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Letter to the Editor

The p53 family inhibitor ΔNp73 interferes with multiple developmental programs

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Dear Editor,

Mutations in the p53 tumor suppressor gene are observed in up to 50% of all tumors and represent one of the most common genetic alterations in cancer. The high rate of spontaneous tumor development in p53-deficient mice was therefore not unexpected.[1] However, considering the central role of p53 as a regulator of cellular proliferation and homeostasis, as well as cell death and premature senescence, the viability of p53 null mice and the abscence of developmental defects came as a surprise.[1] Apart from neural tube closure defects in approximately 16% of female p53 null mice and a reduced reproductive capacity of both genders no *in vivo* developmental abnormalities have been reported.[2] This is in striking contrast to the phenotype of p53 depletion in *Xenopus* embryos which leads to gastrulation failure and defects in mesoderm formation due to impaired TGF-β/Nodal/activin gene responses.[3] A possible explanation for the different p53 knockout phenotype in *Xenopus* and mice is that p53 is only one member of a family of structurally and functionally related genes. In addition to p53, early mouse embryos express the p53 family members p63 and p73 that might compensate for the loss of p53, while in *Xenopus* p53 is solely responsible for early embryogenesis as p73 is not found in the lower vertebrates and *Xenopus* p63 is only expressed at later stages during organogenesis.[3]

We know from mouse knockouts of the p63 and p73 genes that both are critical for developmental processes. p63 null mice are born alive but die shortly after birth due to severe defects in their limb, craniofacial and epithelial development.[4,5] In contrast, p73 appears critical for aspects of neurogenesis, pheromone signaling, and reproduction, and the control of inflammatory responses.[6] Most of the p73-null mice die within 2 month after birth due to chronic infections and only about 25% survive to adulthood.[6,7] However, there have been no reports on the *in vivo* effects of homozygous compound knockouts of the p53 family members.

All p53 family genes have been shown to generate transactivation-defective ΔN-isoforms lacking the aminoterminal transactivation domain.[6,8–11] These ΔN-isoforms are generated either by the use of alternative intronic promoters or by means of alternative splicing.[12–14] Since the ΔN-isoforms retain their DNA-binding capacity, they bind to the promoters of target genes and act as transdominant-negative inhibitors of the full-length isoforms.[15] As shown in Figure 1a, N-terminally truncated p73α generated by both alternative splicing (ΔN^{AS}) or alternative promoter usage (ΔN^{AP}) functions as a potent inhibitor of all the major transactivating p53 family members (p53, TAp63 and TAp73).

The transactivation function of p53 family members is critical for various differentiation processes such as myogenic differentiation of myoblasts or neuronal differentiation of neuroblastoma cells in response to all-*trans* retinoic acid (ATRA).[16–18] Furthermore, exogenous expression of TAp73 is sufficient to induce morphological and biochemical markers of neuronal differentiation.[18] Whereas TAp73 enhances terminal differentiation of oligodendrocyte precursors, ΔNp73 inhibits this process.[19] During nephrogenesis, ΔNp73 is expressed preferentially in proliferating nephron precursors, whereas TAp73 is predominantly expressed in the differentiation domain of the renal cortex.[20] This spatiotemporal switch from ΔNp73 to TAp73 may play an important role not only in the regulation of terminal differentiation in the developing nephron but suggests general antagonistic roles in differentiation control for the different p53 family proteins.

In order to define the role of the p53 family inhibitor ΔNp73 in cellular differentiation and embryogenesis we analyzed the effects of deregulated ΔNp73 expression using both cell culture models for cellular differentiation and transgenic mice carrying a conditional ΔNp73 transgene. In murine C2C12 myoblasts myogenic differentiation induced by growth factor withdrawal is associated with increasing expression of all three p53 family members (Figure 1b). Whereas p53 transcription progressively increases during the first 36 h, expression of TAp63 and TAp73 peaks between 6 and 12 h of differentiation. The TAp73 expression changes are specific and it has been shown, that TAp73 expression is actively repressed by the δEF1/ZEB repressor in proliferating C2C12 myoblasts and activated during differentiation by the muscle regulatory factors MyoD, myogenin, Myf5 and Myf6.[17] To inhibit the transactivation function of all three p53 family members during this differentiation program, we stably transduced C2C12 myoblasts with ΔNp73α by retroviral gene transfer. Whereas cells transduced with an empty retroviral vector (mock) arrest, elongate, align and fuse to form multinuclear myotubes that stain positive for myosin heavy chain (myHC) as a marker for differentiated muscle cells, ΔNp73α expressing C2C12 cells fail to differentiate (Figure 1c). Similar results were obtained in primary human and murine myoblasts (data not shown). Although p53-null mice have no muscle phenotype, it has been previously shown that myogenic differentiation is reduced by loss or inhibition of p53 due to defective induction of the retinoblastoma protein RB.[16] The differentiation defect induced by transdominant-negative p53 (p53DD), however, is subtle compared to the complete block of myogenic differentiation

observed with the pan-p53 family inhibitor ΔNp73α (Figure 1d). This supports the hypothesis of functional redundancy within the p53 family with respect to developmental control of myogenesis.

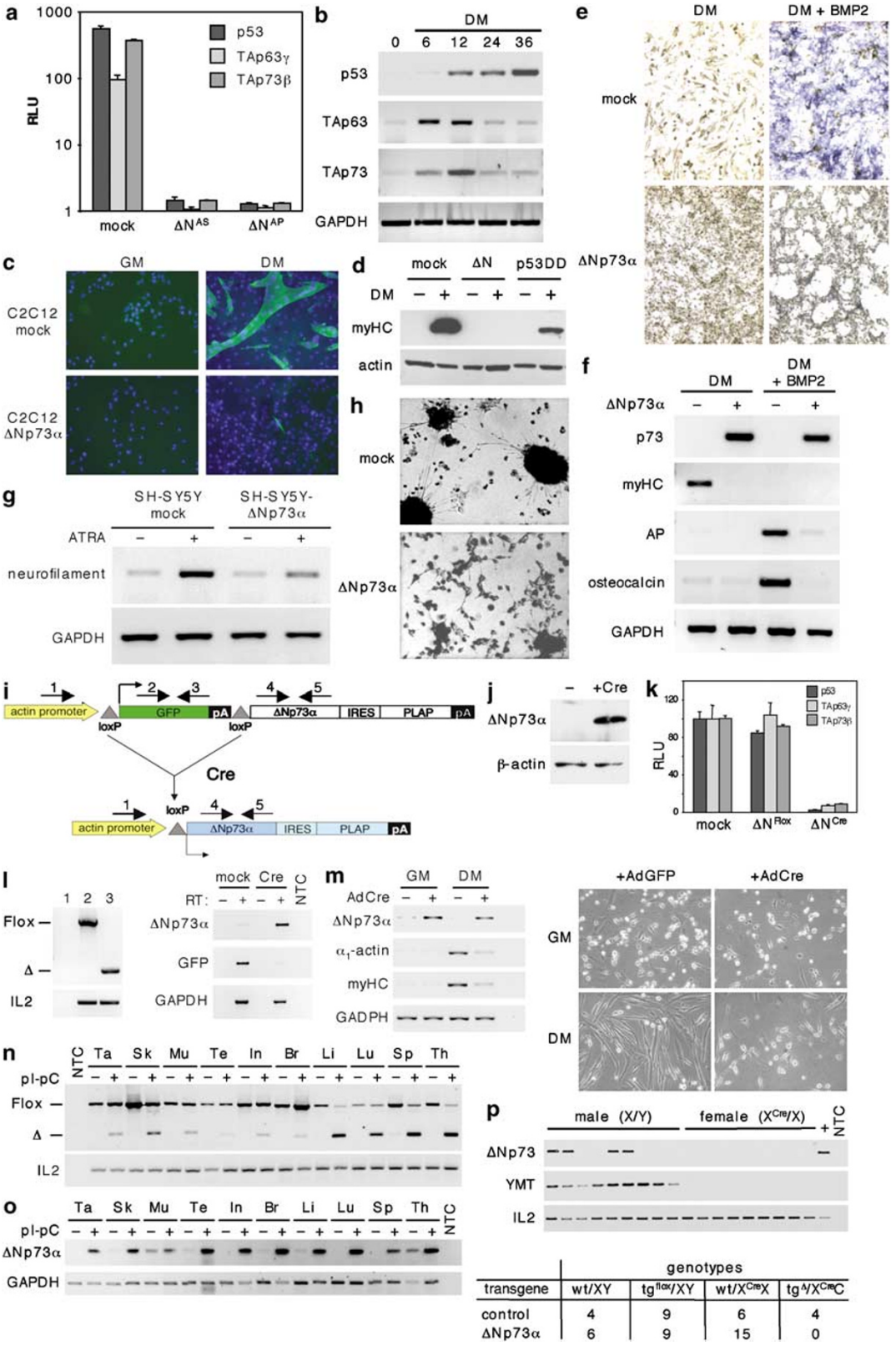
It is known that bone morphogenetic protein-2 (BMP2) converts the myogenic differentiation pathway of C2C12 myoblasts into that of osteoblast lineage.[21] Consistently, differentiation of mock myoblasts in the presence of BMP2 almost completely inhibited the formation of multinucleated, myHC-expressing myotubes, and induced the appearance of numerous alkaline phosphatase (ALP)- and osteocalcin-positive cells (Figure 1e and f). ΔNp73α interfered not only with the myogenic differentiation program but also effectively inhibited BMP2-induced conversion into the osteoblast lineage (Figure 1e and f).

Furthermore, TAp73 has been implicated in the regulation of neuronal differentiation providing an intriguing epxlanation for the neurological phenotype of p73-deficient mice.[22] Both induction of endogenous p73 by ATRA as well as ectopic expression of TAp73 isoforms have been shown to induce morphological and biochemical markers of neuronal differentiation in N1E-115 neuroblastoma cells.[18] Here we show, that ΔNp73α efficiently blocks ATRA-induced differentiation of SH-SY5Y neuroblastoma cells. Whereas ATRA-treatment induced extension of multiple neurites as a morphological marker and expression of neurofilament as a biochemical marker of neuronal differentiation in mock cells, this was significantly reduced in ΔNp73α transfectants (Figure 1g and h). These data clearly show that the p53 family inhibitor ΔNp73α is a potent repressor of differentiation in multiple experimental settings including myogenic, osteoblastic and neuronal differentiation.

To analyze the effect of p53 family inhibition *in vivo* we generated mice transgenic for the ΔNp73α-isoform obtained by alternative splicing of exon 2. We first attempted to create transgenic mice using the ubiquitously active HMG-CoA-reductase promoter, but repeatedly failed to obtain founder mice that expressed the transgene although other transgenic lines were readily obtained with this promoter. Considering that ΔNp73α might interfere with some essential developmental processes we cloned a conditional ΔNp73α transgene construct (Figure 1i) consisting of the broadly active β-actin promoter, followed by a GFP (green fluorescence protein)-stop cassette flanked by two loxP sites and preceeding a ΔNp73α cDNA coupled to a PLAP (human placenta-like ALP) cDNA via an internal ribosomal entry site (IRES). Excision of the GFP expression cassette by Cre recombinase efficiently induced ΔNp73α protein expression in transfected H1299 cells (Figure 1j). In the inactive state the transgene failed to inhibit transactivation of a p53-dependent reporter construct

Figure 1 ΔNp73 inhibits multiple cellular differentiation processes. (**a**) The transactivation function of the three p53 family members (p53, TAp63γ, TAp73β) is inhibited by coexpression of ΔNp73α isoforms generated by both alternative splicing of exon 2 (ΔN^{AS}) or alternative promoter usage (ΔN^{AP}). In detail, H1299 cells were cotransfected with 200 ng luciferase reporter plasmid containing p53 consensus binding sites,[25] 50 ng p53, TAp63γ or TAp73β expression plasmid[26] and 400 ng of the indicated ΔNp73α plasmid.[27] Luciferase activity was determined 48 h after transfection. (**b**) C2C12 cells were induced to differentiate into myotubes by incubation in medium containing 2% horse serum. RNA was isolated from the cells at the indicated time points (h) and expression of p53, TAp63 and TAp73 was measured by semiquantitative RT-PCR. Expression of the housekeeping gene GAPDH is shown as a control. (**c**) C2C12 cells were transduced with retrovirus generated by transient transfection of the amphotropic packaging cell line PT67 (Becton Dickinson) with either empty vector (mock) or the pQCXIP-ΔNp73α vector. Transduced cells were selected in growth medium containing 1.5 μg/ml puromycin. Cells were either grown in growth medium or differentiated for 5 days with 2% horse serum. Differentiation into multinuclear myotubes was analyzed by immunofluorescence staining for myosin heavy chain (MF 20 antibody, Developmental Studies Hybridoma Bank). Nuclei were counterstained with DAPI. (**d**) C2C12 myoblasts were stably transduced with retroviral vectors (mock, ΔNp73α or dominant-negative p53 p53DD) and analyzed for myosin heavy chain (myHC) expression by Western blot before and after induction of differentiation. (**e**) C2C12 myoblasts (mock or ΔNp73α) were differentiated for 5 days in medium containing 5% FCS in the absence or presence of 300 ng/ml BMP2 (kindly provided by Walter Sebald, University of Würzburg, Germany) and stained for the osteoblast marker gene alkaline phosphatase as described.[21] (**f**) C2C12 myoblasts treated as in (**e**) were analyzed by semiquantitative RT-PCR for expression of muscle (myHC) or osteoblast (alkaline phosphatase, AP; osteocalcin) marker genes. (**g** and **h**) SH-SY5Y neuroblastoma cells were transfected with pQCXIP (mock) or pQCXIP-ΔNp73α plasmids and selected in 1 μg/ml puromycin. Cells were induced to differentiate in 1 μM all-*trans* retinoic acid (ATRA), analyzed for (**g**) expression of the neuronal marker gene neurofilament by semiquantitative RT-PCR and (**h**) neurite extension by phase contrast microscopy. Sequences of primers used for RT-PCR are available upon request. (**i**) Diagram of the conditional ΔNp73α transgene construct. In the uninduced state the broadly active β-actin promoter drives expression of a GFP-stop cassette flanked by loxP sites. Recombination induced by Cre results in excision of the GFP-stop cassette and places the ΔNp73α cDNA (ΔNp73α isoform generated by alternative splicing of exon 2) under the control of the β-actin promoter. Expression of ΔNp73α is coupled to a human placenta-like alkaline phosphatase cDNA via an IRES sequence. Binding sites for primers used in subsequent PCR and RT-PCR analyses are indicated. (**j**) H1299 cells were transfected with the transgene construct by electroporation and infected with 50 moi of Cre-expressing adenovirus (+Cre; kindly provided by Anton Berns) 24 h later to induce recombination. Expression of ΔNp73α and β-actin was assessed by Western blot (anti-p73 ER15, Merck Biosciences; anti-actin, Abcam). (**k**) The GFP-stop cassette in the inactive ΔNp73α transgene plasmid (ΔN^{flox}) was excised with recombinant Cre enzyme (Becton Dickinson) *in vitro* resulting in the active ΔN^{Cre} plasmid. Inhibition of the transactivation function of the three p53 family members (p53, TAp63γ, TAp73β) was analyzed by coexpression of either ΔN^{flox} or ΔN^{Cre} as outlined in Figure 1a. Reporter activity in the presence of p53, TAp63γ and TAp73β alone was set as 100. The actual transactivation potential is shown in Figure 1a. (**l**) Recombination of the $\Delta Np73\alpha^{flox}$ transgene can be induced *in vitro* by expression of Cre. Dermal fibroblasts were isolated from $\Delta Np73\alpha^{flox}$ transgenic mice generated by pronuclear injection of the $\Delta Np73\alpha^{flox}$ transgene construct into mouse embryos. Recombination was induced by infection with 100 moi of Adeno-Cre. Left panel: recombination efficiency was analyzed by PCR using primers 1/5. 1, no template control (NTC); 2, uninfected; 3, Cre-infected. IL2 was amplified as a control. Right panel: Expression of GFP (primers 2/3) or ΔNp73α (primers 4/5) was analyzed by semiquantitative RT-PCR on RNA isolated from mock- or Cre-infected transgenic fibroblasts. Controls without reverse transcriptase (-RT) are indicated. (**m**) Muscle satellite cells were isolated from $\Delta Np73\alpha^{flox}$ transgenic mice as previously described[16] and cultured in Ham's F10 medium supplemented with 20% FCS and 2 ng/ml bFGF (Invitrogen). Cells were infected with 100 moi Adeno-Cre or Adeno-GFP as a control and either incubated in growth medium (GM) or induced to differentiate for 30 h in differentiation medium (DM) containing 2% horse serum (left panel: phase-contrast microscopic images; right panel: semiquantitative RT-PCR analysis of ΔNp73α, α_1-actin, myosin heavy chain (myHC) and GAPDH expression). (**n** and **o**) Recombination of the $\Delta Np73\alpha^{flox}$ transgene can be induced *in vivo*. $\Delta Np73\alpha^{flox}$ transgenic mice were crossed to Mx1-Cre mice.[23] Cre expression in $\Delta Np73\alpha^{flox}$/Mx1-Cre double transgenic offspring mice was left uninduced (–) or induced (+) by three intraperitoneal injection of pI-pC (250 μg) at 2-day intervals. At 5 days after the last injection (**n**) DNA and (**o**) RNA was isolated from various tissues and analyzed for recombination efficiency by PCR with primers 1/5 and for ΔNp73α expression by RT-PCR with primers 4/5. Ta, tail; Sk, skin; Mu, muscle; Te, testis; In, intestine; Br, brain; Li, liver; Lu, lung; Sp, spleen; Th, thymus; NTC, no template control. (**p**) Female $\Delta Np73\alpha^{flox}$ transgenic mice were crossed to male CMV-Cre mice (X^{Cre}/Y). DNA from offspring mice was analyzed for the ΔNp73α transgene by PCR with primers 4/5, for the Y-chromosome (YMT) and for IL2 as a control. The genotypes for 30 newborn mice are given in the table. Results of equivalent crosses of male CMV-Cre mice with females of a different conditional transgenic line are included as a control

by the various p53 family members indicating that expression is tightly controlled. *In vitro* recombination of the construct by recombinant Cre enzyme efficiently activated its transdominant-negative function (Figure 1k). Using the conditional construct for pronuclear injection we succeeded in obtaining the transgenic line ΔNp73α^{flox}. Dermal fibroblasts isolated



transgene	genotypes wt/XY	tg^{flox}/XY	wt/X^{Cre}X	tg^{Δ}/X^{Cre}C
control	4	9	6	4
ΔNp73α	6	9	15	0

Figure 1

from ΔNp73α^{flox} mice undergo excision of the GFP-stop cassette following adenoviral delivery of Cre (Figure 1l, left panel). As shown by RT-PCR, excision of the GFP-stop cassette results in a shift from GFP to ΔNp73α expression (Figure 1l, right panel). Only low background levels of ΔNp73α were detected in the uninduced state. Muscle satellite cells isolated from transgenic mice undergo terminal differentiation into multinuclear myotubes within 30 h of serum withdrawal. Induction of ΔNp73α by Adeno-Cre inhibits execution of the myogenic differentiation program (Figure 1m) and correlates with defective expression of skeletal muscle marker genes such as α_1-actin or myHC. This experiment shows that ΔNp73α expression is tightly repressed in the absence of Cre so that myogenic differentiation can proceed efficiently. It further shows that the inhibitory function of ΔNp73α on myogenic differentiation can be rapidly induced in primary muscle precursor cells and that it functions as predicted from our previous experiments in C2C12 myoblasts.

Regulation of ΔNp73α expression by Cre recombinase allows to specifically inhibit p53 family activity in a tissue-specific or time-controlled fashion. The conditional system for expression of ΔNp73α therefore provides a valuable model for studies aimed at assessing the role of the p53 family in cellular differentiation, tissue regeneration, embryonic development and tumorigenesis. To test if the transgene can be activated *in vivo*, we crossed ΔNp73α^{flox} mice to Mx1-Cre mice that express Cre under control of the interferon-responsive promoter of the *Mx1* gene.[23] Transient activation of the Mx1 promoter by intraperitoneal injections of the interferon inducer polyinosinic-polycytidylic acid (pI-pC) induces excision of the floxed GFP-stop cassette in a wide range of tissues resulting in efficient induction of ΔNp73α (Figure 1n and o). Recombination efficiency varies depending on the tissue analyzed from less than 10% in the brain to more than 80% in the liver. This is in agreement with the recombination efficiencies reported for other floxed DNA sequences using the Mx1-Cre transgenic line.[23] To induce ΔNp73α expression during embryonic development, ΔNp73α-transgenic mice were crossed to Cre-deleter mice carrying a CMV-promoter driven Cre expression cassette on the X-chromosome.[24] In this strain expression of Cre has been shown to occur before implantation during early embryogenesis. Owing to the X-chromosomal location of the Cre-transgene, transgene transmission through males is restricted to female offspring. From matings of ΔNp73α^{flox} transgenic mice to male Cre-deleter mice we obtained male ΔNp73-transgenic offspring at the expected Mendelian ratio. Consistent with the absence of a Cre allele in male offspring, the ΔNp73α transgene in these mice contained the floxed GFP-stop cassette. In contrast, no female ΔNp73α transgenic (i.e. ΔNp73α/Cre double transgenic) offspring were obtained from these matings. As a control, female transgenic mice with an excised GFP-stop cassette were readily obtained in parallel experiments with a different transgenic line. These data indicate that activation of ΔNp73α during early embryogenesis interferes with essential steps of embryonic development (Figure 1p). It remains to be seen at which stage of development ΔNp73 interferes with embryogenesis and whether the phenotype resembles the phenotype of p53 knockdown in early *Xenopus* embryos. However, the phenotype of p53 family inhibition by ΔNp73α is more severe than any of the reported homozygous knockouts of single p53 family members and suggests significant functional redundancy and cooperativity within the p53 family in the coordination of embryonic development. Deregulated expression of the pan-p53 family inhibitor ΔNp73α therefore provides a first glance at the putative phenotype of a homozygous compound knockout of all three p53 family members.

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