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Letter to the Editor

ΔNp73 stabilises TAp73 proteins but compromises their function due to inhibitory hetero-oligomer formation

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Dear Editor,

TP73 shares significant structural homology with TP53.[1] This predicts functional similarities for the regulation of cell cycle and apoptosis. Indeed, TAp73 becomes transcriptionally activated upon DNA-damaging agents and oncogenes independent of p53[1,2] and ectopic TAp73 transactivates many p53 target genes involved in cell cycle arrest and apoptosis.[1] Also, intact TAp73 function is an important determinant of cellular sensitivity to anticancer agents.[3] However, the roles of TP53 and TP73 in mammalian tumorigenesis seem to be fundamentally different. In contrast to TP53, TP73 is almost never targeted by inactivating mutations in human tumors.[1] To the contrary, many different types of cancers overexpress TP73 (reviewed in Moll *et al.*[4]). Moreover, TP73 knockout mice lack a cancer phenotype but exhibit a developmental phenotype.[5] These findings are inconsistent with a suppressor function of TP73. The different roles of TP53 and TP73 in tumorigenesis might be based on the fact that TP73 gives rise to several different N- and C-terminal isoforms with opposing functions (reviewed in Moll *et al.*[4]), while p53 encodes a single suppressor protein. Aside from the transactivation-competent proapoptotic TAp73, four N-terminally truncated, dominant-negative isoforms are often concomitantly overexpressed in human cancers. ΔN′p73, ΔNp73, Ex2p73 and Ex2/3p73 (collectively called ΔTAp73) lack the transactivation domain and are generated via alternate splicing of the P1 promoter transcript[6–8] or via the P2 promoter.[9] ΔN and ΔN′p73 transcripts produce identical proteins.

ΔTAp73 isoforms lack transactivation function and fail to induce cell cycle arrest and apoptosis.[9–12] They act as powerful inhibitors of p53 and TAp73 since they retain their DNA-binding and tetramerization competence.[7–9,11,13,14] In cultured cells, ΔNp73 abrogates the suppressive activity of p53 and TAp73 and inactivates their ability to induce apoptosis and cell cycle arrest.[9] ΔNp73 overexpression results in malignant transformation of immortalized NIH3T3 fibroblasts[8] and also promotes immortalization in *primary* cells and cooperates with oncogenic Ras in driving their transformation *in vivo*.[15] Of note, human tumor data support the notion that upregulation of ΔTAp73 alleviates the selection pressure to mutate p53.[9] In gynecological cancers, significantly higher expression levels of ΔTAp73 are found in p53 wild-type than in p53 mutant tumors. Moreover, the prevalence of upregulated ΔN and ΔN′p73 is higher in p53 wild-type than in p53 mutant tumors, while no such difference was seen for TAp73 upregulation.[9,16] Also, ΔNp73 was an independent clinical prognostic marker for poor survival in neuroblastoma patients.[17] Thus, there is mounting evidence that ΔTAp73 isoforms might act as pathophysiologically relevant oncogenes, possibly outcompeting the opposing action of TAp73. This would readily explain the paucity of p73 mutations in cancers.

Previously, we and others demonstrated physical interaction between oncogenic and antioncogenic family members as a transdominant mechanism inhibiting the suppressor functions of wtp53 and TAp73.[9,11,14] Mixed protein complexes were found between endogenous ΔNp73α or β and either wtp53, TAp73α or β in human tumors and cultured tumor cells.[9,11,14] The stoichiometric ratio of TA/ΔNp73 could be a determinant in tumor formation. A small decrease in this ratio might be sufficient to convert TP73 from a tumor suppressor to an oncogene. Promoter competition by ΔNp73 at TAp73/p53 response elements is another transdominant mechanism.[11,12] Highly prevalent tumor-specific upregulation of ΔNp73 has already been found in several studies.[8,9,16–19] For example, we reported that ΔNp73/ΔN′p73 transcripts are overexpressed in 73% of 37 gynecological cancers and ΔN′p73 transcripts are upregulated in 87% of 100 ovarian cancers.[16]

Since inhibitory interactions of two proteins often lead to their stabilization, we asked whether ΔNp73 can affect TAp73 protein levels. First, we determined whether the variant N- or C-terminal region of a given p73 isoform impacts on its own steady-state level by determining the half-lives of human TAp73 and ΔTAp73α and β proteins in isolation (Figure 1a). To circumvent the problem of differential sensitivities of different isoform-specific antibodies, we transfected Flag-tagged p73 isoforms into p53 null H1299, pulsed with protein synthesis inhibitor cycloheximide (CHX) and chased protein levels over 6 h by Flag immunoblots. This analysis indicated that differences exist between C-terminal α- and β-isoforms, with α-forms generally being more stable than β-forms (up to three-fold, see ΔNp73α *versus* ΔNp73β) with the exception of Ex2p73, where the β-form is twice as stable as the α-form. Significantly, oncogenic ΔNp73α is the most stable of all eight proteins, with a half-life of 4 h. The N-terminus also modulates protein stability as seen among the α-subgroup, where differences range up to 5.3-fold (ΔNp73α *versus* Ex2p73α with 4 and 0.75 h, respectively). The shortest lived isoform is Ex2p73α, with a half-life of only 45 min. The N-terminus has only minimal influence among the β-subgroup (range between 1 and 1.5 h). To compare stabilities of endogenous p73 isoforms, H1299 and RKO cells were treated with CHX and

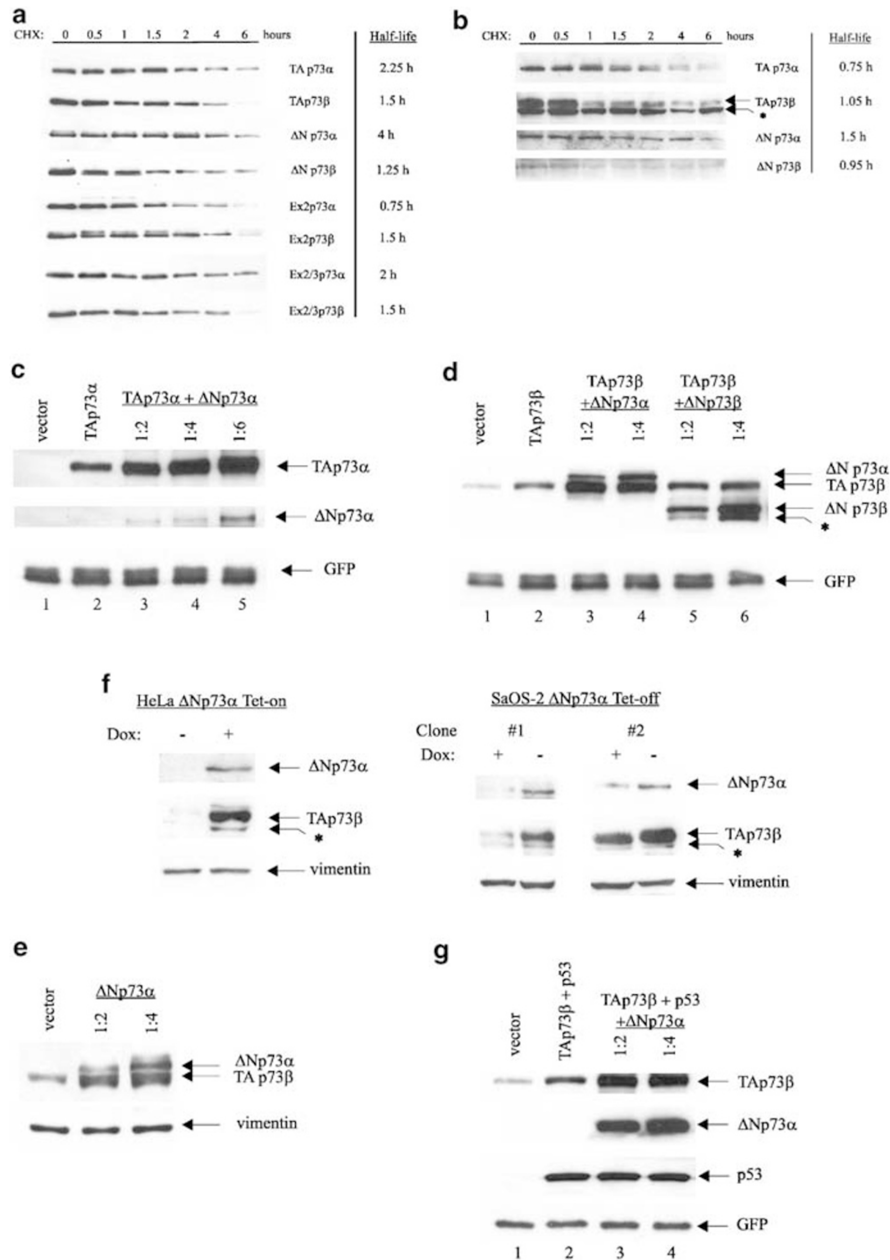

Figure 1 ΔNp73 stabilizes TAp73 proteins. (**a**) Half-lives of individual ectopic p73 isoforms. H1299 cells were transfected with 1 μg of the individual flag-tagged plasmids. After 24 h, cells were treated with CHX (75 μg/ml) for the indicated times and rates of degradation were assessed by immunoblotting with anti-Flag. (**b**) Half-lives of endogenous p73 isoforms in RKO and H1299 cells. Cells were treated with CHX as in (**a**) and lysates were immunobotted. Equal amount of total protein per lane was loaded in all blots. Asterisk denotes a nonspecific band. Band intensities were determined by densitometry and the half-lives were calculated using the EnzFitter program. The results are the average of three independent experiments. (**b**–**g**) C-terminally specific antibody *ER-15* was used to detect the α forms and *GC-15* to detect the β-forms. 5B429, raised against the N-terminus of human p73 and recognizing TAp73 forms, does not crossreact with ΔNp73 or β. For unequivocal identification of p73 isoforms, control cell lysates transfected with the corresponding isoform plasmids were run side by side on the same gels. Moreover, anti-Flag monoclonal was used to detect the flag-tagged isoforms. In many cases anti-Flag and an anti-p73 antibody were used in sequence and the results were the same. (**c**–**g**) Accumulation of p73 is induced by coexpressed ΔNp73. (**c**,**d**) Stabilization of ectopic TAp73α by ectopic ΔNp73α. (**c**) H1299 cells transfected with empty vector or TAp73α alone, or together with increasing amounts of ΔNp73α. (**d**) H1299 cells transfected with empty vector or TAp73β alone, or with increasing amounts of ΔNp73α or ΔNp73β. Cotransfected GFP was used to normalize loading in (**c**, **d** and **g**). Lysates (harvested after 20 h; about 20 μg of protein) were immunoblotted in (**c**) for TAp73α with 5B429 and with anti-Flag for ΔNp73α and levels, and in (**d**) with GC-15 for TAp73β and with anti-Flag for ΔNp73α and β levels. Asterisk denotes a nonspecific band in (**d** and **f**). (**e**) Stabilization of endogenous TAp73β by ectopic ΔNp73α. U2OS cells were transfected with 2 μg of empty vector or increasing amounts of ΔNp73α. α-vimentin was used as loading control (about 20 μg per lane). Immunoblots with GC-15 and reblot with anti-Flag. (**f**) Stabilization of endogenous TAp73β by inducible expression of ΔNp73α. Left: Stable HeLa/ΔNp73α Tet-on cells were induced to express Flag-tagged ΔNp73α by adding doxycycline into the medium. After 24 h, induction was determined by immunoblotting (15 μg) with anti-Flag. The accumulation of TAp73β was determined by reblotting with GC-15. Right. Independent stable SaOs-2/ΔNp73α Tet-off clones were induced to express flag-tagged ΔNp73α by removing doxycycline from the medium for 24 h. (**g**) Wild-type p53 is not stabilized by ΔNp73α. H1299 cell lysates transfected with 2 μg of either empty vector or TAp73β plus p53 alone, or together with increasing amounts of ΔNp73α. TAp73β was immunoblotted with GC-15, ΔNp73α with anti-Flag and p53 with DO-1

their degradation was followed (Figure 1b). The endogenous half-lives were shorter but generally supported the trend seen with ectopic p73 isoforms. Endogenous ΔNp73α again showed the longest half-life of ~1.5 h. Taken together, the N- and C-terminal variant regions moderately influence the stability of p73 proteins. Also, the relative longevity of ΔNp73 *versus* TAp73 proteins, when in isolation, supports previous anecdotal observations that ΔNp73 proteins are more stable than TAp73 in tumors.[13,19]

We next tested whether ΔNp73 proteins can induce stabilization of TAp73 proteins. H1299 cells were transfected with either TAp73α or β alone, or cotransfected with the same amount of TAp73α or β plus increasing amounts of ΔNp73α or ΔNp73β. Indeed, the steady-state levels of TAp73α (Figure 1c) and TAp73β (Figure 1d) increased proportionally with cotransfected ΔNp73α or β. ΔNp73α has a much stronger stabilizing effect towards TAp73 proteins than does ΔNp73β (Figure 1d, compare lanes 2–4 *versus* 2, 5 and 6 and data not shown). Identical data were obtained for endogenous TAp73 proteins in U2OS, H1299 and Phoenix cells (Figure 1e and data not shown). Marked TAp73β stabilization was also observed upon induction of ΔNp73α in Tet-inducible cell systems, such as HeLa Tet-on cells (Figure 1f, left) and in three independently derived SaOs-2 Tet-off clones (Figure 1f, right and data not shown). Thus, TAp73 proteins are stabilized by coexpressed ΔNp73. Moreover, ΔNp73α confers the main

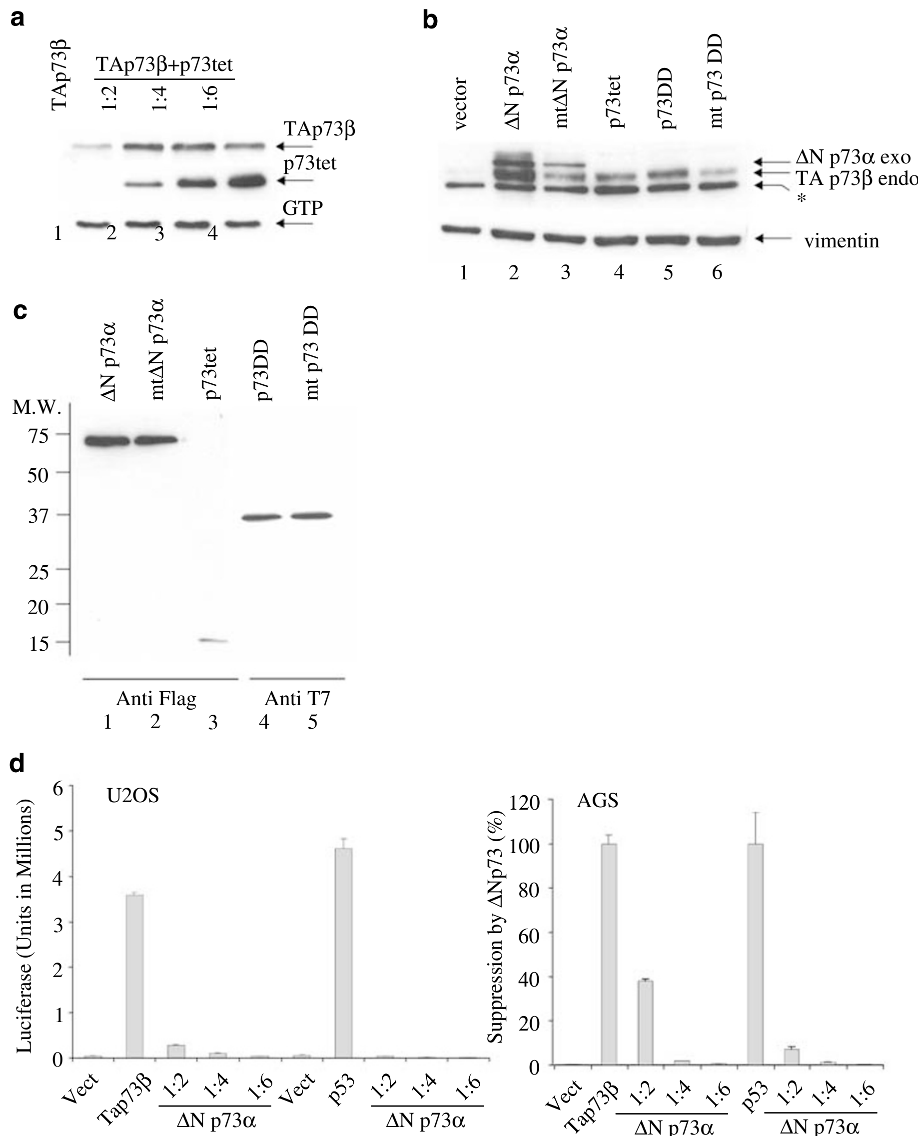

Figure 2 The tetramerization domain is necessary for stabilization of TAp73 by ΔNp73α. (**a**) H1299 cells were transfected with TAp73β alone or with increasing amounts of the p73tet plasmid. Transfected lysates (about 20 μg protein, normalized by GFP coexpression) were immunoblotted for TAp73β with GC-15, and for tet fragment with anti-Flag. (**b**) H1299 cells were transfected with 2 μg of empty vector or the indicated plasmids (ΔNp73α, mtΔNp73α, p73tet, p73DD and mtp73DD) (2 μg each). After 24 h, total cell lysates (20 μg) were immunoblotted with GC-15 and anti-Flag (upper panel). Membrane was reblotted with α-vimentin as a loading control. Asterisk denotes a nonspecific band. (**c**) Verifying expression of plasmids from panel (**b**) Immunoblot (20 μg) with anti-Flag or with T7 Tag antibody. (**d**) ΔNp73α is a dominant-negative inhibitor of the transcriptional activity of wild-type p53 and TAp73β. TAp73 reporter assay. U2OS osteosarcoma and AGS gastric carcinoma cells were transfected with the p53/TAp73-responsive reporter PG13-Luc and either empty vector, TAp73β or p53 alone or cotransfected with ΔNp73α. ΔNp73α mediates a dose-dependent suppression. Average ± S.D. of three independent experiments.

stabilization, possibly because the levels of ΔNp73α are often higher than ΔNp73 which might in turn be related to the longer half life of ΔNp73α compared to β. Given the homology between TP53 and TP73 and the existence of mixed protein complexes between p53 and ΔNp73α,[6,9,11,14] it is conceivable, at least in theory, that p53 protein levels could also be stabilized by ΔNp73α. However, p53 protein levels failed to accumulate despite increasing amounts of co-expressed ΔNp73α Figure 1g, compare lanes 2–4, while TAp73β levels were induced to accumulate in the same cells. This differential is likely related to different degradation pathways. While p53 is degradated by binding to its E3 ligase MDM2, MDM2 binding to p73 disrupts its interaction with p300/CBP, but does not mediate p73 degradation.[20–22] Instead, p73 degradation is linked to SUMO-1 modification which renders it proteasome-degradable.[23] It is possible that hetero-oligmerization prevents sumoylation of p73, thereby stabilizing it.

ΔNp73 strongly inhibits the transactivation function of p53 and TAp73 and this largely depends on forming mixed protein–protein complexes.[9,11,14,15] For example, we found that tetramerization-deficient mtΔNp73(L322P) almost completely abrogates the inhibitory function of ΔNp73 towards p53-mediated transactivation[9] and abrogates the immortalization function of ΔNp73 in primary fibroblasts.[15] We therefore considered that the ability of ΔNp73 to form a heterocomplex with TAp73 could be critical to its ability to mediate accumulation of TAp73 proteins. If this were the case, it would link ΔNp73-mediated TAp73 accumulation to its concomitant functional inhibition, that is, in such an inhibitory complex TAp73 accumulates but is inactive. Indeed, after cotransfecting a constant amount of TAp73β plasmid with increasing ratios of a p73 tetramerization domain plasmid (aa 313–404, called *p73tet*), TAp73β levels markedly accumulated and rapidly reached saturation at a 1 : 2 ratio (Figure 2a). Identical results were seen with endogenous TAp73β protein (Figure 2b, lanes 1and 4). Also, cells expressing the C-terminal fragment of human p73α (aa 327–636), comprised of the tetramerization and the SAM domains (called p73DD[2]) accumulated endogenous TAp73β. In contrast, its mutant version (mt p73DD), harboring a loss-of-function L371P mutation in the tetramerization domain, had lost much of this stabilizing activity (Figure 2b, lanes 5 and 6). Finally, while coexpression of full-length ΔNp73α induced the expected accumulation of endogenous TAp73β, its mutant counterpart mtΔNp73(L322P), which carries a mutation in the tetramerization domain that inactivates its transdominant function,[9] was able to do so only poorly (Figure 2b, lanes 1–3). Figure 2c shows that these polypeptides and their mutant counterparts were expressed at equal levels. Taken together, these data show that the tetramerization domain is the minimal domain that is necessary and sufficient for ΔNp73 to induce accumulation of TAp73. However, other region(s) of ΔNp73 may play an additional minor role, since tetramerization mutants do not completely revert this phenotype. Importantly, the ability of ΔNp73 to mediate TAp73 accumulation clearly correlates with its functional activity as a dominant-negative inhibitor of TAp73. To confirm that ΔNp73α is an inhibitor of TAp73α and β (and wtp53), we performed reporter assays with TAp73, p53 and a TAp73/p53-responsive luciferase reporter in the absence or presence of an increasing ratio of ΔNp73α. As expected, ΔNp73α completely suppressed the transcriptional activity of TAp73β and wt p53 in U2OS and AGS cells (both are wild type for p53) Figure 2d). Thus, these data show that the TAp73 protein, although stabilized by ΔNp73, is functionally compromised or inactive. It has been proposed that ΔTAp73 inhibits the function of TAp73 and p53 in different ways: TAp73 is inhibited mainly at the oligomerization level, forming an inactive hetero-oligomer.[9,14] In the case of p53, in addition to forming inactive heterocomplexes, ΔTAp73 also exerts a dominant-negative effect by displacing p53 from its promoters.[11]

In summary, we show here that ΔNp73 isoforms stabilize TAp73 proteins but, in doing so, inhibit their transactivation function. Individual p73 isoforms with variant N- and C-termini differ only moderately in their half-lives. However, when coexpressed, TAp73α and β proteins become markedly stabilized by ΔNp73α. Similar results were seen with ΔNp73β, albeit the effect is weaker. In contrast, p53 protein fails to accumulate via ΔNp73. Using tetramerization-deficient mutants, we show that the ability of ΔNp73 to mediate TAp73 accumulation largely depends on its tetramerization domain and correlates with its ability to function as a dominant-negative inhibitor of TAp73. In the ongoing debate whether TAp73 is a relevant tumor suppressor, we suggest that increased TAp73 protein levels should be interpreted with caution when levels are the only criteria that can be used to deduce TAp73 activity. This is particularly the case in primary tumors where functional studies are not possible.

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