. Our finding could provide a framework for the fact that p73 is frequently overexpressed in human tumors. Our stable clones will be a helpful tool in future studies aimed at determining the functional relevance of constitutive p73 overexpression in tumors.

*Acknowledgment*—We thank William G. Kaelin for sharing previously unpublished reagents and data relevant to this work and for useful comments.

*Note Added in Proof*—SaOs-2 cells do not express p63 (Ratovitski, E. A., Pattarajan, M., Hibi, K., Trink, B., Yamaguchi, K., Sidransky, D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1817–1822)

[2] A. Zaika and U. Moll, unpublished observation.

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# ΔNp73, a Dominant Negative Inhibitor of wild-type p53 and TA-p73, is Upregulated in Human Tumours

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**Abstract**

The p53 family member p73 has significant homology to p53, but tumour-associated upregulation of p73 and genetic data from human tumours and p73-deficient mice rule out a classical Knudson-type tumour suppressor role. We report that the human TP73 gene gives rise to an N-terminally truncated isoform, ΔNp73, which is derived from an alternative promoter. ΔNp73 is frequently overexpressed in a variety of primary human cancers with upregulated p73. Functional studies indicate that ΔNp73α, which lacks the transactivation domain of full length p73 (TA-p73), acts as a dominant negative inihibitor of both wild-type p53 and transactivation-competent TA-p73. Moreover, ΔNp73α counteracts apoptosis and tumour cell growth suppression induced by wild type p53 and TA-p73. The underlying mechanism of inhibition is heterocomplex formation between ΔNp73α and e.g. wild type p53. Thus, ΔNp73 mediates a novel inactivation mechanism of wild-type p53 and TA-p73 via a dominant-negative family network. Increased expression of ΔNp73 appears to bestow oncogenic activity upon the TP73 gene, a trait that is selected for in human cancers.

## Introduction

The p53 family member p73 has significant homology to the p53 tumor suppressor. Human full length p73 (TA-p73) shares 63% amino acid identity with the DNA-binding region of TP53 including conservation of all DNA contact residues, as well as 38% and 29% identity with the tetramerization domain and transactivation domain, respectively[1]. Ectopically overexpressed TA-p73α and β (two C-terminal splice variants) largely mimic p53 activities including the induction of apoptosis, cell cycle arrest and the transactivation of an overlapping set of target genes[2,3]. Moreover, deregulation of oncogenes E2F1, cMyc and E1A induces apoptosis in tumour cells in a p53-independent manner by transcriptionally inducing and activating endogenous TA-p73 proteins[4-7]. Furthermore, endogenous TA-p73 is activated to mediate apoptosis by a restricted spectrum of DNA damage such as cisplatin, taxol and γ-irradiation via a pathway that depends on the non-receptor tyrosine kinase c-abl [8-10]. Thus, TA-p73 might function synergistically with p53 in a tumour surveillance pathway. However, despite this homology, data from human tumours and p73-deficient mice argue against a classical Knudson-type tumour suppressor role for the TP73 gene. TP73-deficient mice lack a spontaneous tumour phenotype[11] and inactivating mutations in human tumours are extremely rare (over 900 tumours analyzed to date) (reviewed in Ref. 12). Moreover, while all normal human tissues studied express very low levels of p73, multiple primary tumour types and tumour cell lines overexpress p73, including cancers of the breast, lung, esophagus, stomach,

colon, bladder, ovary, liver, bile ducts, ependymal lining, myelogenous leukemia and neuroblastoma (reviewed in Ref. 12). To date, most studies identifying p73 overexpression in primary human tumours have examined total levels of p73 with a few exceptions that specifically measured TA-p73[13,14] or Ex2Del p73[15]. Importantly, in mouse, an N-terminally truncated ΔNp73, generated from an alternative promoter in Intron 3, plays an essential anti-apoptotic role during p53-driven developmental neuronal death *in vivo* by acting as a dominant negative inhibitor of p53[16]. We therefore sought the human counterpart of ΔNp73 and examined its potential role in cancer.

**The human TP73 gene can produce ΔNp73**

Mouse ΔNp73 differs from TA-p73 by a novel Exon 3', which replaces the first 3 Exons, and is spliced in frame to Exon 4 of the TP73 gene[11]. By sequence alignment of a human genomic BAC clone containing TP73 (GenBank Accession Nr. AL 136528), we identified a region with 77% identity to the 5'UTR of mouse ΔNp73 mRNA (Genbank Accession Nr. Y 19235). This allowed us to predict the human Exon 3' and design isoform-specific primers for human ΔNp73. Full length ΔNp73α cDNA, spanning Exons 3'-14 and including 220 bases of 5'UTR and 103 bases of 3'UTR, was cloned by RT-PCR from total RNA of human placenta and MDA 231 breast cancer cells and sequence confirmed (Fig. 1a). Human Exon 3' consists of 13 unique amino acids with almost complete identity to mouse Exon 3' (12 of 13 residues are identical) (Fig. 1a). Human Intron 3 contains the predicted TATA box 30 nt upstream of the transcriptional start site, which is located 7.6 kb downstream of Exon 3.

**Analysis of ΔNp73 and TA-p73 expression in human tumours**

Unique cDNA primers were designed for the specific amplification of ΔNp73 from tissues by semiquantitative RT-PCR (Fig. 1b). We then determined the expression levels of ΔNp73 in 52 breast cancers and compared them to 8 unrelated normal breast tissues (Fig. 2a). All but one normal breast tissues showed either non-detectable or very low levels of ΔNp73. In contrast, 16 of 52 breast cancers (31%) expressed ΔNp73 levels that were between 6 and 44-fold higher than the normal tissue average (indicated by red line in Fig. 2a). Since we previously showed that breast cancers also overexpress TA-p73[14], we next used isoform-specific semiquantitative RT-PCR to measure ΔNp73 and TA-p73 simultaneously. Among these 16 cancers with a 6 to 44-fold increase of ΔNp73, 12 cancers showed much higher upregulation of ΔNp73 than of TA-p73 (data not shown).

We next analyzed a spectrum of tumours that were matched with the patients' normal tissues of origin (Table 1; examples in Fig. 2b). Of 16 matched cancer pairs (ovarian, breast, cervix, kidney and colon cancer) and 1 large benign ovarian tumour (serous cystadenoma), ΔNp73 was specifically upregulated 3 to 78-fold in 10 tumours (63%) (Table 1, second column), while TA-p73 was upregulated 3 to 155-fold in 7 tumours (41%) (third column), compared to their respective normal tissues of origin. Importantly, when upregulation of ΔNp73 and TA-p73 in a given tumour is analyzed more closely, in 7 of the 10 tumours (70%) ΔNp73 is upregulated disproportionately to a far greater degree than TA-p73 upregulation (fourth column). These include 4 ovarian tumours, 2 breast cancers and

1 cervical cancer. Their excessive rise in ΔNp73 compared to their rise in TA-p73 ranges from 5 to 16-fold. Four cancers within this group exhibited exclusive upregulation of ΔNp73 (tumours Nr. 5, 6, 8 and 10). Only 2 tumours exhibited an inverse ratio with an excessive TA-p73 rise compared to their ΔNp73 rise (tumours Nr. 3 and 7). One additional tumour (Nr. 9) with concomitant upregulation of both isoforms could not be quantitated because the corresponding normal tissue levels were undetectably low (ND). Furthermore, among the remaining 7 tumours that did not upregulate ΔNp73, 2 of 4 tumours that we analyzed (Nr. 11 and 17) showed tumour-specific upregulation of Ex2Del p73 instead of ΔNp73. Both of those tumours also failed to upregulate TA-p73. Ex2Del p73 is a dominant negative isoform of p73 lacking the transactivation domain, which is generated from the TA promoter by splicing out Exon 2. It has been previously shown to be upregulated in some ovarian cancers and breast cancer cell lines1[1,15,17]. Thus, a total of 9 tumours in our series (53%) exhibit either exclusive or excessive upregulation of dominant negative p73. Moreover, of the 14 tumours available for p53 mutational analysis by immunocytochemistry, 10 tumours had undetectable p53 levels, suggesting wild-type status, while 4 tumours showed nuclear overexpression, suggesting p53 mutation. This estimated mutational rate (29%) is in good agreement with the reported rates of p53 mutations in these tumor types (about 30 %)[18]. Thus, it appears that in a total of 8 analyzable tumours with disproportional upregulation of a dominant negative p73 isoform (7 tumours with ΔNp73 and 1 tumour with Ex2Del), 7 tumours likely harbored a wild type p53 genotype, while only 1 tumour exhibited a concomitant p53 mutation (fifth column).

**ΔNp73 is an Efficient Dominant Negative Inhibitor of wild-type p53 and TA-p73 function**

To test the hypothesis that human ΔNp73 is a dominant negative inhibitor of human wild-type p53 and TA-p73, we first performed reporter assays with expression plasmids for wild-type p53, TA-p73α and β and a p53/TA-p73-responsive Luciferase reporter in the presence or absence of ΔNp73α in p53 *null* H1299 cells and SaOs2 cells (Fig. 3 and data not shown). ΔNp73α exhibited a dose-dependent, complete suppression of the transcriptional activity of wild-type p53 and TA-p73α and β (Fig. 3a and data not shown). Moreover, a molar ratio of 1:1 between ΔNp73α and wild-type p53 potently inhibited p53 activity, yielding a reduction by 92% (Fig. 3b). In comparison, a 1:1 molar ratio reduced TA-p73β activity by 62%, although a 3-fold molar excess of ΔNp73α completely blocked TA-p73β activity (97% reduction) (Fig. 3b). The latter suggests that ΔNp73α is a stronger inhibitor of wild-type p53 than of TA-p73β. Furthermore, ΔNp73α also efficiently suppresses endogenous target gene products of wild-type p53 and TA-p73 (Fig. 3c). In HeLa and H1299 cells, transfection of wild-type p53 or TA-p73 induces endogenous HDM2, 14-3-3σ and p21Waf1 compared to basal levels seen with empty vector. However, the concomitant expression of ΔNp73α strongly suppresses each of these response gene products (Fig. 3c, compare lanes 2 and 3 and lanes 4 and 5).

Moreover, ΔNp73α is a strong inhibitor of apoptosis induced by wild-type p53 and TA-p73 (Fig. 4a). HeLa and SaOs2 cells undergo wild-type p53- and TA-p73 dependent cell death as assessed by Annexin V staining and TUNEL assay. This apoptotic activity is completely abolished by co-expression of ΔNp73α (Fig. 4a and data not shown). The

inhibitory action of ΔNp73α is dependent on the presence of transcription-competent wtp53 and TA-p73, since ΔNp73α alone cannot affect apoptosis. Furthermore, in agreement with the above results, ΔNp73α is an inhibitor of colony suppression mediated by wild-type p53 and TA-p73 (Fig. 4b and Table 2). Reintroduction of wild type-p53 and TA-p73 suppresses growth of SaOs2 cells[2,19] and this suppression is thought to be largely due to apoptosis[20]. In keeping with these results, transfection of wild-type p53 strongly suppresses macroscopic colony formation of SaOs2 cells compared to many visible colonies with vector backbone alone (4 foci for wild-type p53 versus 1778 foci for vector control). In contrast, co-expression of ΔNp73α together with wild-type p53 at a 1:1 molar ratio counteracts this effect, leading to a 12.5-fold increase in the number of colonies from 4 to 51. Likewise, TA-p73α, although not quite as potent as wild-type p53, suppresses colony formation (82 foci)[2], but co-expression of ΔNp73α together with TA-p73α again antagonizes this effect and increases the number of macroscopic colonies by 8.1-fold to 669 foci. The higher rescue ability of ΔNp73α with respect to wild-type p53 is reminiscent of its stronger inhibition of wild-type p53-mediated transactivation compared to TA-p73 -mediated transactivation (Fig. 3b). Entirely consistent with this finding were data from a subsequent p53 expression analysis of surviving colonies. A complete loss of p53 protein expression was found in 2 of 2 randomly picked and expanded colonies that were derived from plates transfected with wild-type p53 alone (Fig. 4c). This is in agreement with the fact that wild-type p53 expression is incompatible with the outgrowth of colonies in such an assay and the rare colonies that do grow escape because they have lost wild-type p53 expression[21]. In contrast, all 3 randomly picked colonies from plates cotransfected with wild-type p53 and ΔNp73α had detectable levels of p53 protein (Fig. 4c), indicating that ΔNp73α neutralizes the growth suppressive effect of wild-type p53, thereby removing the selection pressure to delete the wild-type p53 plasmid. Thus, ΔNp73α is able to counteract p53 and TA-p73 – induced colony suppression in transformed human cells.

**ΔNp73 inhibits wild-type p53 and TA-p73 function by heterocomplex formation**

One explanation for this dominant negative effect is a direct physical interaction between ΔNp73 and either wild-type p53 or TA-p73 proteins, analogous to the dominant negative mode of action of mutant p53 proteins towards wild-type p53. To test this hypothesis directly, lysates prepared from p53 *null* SaOs2 cells cotransfected with wild-type p53 and ΔNp73α were immunoprecipitated with monoclonal antibody, ER15, which recognizes ΔNp73α. Immunoblot analysis with an antibody specific for p53 (CM1) revealed a complex of the 2 proteins (Fig. 5a, left lane). As a control, no such complex was seen in SaOs2 cells transfected with ΔNp73α alone and immunoprecipitated with ER15 (Fig. 5a, center lane), indicating the specificity of the detection. Of note, TA-p73 isoforms are unable to form a protein complex with wild type p53[16,22-24], excluding the possibility that the observed p53 band was co-immunoprecipitated via the endogenous TA-p73 protein of SaOs2 cells. Moreover, a similar complex was seen in wild-type p53 expressing human U2OS cells after transfection with ΔNp73α alone. Fig. 5b shows a specific complex between endogenous wild-type p53 and ectopic ΔNp73α that was immunoprecipitated by ER15 (left lane). No such complex is seen when an irrelevant monoclonal antibody against green fluorescent protein (GFP) is used (right lane). The same specific complex can again be immunoprecipitated from U2OS cells using a monoclonal antibody specific for p53 (421) and immunoblotted with a

polyclonal antibody specific for ΔNp73 that does not crossreact with any TA-p73 proteins (Fig. 5c, left lane). Again, no such complex is found with preimmune mouse IgG (center lane).

**Discussion**

Here we show for the first time that the human TP73 gene produces an N-terminally truncated isoform from an alternative internal promoter and this isoform lacks the transactivation domain. We provide evidence of physical and functional dominant negative interactions between ΔNp73 and 2 family member proteins, p53 and its own transcription-competent product, TA-p73. ΔNp73 is a potent dominant negative inhibitor of both wild-type p53 and full length TA-p73 with respect to transcriptional activation, apoptotic ability and growth suppressor function. We provide the first clinical evidence that ΔNp73 is frequently overexpressed in a variety of primary human cancers types including one third of breast cancers. Importantly, in a group of matched tumors derived from various tissue types, 41% (7 of 17) exhibit either exclusive or excessive upregulation of dominant negative ΔNp73 in a tumor-specific manner, irrespective of a concomitant increase in TA-p73. Of note, the majority of these tumours seem to harbor wild-type p53 status.

*In vitro* responsiveness to oncogenes and select forms of DNA damage might suggest a putative tumour suppressor role of TP73 analogous to TP53. However, tumour-associated overexpression of total p73 and in some cases TA-p73, in conjunction with a conspicuous lack of p73 mutations and the lack of a cancer phenotype in p73-deficient mice provide clear evidence that the TP73 gene is not a target of genetic deletion in tumorigenesis. Instead, the demonstration that a significant number of human tumours specifically select for dominant negative p73 isoforms strongly argues for an oncogenic role of these forms. Preferential expression of ΔNp73 in tumours appears to bestow oncogenic activity upon the TP73 gene that specifically interferes with the tumour suppressor functions of wild-type p53. The existence of this inhibitory family network might also explain the paucity of p73 mutations in human tumors, although the *in vivo* impact of the anti-tumor safeguard role of TA-p73 is unclear. In the developing mouse brain, ΔNp73 is the *predominant* form of TP73 and a powerful inhibitor of p53[16]. *In vivo* studies showed that ΔNp73 plays an essential anti-apoptotic role required to counteract p53-mediated neuronal death during the 'sculpting' of the developing mouse neuronal system[16]. For example, p73-/- mice, missing all forms of p73 including protective ΔNp73, underwent accelerated neuronal death in sympathetic ganglia[16]. Thus, in keeping with a common theme in cancer growth, a developmental inhibitory network, which is essential in normal physiology becomes corrupted in cancer. In support of the idea that ΔNp73 can act as an oncogene, overexpression of ΔN isoforms of p63, a p73 homolog, is found in human cancers including lung cancer, squamous cell carcinoma of the head and neck[25], bladder cancer[26] and nasopharyngeal carcinoma [27]. Importantly, a specific ΔNp63, p40AIS, is oncogenic in nude mice and in the Rat 1a focus formation assay[26].

We show that a physical interaction between ΔNp73 and wild-type p53 proteins is the basis of the functional inhibition. Gel shift assays ruled out a direct competition between ΔNp73α and wild-type p53 at the promoter for specific DNA

binding (data not shown). Thus, heterocomplexes interfere with the specific DNA binding activity of wild-type p53. This mechanism mirrors the ability of many missense p53 mutants in heterozygous tumours which abrogate the function of the remaining wild-type p53 allele in a dominant negative fashion[28,29]. Of note, TA-p73 isoforms are unable to form a protein complex with wild type p53[16,22-24]. Therefore, the formation of a heterocomplex between ΔNp73α and wild-type p53 suggests that the lack of the transactivation domain induces a conformational change in ΔNp73a, allowing mixed complex formation to occur.

Evidence from some human cancers and mouse models indicates that the mutational status of p53 is an important clinical variable for prognosis as well as for guiding the aggressiveness of anticancer therapy. However, currently, p53 mutational status is not widely accepted as a clinical indicator because many studies show that its prognostic power for survival and predictive value for therapeutic response is poor (reviewed in Ref. 30). The discovery of a ΔNp73-based p53/p73 interference network suggests that the p53 status of a tumour should no longer be considered in isolation. The existence of this inhibitory network might account for the inconsistencies of clinical studies that sought to use p53 status as a predictor of outcome. It mandates that we do a careful analysis of the functional consequences of this network *in vivo*. Establishment and clarification of an inhibitory p53/p73 network would have a major clinical impact ranging from fine-tuning the prognostic power of p53 mutation status to rational p53/p73-targeted drug design.

## Methods

### Tumour samples and cell lines

Primary tumours and normal tissues were collected at University Hospital SUNY Stony Brook in compliance with the Institutional Review Board regulations. Freshly harvested tumours (minimum 60% tumour cells) and normal tissues were immediately snap frozen in liquid nitrogen and stored at –80°C until RNA extraction. The human breast cancer cell line MDA 231, the p53 *null* lines H1299 and SaOs2, the wild-type p53 line U2OS and HeLa cells were maintained in DMEM/10% fetal calf serum.

### Semiquantitative RT-PCR assay

Total RNA was extracted from tissues as described[14]. 1 μg of total RNA, 10 pmoles each of a radiolabeled specific upstream primer and a common Exon 4 reverse primer were used in a 10 μl reaction (Titan Kit, Roche, Indianapolis, Indiana) for 25 cycles to ensure linearity of the assay (data not shown) and subjected to Phosphoimage analysis. Primer sequences were 5'-TGC TGT ACG TCG GTG ACC-3' (sense ΔNp73), 5'-CGA CGG CTG CAG AGC GAG-3' (sense TA-p73) and 5'-TCG AAG GTG GAG CTG GGT TG-3' (antisense for both). ΔNp73 and TA-p73 amplicon sizes were 175 bp and 365 bp , respectively. Band intensities were normalized to GAPDH. In matched samples, upregulation in tumours was calculated by dividing normalized $\Delta Np73^{Tumour}$ by $\Delta Np73^{Normal}$, and, likewise, normalized $TA\text{–}p73^{Tumour}$ by $TA\text{–}p73^{Normal}$. To calculate disproportional upregulation of ΔNp73 in tumours, the double fraction $\Delta Np73^{Tumour}/\Delta Np73^{Normal}$ divided by $TA\text{–}p73^{Tumour}/TA\text{–}p73^{Normal}$ was used. Some samples were independently repeated which yielded highly reproducible results. For Ex2Del p73, primers were 5'-GCG CCA GGC CAG CCG GGA CGG AC -3' (sense) and 5'-CGC GGC TGC TCA TCT GGT CCA TGG TGC-3' (antisense) which yielded a 320 bp band. Upregulation was calculated as above.

### Luciferase assays

A pcDNA3-based expression plasmid for ΔNp73α was generated with an N-terminal Flag tag. pcDNA3-Fp53 expressing human Flag tagged wild-type p53, pcDNA3-p73α and β expressing HA-tagged human TA-p73α and β (gift of G. Melino) and reporters PG13-Luc were previously described[4]. H1299 cells and SaOs2 cells were transfected by Fugene (Roche) Luciferase activity was normalized for Renilla Luciferase activity (Promega, Madison Wisconsin).

### Western blot and co-immunoprecipitation analysis

For Fig. 3c, Hela and H1299 cells were transfected with expression plasmids for wild-type p53 (0.5 μg) or TA-p73β (0.5 μg) together with either ΔNp73α (1.5 μg) or empty vector (1.5 μg). This plasmid ratio was verified to yield equal p53 or TA-p73β expression levels, independent of the presence or absence of ΔNp73α. 24 h later total cell lysates were prepared and subjected to immunoblot analysis. Gel loading was normalized for equal vimentin levels (around 20 μg per lane). Antibodies to p73 were the monoclonal ER15 (recognizes amino acids 380-495 of

human p73α isoforms (Oncogene Sci., Cambridge, MA) and the polyclonal anti-ΔNp73 (raised in rabbit against the Exon 3' peptide LYVGDPARHLATA and immunopurified; does not crossreact with p53 or any TA-p73 isoform). Antibodies against p53 (DO-1 and PAb 421), HDM2 (IF2), p21Waf1 and 14-3-3σ were from Oncogene Sci. and Flag antibody (M2)was from Sigma. For normalization of protein loading, blots were reprobed with α-vimentin (BioGenex). For immunoprecipitation, SaOs2 and U2OS cells were transfected with 2.4 μg of the indicated plasmids in single transfections and 0.6 μg of p53 plasmid plus 1.8 μg of ΔNp73α. Twenty-four hours later, 600 μg of lysates were subjected to immunoprecipitation with 1 μg of the indicated antibodies and analyzed by Western blot as described[31].

**Apoptosis assay**

Hela and SaOs2 cells were seeded into 8 well chamber slides and cotransfected with 300 ng of the indicated plasmids using Fugene. Control wells (vector alone) received 600 ng. After 16 h or 24 h, cells were stained with Annexin V or TUNEL respectively, according to the manufacturer's instructions (Roche) and expression was determined by immunofluorescence in duplicate wells. Transfection efficiency was reproducibly about 30% of cells, similar among all constructs and evenly distributed throughout the wells. Annexin V or TUNEL positive cells (494 fields at 40x) and plasmid expressing cells (15 random fields, > 500 cells) were counted and the percentage of apoptosis in transfected cells was determined after correction for background with vector alone.

**Colony suppression assay**

SaOs2 cells in 100 mm plates were transfected by Fugene with 1.5 μg each of the indicated expression plasmids. Vector only plates received 3 μg plasmid. 48 h later, cells were placed under G418 selection (500 μg/ ml) for 21 days, fixed, stained with crystal violet[32], photographed and all foci counted.

**Figure Legends**

**Fig. 1 Gene architecture of human TP73.** **a.** In contrast to TP53 which harbors a single promoter generating a single protein composed of the transactivation domain (TAD), DNA-binding domain (DBD) and tetramerization domain (TD), the TP73 gene is complex and contains 2 promoters. The P1 promoter in the 5'UTR region produces transactivation-competent full length proteins containing the TA domain (TA-p73). The P2 promoter in Intron 3 produces TA-deficient protein(s) (ΔNp73) with dominant-negative function towards TA-p73 and towards wild-type p53. ΔNp73 starts with Exon 3' which consists of 13 unique amino acids that are highly conserved between human and mouse. Another N-terminally truncated p73, Ex2Del, also lacks the TA domain but is created by splicing out Exon 2 from the P1 transcript[1]. The C-terminus of TA-p73 undergoes additional exon splicing which generates β–ϕ isoforms. **b.** Positions of primers used for RT-PCR analysis are indicated.

**Fig. 2 ΔNp73 is frequently overexpressed in a variety of primary human cancers with upregulated p73.** **a.** Upregulation of ΔNp73 transcripts in a series of 52 human breast cancers, compared to 8 unrelated normal breast tissues. ΔNp73-specific semiquantitative RT-PCR assay with total RNA extracted from tissues. Expression levels were standardized using the corresponding GAPDH value of each sample. The fold-induction over the average normal tissue expression (red line) is indicated. The arrow marks the arbitrary cut-off delineating tumours with a 5-fold or higher ΔNp73 expression. Sixteen of 52 breast cancers (31%) express ΔNp73 levels that were between 6 and 44 - fold higher than normal breast tissue. **b.** Examples of the isoform-specific semiquantitative RT-PCR assay for ΔNp73 and TA- p73 in a case of ovarian cancer (Nr. 4 in Table 1) and a case of cervical cancer (Nr. 1 in Table 1), each with their respective normal tissue of origin from the same patient. Although both tumours upregulate TA-p73, their upregulation of ΔNp73 is disproportionately higher. Transcript-specific upstream primers and a common downstream primer in Exon 4 were used (see Fig. 1b). ΔNp73 yields a 175 bp band and TA- p73 a 365 bp band. Lane marked Δ+ contains recombinant ΔNp73. Band intensities were normalized to GAPDH by Phosphoimage analysis and increases calculated as described in methods. Both results were highly reproducible in 2 independent experiments.

**Fig. 3 ΔNp73 is an efficient dominant negative inhibitor of the transcriptional activity of wild-type p53 and TA-p73.** **a.** ΔNp73α-mediated suppression of the wild-type p53 / TA-p73-responsive reporter construct PG13-Luciferase in p53 *null* H1299 cells. Luciferase activity is normalized for Renilla Luc activity. Co-expressed ΔNp73α causes a dose-dependent complete suppression of the transcriptional activity of wild-type p53 and TA-p73β. Molar ratios of wild-type p53 or TAp73 β to ΔNp73α are indicated. Results were similar with TA-p73α. Results are the average +/- s.d. of 3 independent experiments. **b.** Similar experiment as in **a**. Here, the transcriptional activity of TA-p73β and wild-type p53 is set as 100%. The percentage of suppression by ΔNp73α for the indicated molar ratios is shown. ΔNp73α is a more potent transcriptional inhibitor of wild-type p53 than of TA-p73β. Results are the average +/- s.d. of 3 independent experiments. **c.** ΔNp73α suppresses the wild-type p53 and TA-p73β-induced

transactivation of endogenous target genes. p53 *null* H1299 cells were transfected with expression plasmids containing wild-type p53 or TA-p73β together with either empty vector or ΔNp73α at a 1:3 molar ratio. This ratio was determined to yield equal p53 and TAp73β expression levels, independent of the presence or absence of ΔNp73α (data not shown). Transfected crude lysates, normalized for equal protein loading by vimentin, were immunoblotted for HDM2, 14-3-3σ and p21Waf1. Cells in the *Vect* lane

**Fig. 4 ΔNp73α counteracts apoptosis and the suppression of tumour cell growth induced by wild type p53 and TA-p73. a.** Annexin V analysis of apoptosis. HeLa cells were transfected with expression plasmids encoding wild-type p53 or TA-p73β together with either empty vector or ΔNp73α at a 1:1 molar ratio and analyzed after 16 h. As control, ΔNp73α plus empty vector was used. For each construct, the number of expressing cells were determined by immunofluorescence and the apoptotic index of expressing cells is indicated. Results are the average +/- s.d. of 4 independent experiments. Similar results were obtained with SaOs2 cells and with TUNEL analysis. **b.** SaOs2 colony suppression induced by wild type p53 and TA-p73α is inhibited by ΔNp73α. Number of colonies are shown in Table 2.

**Fig. 5 Physical interaction between ΔNp73α and wild type p53 protein. a.** Crude cell lysates of p53 *null* SaOs2 cells, transfected with the indicated expression plasmids, were immunoprecipitated with a monoclonal antibody against ΔNp73α (ER15) and immunoblotted for co-precipitating wild-type p53 with polyclonal CM-1. Lysate only was loaded in the indicated lane. **b.** Crude cell lysates of wild type p53 harboring U2OS cells, transfected with a plasmid encoding ΔNp73α, were immunoprecipitated with ER15 or an irrelevant monoclonal antibody against green fluorescent protein (GFP) and immunoblotted for co-precipitating endogenous p53 with polyclonal CM-1. **c.** Crude cell lysates of U2Os cells, transfected with a plasmid encoding ΔNp73α, were immunoprecipitated with a monoclonal antibody against p53 (421) or irrelevant mouse IgG and immunoblotted for co-precipitating ΔNp73α with polyclonal α–ΔNp73. This antibody is raised and immunopurified against Exon 3' and does not crossreact with TA-p73 isoforms or p53. Lysate only was loaded in the indicated lane.

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**Acknowledgment**

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Fig 1

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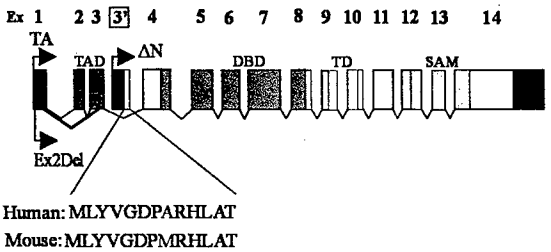
*a*

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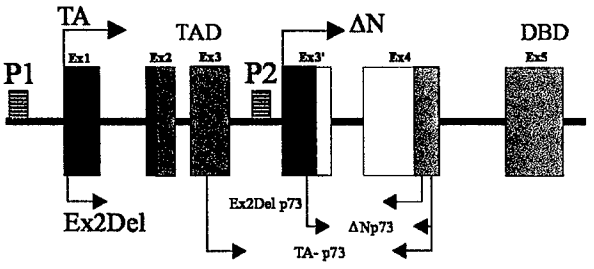

Fig 2

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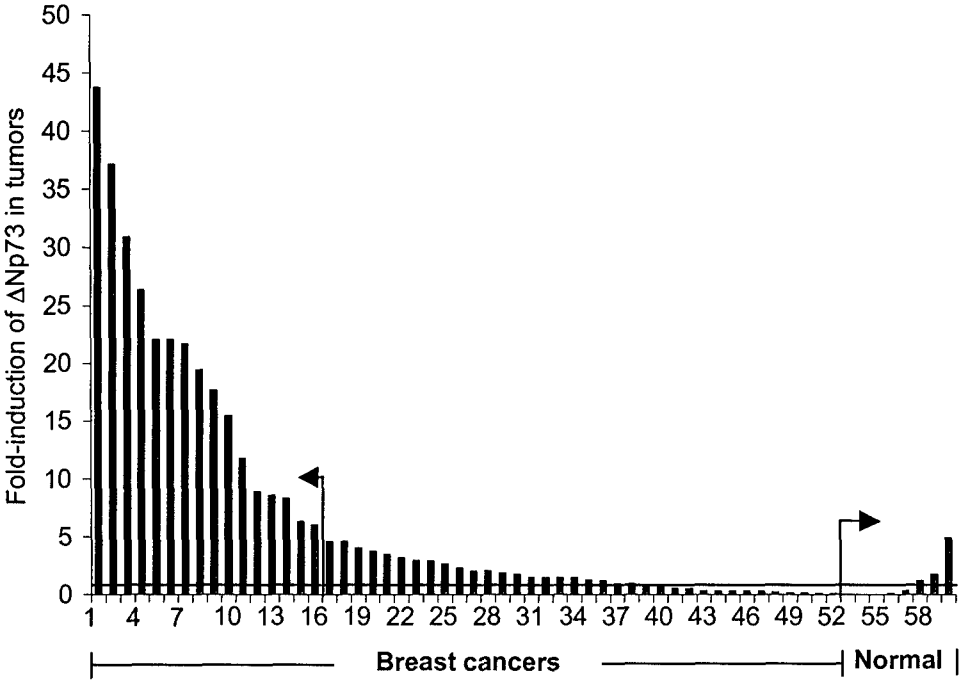

Fig 2b

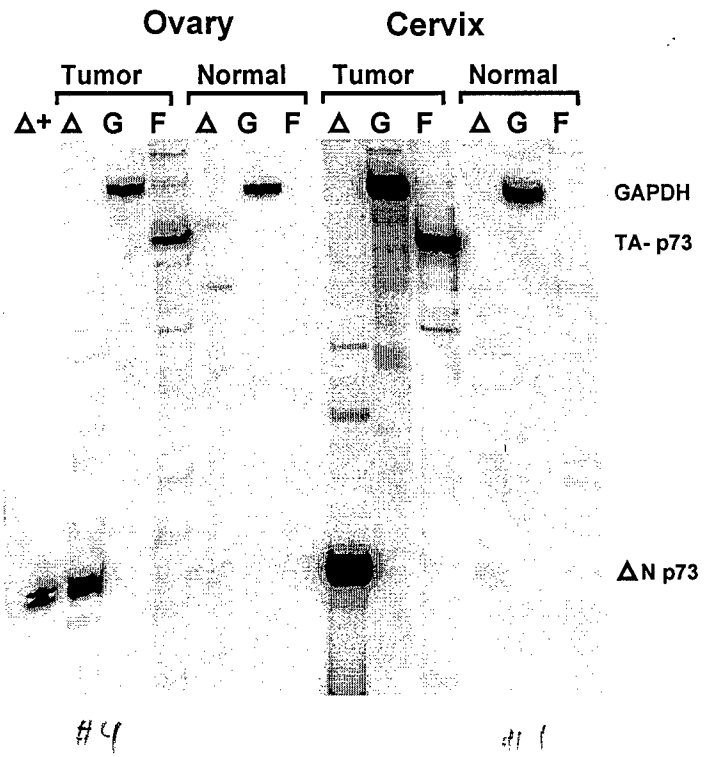
Ovary
Cervix
Tumor
Normal
Tumor
Normal
Δ+ Δ G F Δ G F Δ G F Δ G F
GAPDH
TA- p73
ΔN p73

**Table 1 Expression of ΔNp73 in matched human tumours**

| | Tumour | Increased ΔNp73 Expression In Tumour *vs* Normal | Increased TA-p73 Expression In Tumour *vs* Normal | Disproportional Upregulation of ΔNp73 or Ex2Del *vs* TA-p73 in Tumours | p53 status (estimated by ICC) |
|---|---|---|---|---|---|
| 1 | Cervical Ca # | 78 | 7 | 11 | wt |
| 2 | Ovarian Ca | 36 | 3 | 12 | wt |
| 3 | Cervical Ca | 25 | 155 | - | mut |
| 4 | Ovarian Ca | 21 | 4 | 5 | wt |
| 5 | Breast Ca | 12 | - | 11 | wt |
| 6 | Breast Ca # | 10 | - | 16 | wt |
| 7 | Ovarian Ca | 4 | 10 | - | mut |
| 8 | Ovarian Ca # | yes* | - | yes* | mut |
| 9 | Ovarian Ca | yes* | yes* | ND | wt |
| 10 | Ser. Cystadenoma | 3 | - | 8 | wt |
| 11 | Ovarian Ca | - | - | - / Ex2Del | wt |
| 12 | Breast Ca # | - | 29 | - | wt |
| 13 | Ovarian Ca # | - | - | - | |
| 14 | Breast Ca | - | - | - | wt |
| 15 | Ovarian Ca | - | - | - | mut |
| 16 | Renal Ca | - | - | - | |
| 17 | Colon Ca | - | - | - / Ex2Del | |
| | | 58%. | 41%. | 70%. / 53%. | |

Numbers reflect fold-increase
* Cannot be calculated as fold-increase because level in normal tissue is undetectable
# Average of 2 independent measurements. Values differed by < 10%
Ca carcinoma
Ser. Cystadenoma Serous cystadenoma, a large benign ovarian tumour
ICC immunocytochemistry; negative suggests wild type status; positive suggests mutant status
ND fold-increase cannot be calculated
Ex2Del: These tumours had disproportional upregulation of Ex2Del p73 *vs* TA-p73

Fig 3

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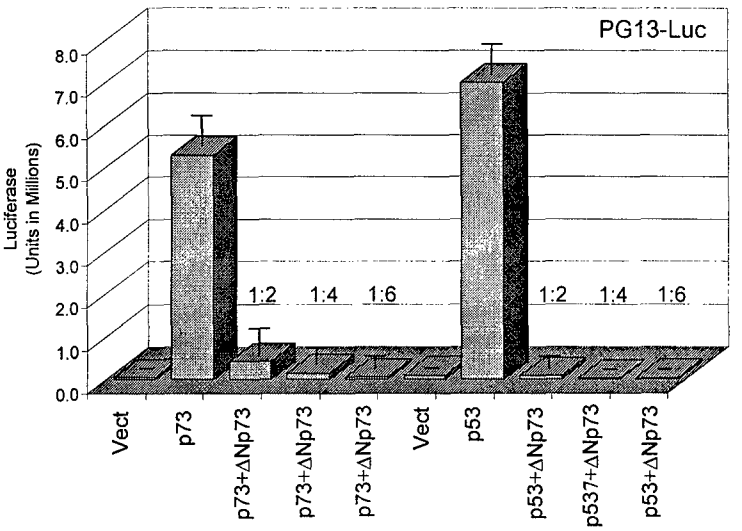


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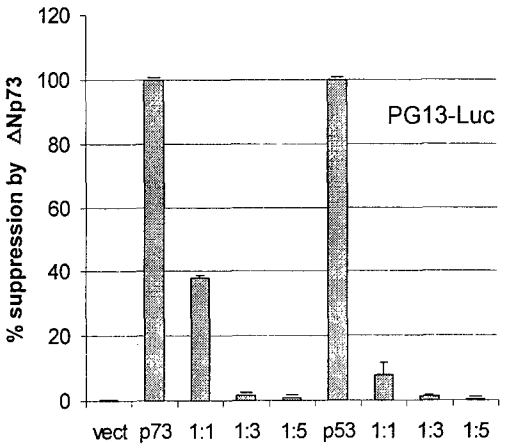

Fig 3

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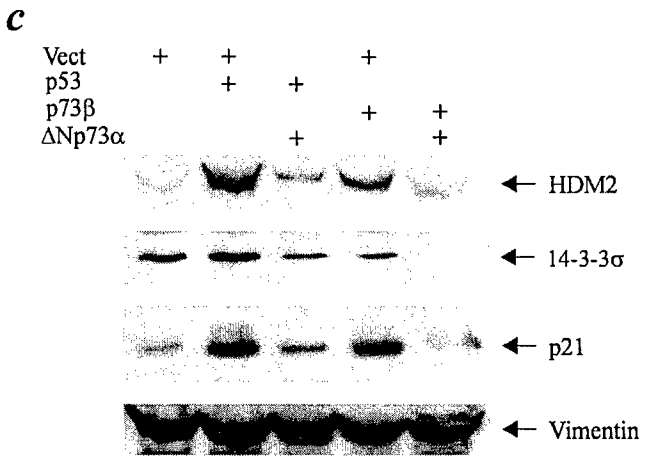

Fig 4

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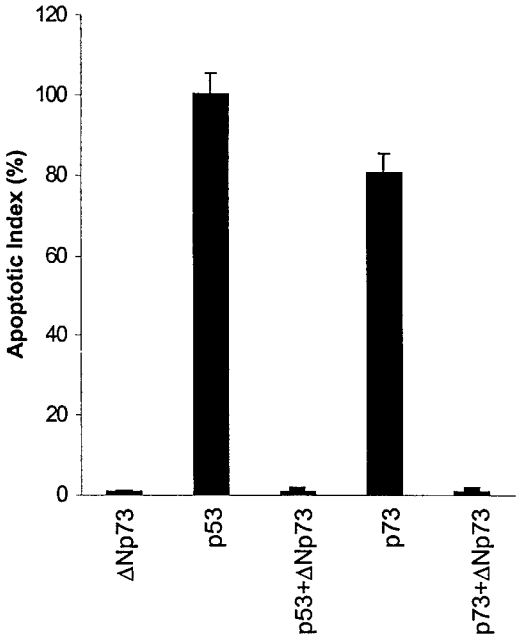

Fig 4

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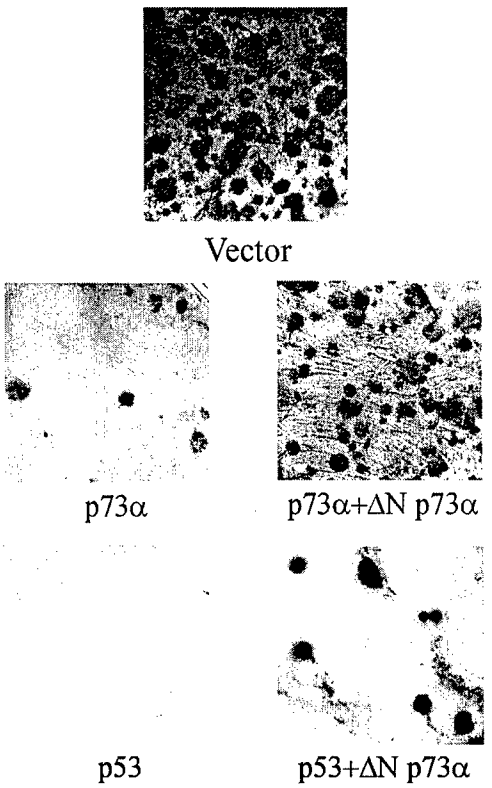
***b***




***c***

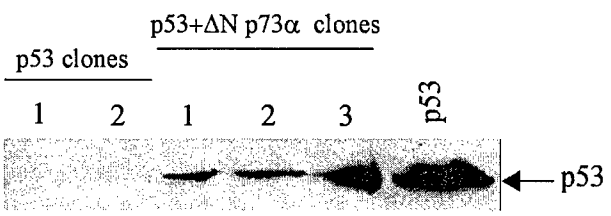
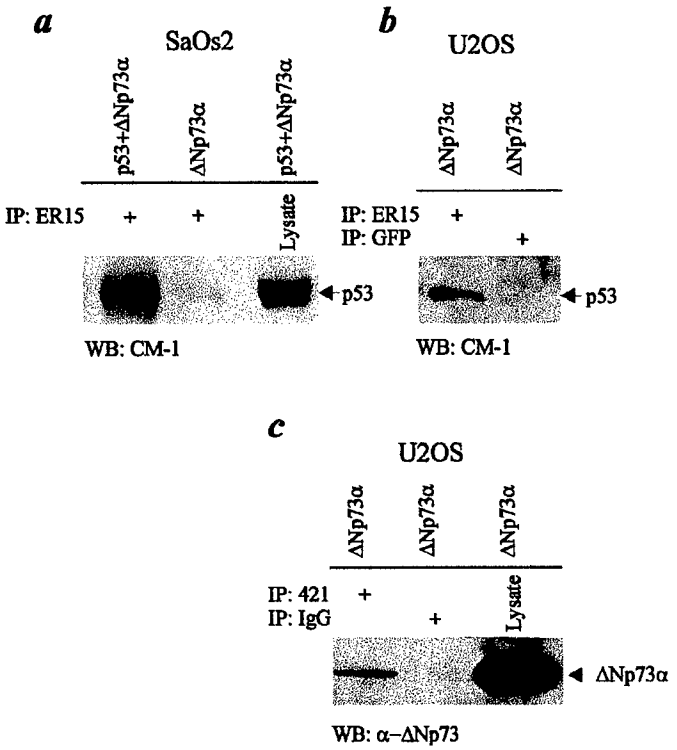


**Table 2 Inhibition of Colony Suppression by ΔNp73**

| Plasmid | Number of foci |
|---|---|
| vect | 1778 |
| wtp53 | 4 |
| wtp53 + ΔNp73 | 51 |
| TAp73α | 82 |
| TAp73α + ΔNp73 | 669 |

Fig 5
unpublished



# p53, p63 and p73 - solos, alliances and feuds among family members

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Key words: p53, p63; p73; transcription factor, tumor suppressor gene, oncogene,

## 1. Introduction

p53 controls a powerful stress response by integrating upstream signals from many types of DNA damage and inappropriate oncogenic stimulation, all of which lead to p53 activation. Activated p53 elicits apoptosis, cell cycle arrest and, in some circumstances, senescence, thereby preventing the formation of tumors. Hence, loss of p53 function is a preeminent finding in most human cancers, whether directly through mutation of p53 itself, the most common mechanism [1], impaired nuclear retention of p53 [2,3], loss of the upstream activator p14ARF through silencing or mutation [4], or amplification of the p53 antagonist HDM2 [5].

Despite the central role of p53 in tumorigenesis - and an intense search by many laboratories - no related genes were found for 20 years. This changed suddenly in 1997, when two novel family members were identified and termed p73 [6] and p63 [7-11]. [Since p63 was cloned independently by multiple groups, it led to a prolific and rather confusing nomenclature: KET, p51A and p51B, p40, p73L, NBP]. Based on their remarkable structural similarity to p53, p63 and p73 generated instant excitement and quick expectations about their biological functions. Four years later we have unearthed striking similarities but also surprising diversities. Both genes give rise to proteins that have *i)* entirely novel functions, and, *ii)* p53-related functions. However, the latter are complex because they can be of an agonistic or antagonistic nature. Both p63 and p73 share over 60% amino acid identity with the DNA-binding region of p53 [and even higher identity amongst themselves], including conservation of all DNA contact and structural residues that are hotspots for p53 mutations in tumors. Even the conditional temperature-sensitive residue Ala143 in p53 is preserved in p63 and p73 [12]. In addition, p73 shows 38% identity with the tetramerization domain of p53 and 29% identity with the transactivation domain [TA] of p53. In vertebrates, the p73 and p63 genes are ancestral to p53 and possibly evolved from a common p63/p73 archetype [6,7].

## 2. Gene architecture of the p53 family

In sharp contrast to the simple gene structure of p53, which is highly conserved from mollusk to human, the structures of the p63 and p73 genes are complex [Figure 1]. Human TP53 has a single promoter which encodes a single protein of 393 amino acids. The three most conserved domains of p53 are the transactivation domain [TA], the specific DNA-binding domain [DBD] and the oligomerization domain [OD]. In contrast, TP63 and TP73 make heavy use of an alternative promoter and alternative splicing [Figure 1]. TP63 and TP73 each have 2 promoters, P1 in the 5'UTR upstream of a non-coding exon 1, and P2 located within the 23 kb spanning Intron 3. P1 and P2 promoters produce two diametrically opposed classes of proteins: those containing the transactivation domain [TAp63, TAp73] and those lacking it [ΔNp63 and ΔNp73].

TA-proteins can mimic p53 function *in vitro* and in cell culture including transactivating many p53 target genes and inducing apoptosis, while ΔN-proteins act as dominant negative inhibitors towards themselves and towards other family members [4, 7,13]. Moreover, TP73 can undergo alternate N-terminal splicing of Exon 2 which again produces a TA-deficient protein called p73 Ex2Del. As if this complexity were not enough, TP73 and TP63 also undergo multiple C - terminal splicing, skipping one or several exons [6 forms for TP73 [called α, β, γ, δ, ε and ϕ with α being full length] [6, 14, 15] and 3 forms for TP63 [α, β and γ] [7]. In some isoforms, exon splicing also leads to unique sequences due to ensuing frameshifts. Splicing of different 'tails' further modulates the p53-like function of TA-proteins. Importantly, in all C-terminal splice and ΔN forms, the DNA-binding domain and tetramerization domain are still preserved. Structurally, the γ forms of TP73 and TP63 most closely resemble p53 itself, featuring just a small C-terminal extension beyond the last 30 amino acid stretch of p53. Surprisingly, though, while TAp63γ [also called p51A] is as powerful as p53 in transactivation and apoptosis assays [7], TAp73γ is rather weak. The α forms of TP73 and TP63 contain an additional highly conserved SAM motif [sterile alpha motif]. SAM motifs are protein interaction modules found in a wide variety of proteins implicated in development. The crystal and solution structure of p73 SAM agree with each other and feature a five-helix bundle fold that is characteristic of all SAM-domain structures [16,17]. Other

SAM-containing proteins are e.g. the ETS transcription factor TEL that plays a role in leukemia, the polycomb group of homeotic transcription factors and the ephrin receptors. Despite predictions of homo- and hetero-oligomerization of SAM-containing proteins, though, p73 SAM appears monomeric by experimental analysis, casting doubt whether this domain mediates interaction of p73 with heterologous proteins [17]. There are also functional differences between TAp73α and TAp63α. While TAp73α is comparable to p53 in transactivation and apoptosis assays, TAp63α [also called p51B] is very weak [7]. The reason for this difference is not obvious from the structure and remains unclear. In general, more structure-function analysis is needed to understand why and how C-terminal variations influence function.

The P2 promoters transcribe truncated forms of TP73 and TP63 that lack the transactivation domain. While ΔNp63 has been shown to occur in human and mouse, ΔNp73 has so far only been reported in mouse. Recently we have also cloned ΔNp73 from human tissues [A. Zaika and U.M. Moll, unpubl. results]. Most importantly, these ΔNp73 and ΔNp63 proteins behave in a dominant negative fashion towards their own TA-proteins and towards p53 *in vivo* in the mouse and in transfected human cells. Strikingly, squamous cell carcinoma of the skin produces high levels of ΔNp63α. Moreover, the TP63 locus was contained within a frequently amplified region in this cancer type [18]. Furthermore, ΔNp73 is the predominant TP73 product in the developing mouse nervous system [4,13] [see below].

In summary, by using alternate promoters and exon splicing, TP73 and TP63 genes can generate an impressive modular complexity by combining a specific 'head' with a particular 'tail'. In practice, this means that our understanding of their biological roles will greatly depend on knowing which forms get expressed under what circumstances.

**3. TP63 and TP73 play important roles in development and differentiation.**

Both genes play important and, despite their structural similarity, surprisingly unique roles in mouse and human development. This is powerfully revealed by the striking developmental phenotypes of p63- and p73-deficient mice [4,19,20].

**TP63**

TP63 expression is absolutely essential for limb formation and epidermal morphogenesis [integument, tongue] including the formation of adnexa [teeth, hair, mammary and prostate glands, sweat and lacrimal glands]. p63 null animals show severe limb truncations or even absence of limbs as well as craniofacial malformations. The animals do not survive beyond a few days postnatally. Reminiscent of the knock-out phenotype in mice, heterozygous germ line mutations of p63 in humans cause the rare autosomal dominant developmental disorder EEC [ Ectrodactyly, Ectodermal Dysplasia, Facial Clefts]. The p63 mutations found in EEC patients are typically missense mutations within the DNA binding domain. These EEC mutations inhibit DNA binding of the TAp63 forms. Conversely, EEC mutations in ΔNp63 proteins cause a loss of their dominant negative properties towards p53 and TAp63γ [21]. Importantly, basal cells of normal human epithelium including the epidermis strongly express p63 proteins, predominantly the ΔNp63 isotype [7], but lose them as soon as these cells withdraw from the stem cell compartment [22]. Consistent with this notion, keratinocyte differentiation is associated with the disappearance of ΔNp63 isotypes [23,24]. Together, this data clearly establishes a fundamental role of p63 in the biology of the keratinocyte stem cell and the apical ectodermal ridge of the limb bud [22]. This role is likely one in stem cell self renewal rather than in stem cell differentiation into stratified epithelium, although this remains a matter of controversy [19,20]. What appears clearer is that p63 is probably not simply required for the proliferative capacity of stem cells, since their immediate progeny, the TAC cells, are equally proliferative but have already lost p63 expression [22].

**TP73**

TP73 has varied but distinct developmental roles. TP73 expression is required for neurogenesis of specific neural structures, for pheromonal signaling and for normal fluid dynamics of cerebrospinal fluid and the respiratory mucosa [4]. p73 null animals exhibit highly site-specific hippocampal dysgenesis. The hippocampus is central to learning and memory and continues to develop throughout adulthood. The basis

of hippocampal dysgenesis in p73-deficient mice is the selective loss of large bi-polar neurons called Cajal-Retzius located in the marginal zone of the cortex and the molecular layers of the hippocampus. These CR neurons co-express p73 and the secretory glycoprotein reelin, which is essential for neuronal migration in the cortex [4]. In addition, p73 null mice suffer from hydrocephalus, probably due to hypersecretion of cerebrospinal fluid by the choroid plexus, and from purulent pan-infections of the respiratory mucosa, likely due to mucus hypersecretion. Moreover, the animals show abnormal reproductive and social behavior which in rodents is governed by pheromone sensory pathways. This abnormality could be related to dysfunction of the vomeronasal organ which normally expresses high levels of p73.

**Role of ΔNp73 in mouse development.** ΔNp73 is the predominant form in the developing mouse brain [7,13]. In situ-hybridization reveals strong ΔNp73 expression in E12.5 d fetal mouse brain in the preplate layer, bed nucleus of stria terminalis, choroid plexus and the preoptic area [4]. Moreover, it is the *only* form of p73 found in mouse brain and the sympathetic superior cervical ganglia in P10 neonatal mice [13].

Functional studies and knock out mice showed that ΔNp73 plays an essential anti-apoptotic role *in vivo*. ΔNp73 is required to counteract p53-mediated neuronal death during the normal 'sculpting' of the developing mouse neuronal system [13]. Withdrawal of NGF, an obligate survival factor for mouse sympathetic neurons, leads to p53 induction and p53-dependent cell death. Conversely, NGF withdrawal leads to a *decrease* of ΔNp73. Importantly, sympathetic neurons are *rescued* from cell death after NGF withdrawal when ΔNp73 levels are maintained by viral delivery. Likewise, sympathetic neurons are rescued from Adp53-mediated neuronal death by co-infected AdΔNp73. In pull-down assays, mixed protein complexes of p53/ΔNp73 were demonstrated, suggesting a biochemical basis for the transdominance. Together, this data firmly puts ΔNp73 downstream of NGF in the NGF survival pathway. It also explains why p73-/- mice, missing *all* forms of p73 including protective ΔNp73, undergo accelerated neuronal death in postnatal superior cervical ganglia [13].

In tissue culture models, p73 also plays a role in differentiation of several cell lineages. TP73 expression increases during retinoic acid-induced and spontaneous differentiation of neuroblastoma cells [25, 26]. Also, ectopic TAp73β, but not p53, induce morphologic and biochemical markers of neuroblastoma differentiation [25]. Moreover, expression of specific C-terminal isoforms correlate with normal myeloid differentiation. p73α and β are associated with normal myeloid differentiation, while p73 γ, δ, ε and θ are associated with leukemic blasts. In fact, p73ε is specific for leukemic blast cells [27]. Similarly, TAp73 γ and δ may play a role in the terminal differentiation of human skin keratinocytes [28]. This suggests a p73 - specific differentiation role that is not shared by p53 and for the most part not shared by p63 either.

p53 has an important developmental role in early mouse embryogenesis [E7-8 d], as revealed when the autoregulatory feedback loop with MDM2 is removed and p53 levels remain uncontrolled [29,30]. Nevertheless, in stark contrast to TP63 and TP73 null mice, TP53 null mice make it through development with essentially no problems [with the exception of rare exencephaly in females]. A commonly offered explanation is that p53 functions are covered by redundant p63 and p73 functions. At least in theory, this idea could now be tested, although generating double or even triple knockouts might be a daunting task. The concept of substitution, however, is inconsistent with the finding that ΔN isoforms, rather than TA isoforms, are the predominant proteins of TP63 and TP73 during development. Indeed, the very fact that TP63 and TP73-deficient mice have a phenotype could be viewed as evidence for the important *in vivo* role of ΔN isoforms during development, since conversely p53 cannot substitute for those forms.

Of note, p73-deficient mice lack *spontaneous* tumor formation, even after a 2 year observation period [4]. While the tumor rate after mutagenic challenge or the tumor rate of double p53/p73 null mice is currently unknown, this result is another clear difference between p53 and the other family members. It indicates that if TP73 and TP63 do have a role in tumor formation, it might be a complex one which is probably not revealed by simply eliminating the entire gene.

**4. p63 and p73 expression in normal human tissues.**
p73 gene expression occurs at very low levels in all normal human tissues studied [26, 31]. It is not readily detectable by Northern blots and immunoblots. p63, mainly its ΔN form, is readily detectable at the protein level. p63 expression is restricted to the nuclei of basal cells of normal epithelia [skin, prostate, urothelium, ectocervix, vagina] [7].

**5. TAp73 and TAp63 function during forced overexpression.**
In general, many functional parallels are found among p53, TAp73 and TAp63 on the one hand, and between ΔNp73 and ΔNp63 on the other hand. The two groups, however, behave antithetically. Overall, far fewer studies have been reported on p63 than on p73.

When ectopically overexpressed in cell culture, p73α and β closely mimic the transcriptional activity and biological function of p53. p73β, and to a lesser extent p73α, bind to canonical p53 DNA-binding sites and transactivate many p53-responsive promoters [32-35], although relative efficiencies on a given p53-target promoter may differ from p53 and also differs among various C-terminal isoforms of TAp73 and TAp63 [34,35]. In reporter assays, p73-responsive promoters include well known p53 target genes involved in anti-proliferative and pro-apoptotic cellular stress responses such as p21WAF1,14-3-3σ, GADD45, BTG2, PIGs [34], ribonucleotide reductase p53R2 [36] and IGFBP3 [37]. Bax transactivation is controversial [34,37]. TAp73α and β also induces MDM2. While there are probably still dozens of common targets that have not yet been described or discovered, it will be important to identify p73-preferred or even p73-specific targets. One example may be the Aquaporin 3 gene, a glycerol and solute transporter, which is greatly preferred by the p73β isoform compared to poor activation by p73α and p53 [38]. The physiologic significance of this relationship, however, is currently unclear. Conversely, ectopic p73 overexpression leads to transcriptional repression of VEGF at the mRNA and protein level, analogous to the ability of p53 to transcriptionally suppress VEGF [39].

Ectopic p73 promotes apoptosis in human tumor cell lines independent of their p53 status [6, 32]. In fact, in a subset of cancer cell lines p73β is more efficient in inducing apoptosis than p53 itself [40]. Major potency differences exist among the C-terminal isoforms. Overexpression of p73 α, β and δ suppresses focus formation of p53-deficient Saos-2 cells, while p73 γ fails or suppresses only very poorly [14, 32, 40]. Similarly, TAp63α lacks significant transcriptional and apoptotic ability, while TAp63γ is very potent in both [7].

Like p53, p73 utilizes p300 /CBP as its coactivator by forming a complex with the CH1 domain [aa 350-450] of p300/CBP [41]. In contrast to p53, however, p73 does not require acetylation by p300 to become transcriptionally stimulated [42].

**6. Regulation of p73 and p63 protein stability and transcriptional activity.**
Proteasomes are implicated in the turnover of p73 proteins since proteasome inhibitors stabilize p73 isoforms. In sharp contrast to p53, however, this turnover is not mediated by MDM2. One important open question is whether an as yet undefined MDM2-like protein exists for p73, or alternatively, whether p73 stability is not specifically regulated by a dedicated E3 ligase. The molecular basis for the MDM2 resistance of p73 was found by systematic motif swapping. Region 92-112 of p53, which is absent in p73, was identified to confer MDM2 degradability to p53 [43]. p73 protein is also resistant to HPV E6, which together with E6-AP mediates hyperactive degradation of p53 in HPV infected cells [44,45]. This relationship might have some bearing in tumors with increased p73 expression [see below]. And just as MDM2 does not mediate p73 degradation, p19ARF, which stabilizes p53 levels by antagonizing the degrading action of MDM2, has not been shown to stabilize p73 protein. One potential consequence of the differential MDM2 sensitivity between p53 and p73 was seen in tissue culture: ectopic co-expression of p73 leads to a *selective* decrease of ectopic p53 and of endogenous induced p53, because p53 is susceptible to MDM2, while p73 is not [46]. This suggests a potential downmodulation of p53 by high levels of TAp73, an interesting family twist to keep in mind with respect to tumor formation. On a transcriptional level, however, the negative feedback regulation between the two genes is preserved.

MDM2 is transcriptionally activated by p73 and, in turn, negatively regulates the transcriptional ability of p73, just as it functions towards p53 [44, 47, 48]. However, the mechanism is again distinct from p53. MDM2 disrupts the interaction of p73 - but not of p53 - with p300/CBP by competing with p73 for binding to the N-terminus of p300/CBP [48]. It is currently not known whether p63 has a similar relationship with p300/CBP.

Proteasome inhibitors also stabilize overall levels of p63. In an additional family twist, however, the stability of ΔNp63 isoforms may also be regulated independently of proteasomes and in fact, be promoted by physical complex formation with wild type p53. A protein-protein complex between ΔNp63α and p53, mediated by both DNA binding domains, can form in cells. Moreover, p53 overexpression reveals p53-dependent degradation of ΔNp63 via a caspase-1 specific pathway [49]. This result may explain the observation that UV irradiation of cultured keratinocytes suppresses ΔNp63 levels [50]. A check-and-balance system may exist: while ΔNp63 is a transcriptional inhibitor of p53, p53 is a stability inhibitor of ΔNp63. This relationship also points towards another level of intimate functional cross-talk among p53 family members, a theme that will surface again and again.

**7. Posttranslational modifications during activation.**

p53 stabilization and activation by genotoxic stress is associated with multiple posttranslational modifications at the N-and C-termini of p53 *in vivo*. In close temporal relationship to stress, the N-terminus undergoes heavy phosphorylation [Ser 15, 20, 33, 37, 46 and Thr 18, 81], which is thought to stabilize the protein by interfering with MDM2 binding, thereby disrupting the constitutively targeted degradation. The C-terminus also undergoes site specific phosphorylation [Ser315, 392], acetylation [Lys 320, 373 and 382] and sumoylation [Lys386]. The C-terminal modifications are thought to activate the transcriptional activity of p53 [51]. So-called stress kinases [e.g. ATM, ATR, Chk2] which detect genotoxic stress and initiate signal transduction are *in vivo* kinases for specific p53 Serine residues, while the histone acetyltransferases p300/CBP and PCAF [which at the same time are transcriptional co-activators] acetylate p53.

Modification differences among p53 family members are starting to be worked out. Interestingly, Serine phosphorylation has not been reported for p73 and p63. Instead, p73α undergoes phosphorylation at Tyr 99 by c-abl in response to γ–IR which in turn activates p73 for apoptosis [52,53]. This is due to a direct interaction between the PXXP motifs of p73 and the SH3 domain of c-abl. Interestingly, Tyr 99 phosphorylation activates p73 but does not stabilize the protein. On the other hand, cisplatin also activates p73 function and stabilizes the protein but does not Tyrosine-phosphorylate it. Sumoylation of C-terminal Lys 627 occurs specifically in p73α but not in p73β *in vitro*. However, in contrast to sumoylation of p53, which activates its transcriptional activity, sumoylation of p73 promotes its degradation [54]. p63 does not have PXXP motifs and modification studies for p63 have not been reported.

## Role of p73 and p63 in tumors

**8. p73 is not a classic Knudson-type tumor suppressor.**

p73 maps to chrom 1p36.33 which frequently undergoes loss of heterozygosity [LOH] in breast cancer, neuroblastoma and several other human cancers [6]. This fact, in conjunction with the functional similarity to p53, originally led to the proposal that p73 is a tumor suppressor gene [6]. Genetic data on most cancer types - with the notable exception of leukemias and lymphomas - however, exclude p73 as a *classic* Knudson-type tumor suppressor, which by definition is targeted to undergo loss of expression or function during tumorigenesis. To date, in a total of over 900 primary tumors, loss-of function mutations in the p73 ORF are vanishingly rare. Moreover, imprinting of the p73 locus, initially thought to be an epigenetic explanation to satisfy the 2 hit hypothesis [since it would only require one hit of LOH against the transcribed allele], is rather uncommon and if present, varies from tissue to tissue and person to person and does not correlate with p73 expression levels [15, 26, 55, 56]. In fact, in lung, esophageal and renal carcinoma, the second p73 allele is specifically *activated* in tumors [loss of imprinting] [57-59].

As an additional difference from p53, p73 protein fails to be inactivated by most of the major viral oncoproteins that inactivate p53, namely SV40 T antigen [60] and Ad E1B 55 kD [61]. For HPV E6, while clearly not inducing p73 degradation [44, 45, 62], controversy exists whether E6 of low and high risk strains inactivates the transcription function of p73 [62, 63]. However, *some* viral protein products do target p73. p73 transcriptional activity is inhibited by Ad E4orf6 [64] and by HTLV 1 Tax [65]. p63 also fails to interact with SV40 T antigen and the HPV E6 protein [66].

**9. Alteration of p73 expression in human cancer.**

Surprisingly, work from our lab and confirmed by others on multiple primary tumor types and tumor cell lines show that the most common identifiable cancer-specific alteration is an *overexpression* of the wild type p73 gene rather than a loss of expression [6]. This suggests that TP73 plays some role in tumorigenesis. To date, significant prevalence of p73 overexpression has been found in a dozen different tumor types including tumors of breast [15], neuroblastoma [26], lung [58, 67], esophagus [59], stomach [68], colon [69], bladder [70, 71], ovarian cancer [70% of cases in one cohort] [72-74], ependymoma [74], liver cancer [77,77], cholangiocellular carcinoma [78], CML blast crisis and acute myelogenous leukemia [27, 79]. Most studies measure overexpression of full length p73 mRNA [TAp73] by RT-PCR, but a few studies also measure overexpression of TAp73 protein[s], either by immunoblot or immunocytochemistry. For example, we found overexpression of TAp73 transcripts [5 to 25-fold] in 38% of 77 invasive breast cancers relative to normal breast tissue, and in 5 of 7 breast cancer cell lines [13 to 73-fold] [15]. Likewise, we found overexpression of TAp73 transcripts in a subset of neuroblastoma [8 - 80-fold] and in 12 of 14 neuroblastoma cell lines [8 - 90-fold] [26]. A close correlation between p73 mRNA levels and protein levels was shown e.g. in ovarian carcinoma cell lines [72]. In a series of 193 patients with hepatocellular carcinoma, 32% of tumors showed detectable [high] p73 by immunocytochemistry and in situ hybridization, while all normal tissue had undetectable levels [low] [77]. Of note, primary tumors and tumor cell lines with p73 overexpression tend to simultaneously overexpress a complex profile of shorter C-terminal splice variants [p73$\gamma$, $\delta$, $\varepsilon$, and $\phi$], while the normal tissue of origin is limited to the expression of p73$\alpha$ and $\beta$ [15]. Importantly, patients with high global p73 protein expression had a worse survival than patients with undetectable levels [77].

The single exception to this picture seems to be lymphoid malignancies. TP73 has been found to be transcriptionally silenced in some lymphoblastic leukemias and lymphomas due to hypermethylation [80, 81].

Although ΔNp73 has so far only been shown in developing mouse brain [13] and human placenta [A. Zaika and U.M.Moll, unpubl. results], it will be of great interest to determine whether tumors also upregulate dominant negative ΔNp73 proteins. If indeed this is the case, one could envision an important *inhibitory cross-talk* between ΔNp73 and wild type p53 and/or TAp73 products, thus potentially converting an anti-oncogenic synergism into an oncogenic antagonism. p73 Ex2Del might also be expressed in some tumors. Using an upstream RT-PCR primer to the 5' UTR region, p73 Ex2Del was found to be co-expressed with TAp73 in 5 of 10 invasive ovarian cancers and in 3 of 7 ovarian cancer cell lines. None of 6 normal ovarian epithelial cells or 9 borderline ovarian tumors expressed p73 Ex2Del [72]. As predicted, p73 Ex2Del acts as a dominant negative inhibitor towards the transactivation and apoptotic ability of p53 and TAp73$\alpha$ [82].

**10. Alteration of p63 expression in human cancer.**

Currently only a limited analysis of the expression and mutational status of p63 in primary tumors exists. The trend here appears to be rare inactivating mutations but upregulation of dominant negative forms. For example, no p63 mutations were found in 47 bladder cancers [83]. Only 1 missense mutation [Ala148Pro] out of 66 various human tumors and 2 missense mutations in 35 tumor cell lines were found [9].

The human TP63 gene is located on chrom 3p within a region which is frequently amplified in squamous cell carcinomas. Some lung cancers and squamous cell carcinomas of the head and neck show p63 overexpression associated with a modest increase in TP63 copy numbers [18]. [The authors therefore named the amplified locus Amplified In Squamous carcinoma, AIS]. Importantly, although many AIS

isoforms are produced in those tumors, the majority are dominant negative ΔNp63 forms [mainly p40 AIS]. p40AIS acts like an oncogene in nude mice and in Rat1a focus formation assays [18]. Similar findings exist in nasopharyngeal carcinoma [NPC], which almost always have functional wt p53. In 25 primary NPCs, *all* tumor cells overexpressed predominantly ΔNp63, which in normal nasopharyngeal epithelium is limited to proliferating basal and suprabasal cells [84]. Likewise, upregulation of ΔNp63 was found in 30 out of 47 bladder cancers [83]. Interestingly, TAp63 was concomitantly downregulated in 25 of those 47 tumors.

**11. Upstream components that signal to p73 and p63.**
Recent data established that p73 is able to integrate and mediate death stimuli from 3 different tumor surveillance pathways *in vivo*: oncogenes, DNA damage and T-cell receptor hyperactivation. Again, only scant data exist for p63.

**p73 is activated to mediate apoptosis by cellular and viral oncogenes.** We and others recently established that the cellular and viral oncogenes E2F1, cMyc and E1A can induce and activate the endogenous TAp73α and β proteins for target gene transactivation, apoptosis and growth suppression in p53-deficient human tumor cells [85, 86, 87, 88]. E2F1 is a direct transcriptional activator by binding to several E2F1-responsive elements within the P1 promoter of TP73 [86,88]. This is specific for TP73 since E2F1 does not activate the TP63 promoter, suggesting that this promoter is devoid of an E2F1 response element [88]. Since oncogene deregulation of E2F1 and cMyc are one of the most common genetic alterations in human tumors, these findings might provide a physiologic mechanism for TAp73 overexpression in tumors. Taken together, this data establishes another important link between p73 and human cancer.

Moreover, during E2F1-mediated apoptosis in primary mouse embryo fibroblasts [MEF] a striking non-additive cooperation between wild type p53 and p73 exists [88]. While wt MEFs show 77% apoptosis after forced E2F1 expression, p53-/- MEFs [containing p73] and p73-/- MEFs [containing p53], *both* show reduced killing ability after forced E2F1 expression with 12% and 15%, respectively. This excessively weakened killing ability of p73-/- MEFs, despite the presence of wt p53, is consistent with an important *synergistic* but independent signal emanating from TAp73 that cooperates with p53 to induce oncogene-triggered death in a tumor surveillance pathway.

An E2F1 - p73 pathway mediates cell death of circulating peripheral T cells induced by T cell receptor activation. Normal peripheral T cells undergo apoptosis after hyperstimulation of their T- cell receptors. This cell death pathway is mediated via the E2F1 - p73 pathway [87]. Consistent with this notion, E2F1 null mice exhibit a marked disruption of lymphatic homeostasis with increased numbers of T-cells and splenomegaly, suggesting that p73 plays a role in tumor surveillance pathways of lymphoid cells [89, 90]. Moreover, the p73 gene is transcriptionally silenced in acute lymphoblastic leukemia and Burkitt's lymphoma due to hypermethylation [80, 81, 91, 92]. This appears to be restricted to lymphoid tumors, since neither other hematopoietic malignancies nor solid tumors show p73 hypermethylation [80, 91]. Interestingly, in radiation-induced T-cell lymphomas of the mouse, the p73 locus undergoes LOH in 33% of the cases [93]. Thus, in lymphoid tumors p73 shows some genetic features of a classic tumor suppressor gene.

**p73 is activated to mediate apoptosis by a restricted spectrum of DNA damage.** p73 is not activated by UV, Actinomycin D, Doxorubicin and mitomycin C, all of which stabilize and activate p53 [6, 94]. However, endogenous p73 is activated for apoptosis in response to cisplatin, taxol and γ-IR in a pathway that depends on the non-receptor tyrosine kinase c-abl [52, 53, 95]. Conversely, cells deficient in c-abl do not upregulate or activate their p73 and are resistant to killing by cisplatin. Together, this supports a model in which some DNA damage signals are channeled through c-abl to p73. Hence, one would predict that p73-deficient cells should have defective DNA damage checkpoint controls. This seems to be borne out by the observation that p53/p73 double null MEFs are more resistant to killing by cisplatin and Taxol than p53 single null MEFs [96]. Endogenous p73β protein is also rapidly induced by camptothecin

treatment (K. Cheng and U.M. Moll, unpubl. observation). Thus, DNA-damage-dependent activation of p73 might be partly responsible for p53-independent apoptosis.

In one report, ectopic TAp63γ in a mouse erythroleukemia line is rapidly stabilized and induces WAF1 after treatment with UV, γIR or Actinomycin D [97]. Surprisingly though, stabilized TAp63γ was associated with erythroid differentiation rather than apoptosis, as was seen with ectopic p53. Since ectopic TAp63γ, without additional DNA damage, caused apoptosis in baby hamster kidney cells [7], it hints at a functional versatility of TAp63γ to induce differentiation under genotoxic circumstances.

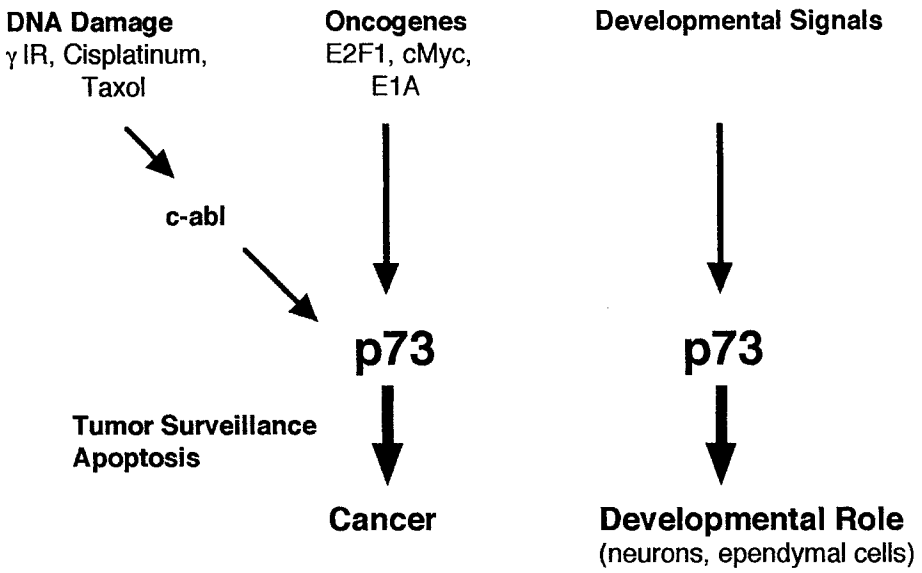


Fig. 2: Proposed model of the biological roles of p73.

**12. More transdominant cross-talk - this time between mutant p53 and TAp73 or TAp63.**
Physical interactions between certain - but not all - human p53 mutants and TAp73 or TAp63 proteins were found in co-immunoprecipitation assays of exogenous and endogenous proteins. Importantly, these interactions correlate with functional transdominance. In contrast, complexes between wild type p53 and p73 are not observed in mammalian cells [13, 33, 98]. Unexpectedly, protein contact occurs between the DNA binding domain [DBD] of mutant p53 and the DBD plus oligomerization domain of p73 [99-101] rather than between the respective oligomerization domains. In co-transfections, mixed heterocomplexes were shown between p53 mutants p53Ala143, p53Leu173, p53His175, p53Cys220, p53Trp248 or p53Gly281 and TAp73 α, β, γ and δ [33, 98, 99,101] or TAp63 [101]. Physiologic complexes were found in 5 tumor cell lines between endogenous mutant p53 and p73 [98, 99]. Functionally, formation of such stable complexes leads to a loss of p73- and p63-mediated transactivation and proapoptotic abilities. Moreover, E2F1-induced p73 transactivation, apoptosis and colony suppression was inhibited by co-expressed p53His175 [86]. One study proposes that the Arg/Pro polymorphism at codon 72 of mutant p53 might be a further biological determinant for binding and inactivation of p73, with 72R mutants of p53 being more inhibitory than 72P mutants [98].

This inhibition mirrors the ability of many transdominant missense p53 mutants to abrogate wild type p53 function [102, 103]. It suggests that in tumors which express both TAp73 and mutant p53 [typically at very high levels due to deficient mdm-2 mediated degradation], the function of TAp73 and TAp63 might be inactivated. Moreover, these functional interactions define a network that could result in a "two-birds-with-one-stone" effect for at least some inactivating p53 mutations. If this occurs in primary human tumors, it might have far-reaching consequences since *i]* it argues for a transdominant inhibition of the

tumor suppressor function of TAp73 isoforms during tumor development, *ii]* it could be the underlying mechanism for the gain-of-function activity of certain p53 mutants, and *iii]* it might further increase chemoresistance in cancer therapy of established tumors. p53 is exceptional among tumor suppressors in that it selects for the overexpression of missense mutants rather than for loss of expression as most other suppressor genes do. This gain-of-function results in increased tumorigenicity compared to p53 null parental cells, increased resistance to cancer agents, and increased genomic instability due to abrogation of the mitotic spindle checkpoint [104-106]. Conceivably, p63 also participates in this network. On the other hand, it should be noted that some p53 mutants clearly are recessive towards TAp73 [e.g. p53His283 and p53Tyr277] [101] and do not interfere with its action.

At least in theory, another transdominant mechanism besides hetero-oligomer formation migh be promoter competition. It is conceivable that ΔNp73 or ΔNp63 homo-oligomers might have a stronger affinity to certain target gene promoters than wild type p53. In those cases, p53 inhibition would occur due to competition at the level of target gene access. Some experimental evidence does exist for this idea. In the wtp53-containing ovarian carcinoma cell line A2780, co-expression of increasing amounts of either TAp73α, β, γ or ε inhibits specific DNA binding and transcriptional activity of p53 in the absence of hetero-oligomer formation [107, 108].

In short, the biological consequences of TP73 and TP63 expression might be diametrically different depending on the concomitant presence or absence of mutant p53 and / or the presence or absence of dominant negative ΔNp73 and ΔNp63 isoforms.

**13. p73 and p63 appear to play a role in cancer - but as an oncogene or as a suppressor gene?**

Overall, evidence is mounting that p73 does play an important role in human tumors *in vivo*. However, the current picture of p73's involvement in human cancer is a puzzling paradox, with the possible exception of lymphoid malignancies. How can we reconcile the p53-compensatory action of TAp73 after DNA-damage or oncogene activation, which is revealed both in primary cells and p53-deficient tumor cells, with the paucity of TP73 mutations and the presence of TAp73 overexpression in many human tumors? One possible interpretation is that although p73 does mount a surveillance response, it is functionally of no consequence and therefore not targeted during tumor development. However, this view might be too simple and disregard the real possibility of 'epigenetic' TAp73 inactivation by dominant negative interference from mutant p53 and ΔN isoforms. An urgent question that needs to be answered is whether tumors upregulate dominant negative ΔNp73. If so, ΔNp73 might specifically antagonize and compromise the function of p53 and TAp73. A similar situation might exist with TP63, given the early data on ΔNp63 overexpression by head and neck squamous carcinomas [18]. Only careful functional and biochemical analysis of such relationships will answer the question whether p73 and p63 do matter in cancer.

**14. Conclusions**

Inactivation of the p53 tumor suppressor is the single most common genetic defect in human cancer. The discovery of two close structural homologs, p63 and p73, generated instant excitement and quick expectations about their biological functions. We now know that in development both genes clearly have novel, p53 independent functions. p63 is involved in epithelial stem cell regeneration and p73 in hippocampal neurogenesis, pheromonal pathways and ependymal cell function. A major future challenge is to determine what role these p53 homologs play in tumor biology. It is already clear that they are not classic Knudson type tumor suppressors. However, the existence of inhibitory versions of both genes and intimate functional cross-talk among all family members might explain the currently conflicting properties, which argue for tumor suppressive and oncogenic roles.

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## Legend

**Fig. 1: Gene Architecture of the p53 Family.** In contrast to the simple structure of the p53 protein which harbors the transactivation domain (TA), the DNA-binding domain (DBD) and the oligomerization domain (OD) as the 3 major modules, the products of TP73 and TP63 are complex and can contain a C-terminal SAM domain. Both genes contain 2 promoters. The P1 promoter in the 5'UTR region produces full length proteins containing the TA domain, while the P2 promoter in Intron 3 produces TA-deficient proteins with dominant-negative functions towards themselves and towards p53. In addition, extensive C-terminal splicing and, in the case of TP73 another N-terminal splice variant Ex2Del, further modulate the p53-like functions of the TA-proteins (see columns).

**Fig. 2: Proposed model of the biological roles of p73.**

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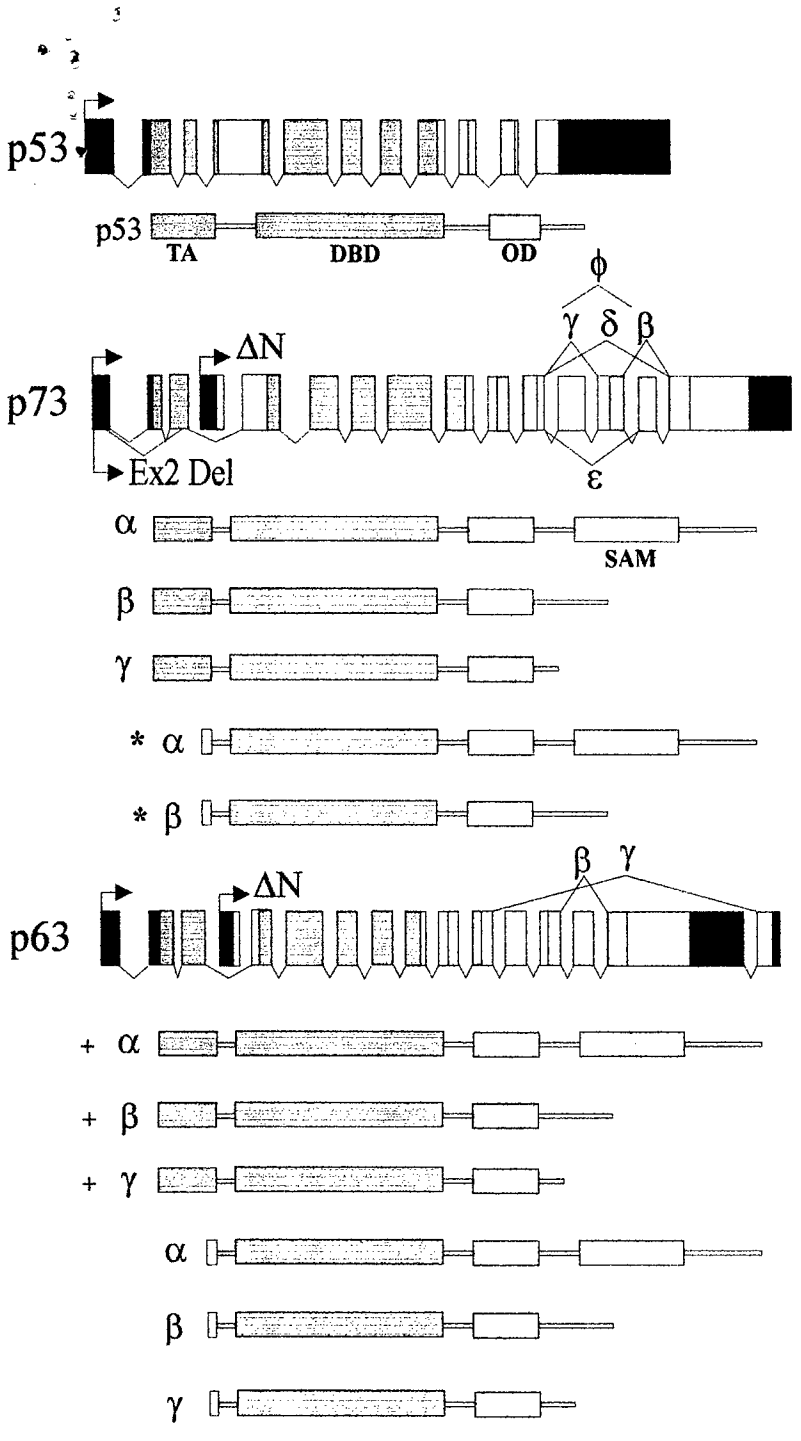


| | TA | Focus Suppression / Apoptosis | DN Behavior |
|---|---|---|---|
| p53 | +++ | +++ | – |
| p73 α | ++ | ++ | – |
| p73 β | +++ | +++ | – |
| p73 γ | (+) | (+) | – |
| p73 * α | – | – | + |
| p73 * β | – | – | + |
| p63 + α | – | – | – |
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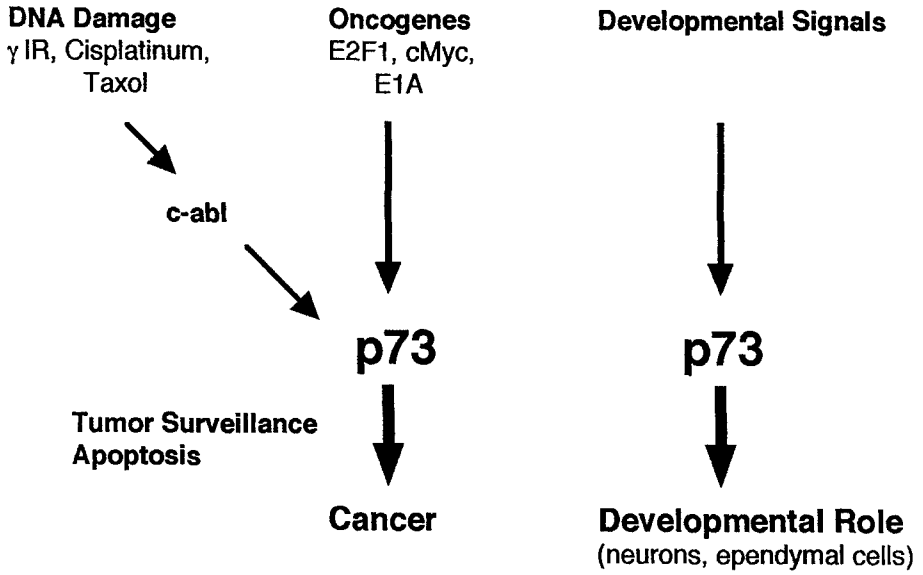


**Fig. 2: Proposed model of the biological roles of p73.**

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

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