Letter to the Editor

Deregulated expression of Δ Np73 α causes early embryonic lethality*Cell Death and Differentiation* (2006) 13, 170–173. doi:10.1038/sj.cdd.4401743; published online 19 August 2005

Dear Editor,

The tumor suppressor protein p53 shares considerable structural and functional homology with its family members p63 and p73. For example, full-length human p73 (TAp73) shares 63% amino-acid identity with the DNA-binding region of p53, conserving all DNA contact residues, and 38% and 29% identity with the p53 tetramerization and transactivation domains, respectively.¹ Human TAp73, or its homolog TAp63, is required for p53-mediated transcriptional activation of apoptotic genes in fibroblasts.² However, neither TAp63 nor TAp73 are inactivated by mutations in human cancers, excluding them from being categorized as classic Knudsen-type tumor suppressors. In fact, p73 has been shown to be overexpressed in many different human cancers. This apparent contradiction in p73 activity may lie in the fact that the p73 gene produces diverse N-terminal isoforms with distinct or even opposing properties. Aside from the pro-apoptotic transactivation-competent TAp73 isoform, the N-terminally truncated transactivation-deficient Δ Np73 is biologically important and might play a role as an oncogene in human cancers. Δ Np73 protein is either generated from the P1 promoter by alternate Exon 3' splicing or via an alternate P2 promoter in Intron 3. Moreover, alternate splicing at the C-terminus results in at least six different C terminal variants α – ϕ (of which α is the full-length version) that are thought to differ in their transcriptional potency but whose biological differences remain to be fully elucidated.³

Importantly, the Δ Np73 isoform is frequently upregulated in various human cancers, including ovarian cancer,⁴ breast and gynecological cancers,⁵ hepatocellular carcinoma,⁶ lung cancer,⁷ gastric cancer,⁸ thyroid cancer⁹ and neuroblastoma.¹⁰ Overexpression of Δ Np73 is an independent prognostic marker for reduced survival in lung cancer patients⁷ and in neuroblastoma patients.¹⁰ In cell-based assays, Δ Np73 α displays oncogenic properties. Δ Np73 α immortalizes primary mouse embryo fibroblasts (MEFs) *in vitro* and cooperates with oncogenic Ras in inducing MEF-derived fibrosarcomas *in vivo*.^{6,11} Together, these results suggest that in cancer tissues, Δ Np73 overexpression plays an oncogenic role and functionally abrogates any concomitant increase in TAp73.

We were interested in further characterizing the role of Δ Np73. In addition to its inhibition of TAp73, Δ Np73 functions as a dominant-negative inhibitor of wild-type p53 and most likely of TAp63, and thus is a potent pan inhibitor of the p53 gene family.^{11,12} There are no studies reported to date examining the *in vivo* effects of homozygous compound

knockouts of individual family members. Although the loss of any one of these genes alone does not interfere with viability, it creates a significantly disrupted phenotype that is gene-specific. Deficiency of p53 leads to a strongly increased tumor susceptibility at young age, but the majority of p53-null mice survive to adulthood, indicating that p53 function is not essential for embryonic development.¹³ Only a small percentage of young, mainly female p53 null mice exhibit neural tube closure defects due to neuronal overgrowth with exencephaly of the midbrain.¹⁴ Interestingly, this exencephalic phenotype resembles that of animals with targeted mutations in other members of the mitochondrial death pathway such as APAF-1,^{15,16} caspase-9¹⁷ and caspase-3.¹⁸ p63-deficient mice, on the other hand, show grave developmental defects in limb and craniofacial formation and epithelial morphogenesis but do survive until a few days after birth.^{19,20} p73 deficient mice exhibit a milder developmental phenotype, with defects specifically within the central nervous system (hippocampus) and the innate immune system. They survive to birth, but most p73-null mice die within 2 months after birth due to infection, with about 25% reaching adulthood.²¹ Aged p63 +/– and p73 +/– mice show a somewhat higher spontaneous tumor predisposition that is about half of the predisposition conferred by a p53 +/– status.²²

Defining the phenotype of compound disruptions in the gene family will be important. Mice with heterozygous compound null alleles of any two of the three family members reveal the cooperativity of family members in tumor suppression.²² However, homozygous compound double or even triple knockout mice have not been reported, possibly because they are difficult or even impossible to generate. Importantly, Δ Np73 acts as a dominant-negative pan inhibitor of p53, TAp73 and TAp63. Thus, we chose overexpression of Δ Np73 as an alternate way to create functional compound knockout animals of the p53 gene family. We, therefore, set out to generate mice overexpressing the α variant of human Δ Np73. Initially, we used constitutive overexpression of Flag-tagged Δ Np73 α driven by a CMV promoter. As negative control, mice expressing an inactive (tetramerization-incompetent but DNA-binding competent) mutant form of Δ Np73 α (L322P) were also created (Figure 1a).^{5,11} These constructs were first verified for protein expression in the p53-null human cancer cell line H1299. As shown in Figure 1b, a clear Flag signal was obtained from cells transfected with wild-type and mutant Δ Np73 constructs, but not from cells transfected with green fluorescent protein (GFP).

Figure 1 Overexpression of $\Delta Np73\alpha$ causes early embryonic lethality. (a) Constructs of Flag-tagged wild-type and mutant $\Delta Np73\alpha$ cDNAs were inserted between a CMV promoter and terminated with a bovine growth hormone polyadenylation signal. Arrows indicate the location and direction of the two PCR primer sets used for genotyping: T7-specific sense primer (5'-aatagcactcactagggagaccc) paired with a $\Delta Np73\alpha$ -specific antisense primer beginning at codon 42 (5'-ggcgagtggtgggcacgctggc) and a $\Delta Np73\alpha$ -specific sense primer beginning at codon 501 (5'-cagggccacgactacagcaccgcg) paired with a BGH-polyA-specific antisense primer (5'-ggcacagctcagggctgatcagcggg). (b) Protein expression in transiently transfected H1299 cells. Cells were transfected with wild-type, mutant $\Delta Np73\alpha$ L322P plasmids or GFP as control. Protein expression was detected by Western blots using an anti-Flag antibody (Sigma). (c) Embryo survival and phenotypes at day E8.5. Zygotes from superovulated, mated C57Bl/6 mice were injected with either wild-type or mutant $\Delta Np73\alpha$ constructs, implanted in pseudo-pregnant mice and harvested 8.5 days later. DNA was prepared from viable embryos and genotyped using the transgene-specific primer pairs described above, and also with BclXI as an internal control for template DNA. (The BclXI primers were sense 5'-agagaacaggactgaggccc and antisense 5'-tcaagctctgatctgtctgcc.) PCRs were 30 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by 7 min at 72°C. Products were identified by ethidium bromide stained 1.5% agarose gels. '—' no template control, '+' transgene plasmid as positive control, * phenotypically abnormal embryo

We then microinjected the CMV-based wild-type $\Delta Np73$ construct into zygotes obtained from superovulated, mated C57Bl/6 mice and implanted them into pseudo-pregnant isogenic mice in an attempt to create $\Delta Np73\alpha$ transgenic mice. However, we could never obtain transgenic litters. We, therefore, repeated the injections and collected embryos at stage E8.5, characterizing them for transgenesis by genomic PCR and phenotypic survival (Figure 1c). Implantation of zygotes injected with the wild-type $\Delta Np73\alpha$ construct resulted in 14 successful implantations. However, by day E8.5, four implantation sites showed complete resorption of their embryos. Moreover, of the 10 remaining viable embryos, only three were normal. The other seven embryos were abnormal: three had begun to resorb, while four were small and developmentally delayed (E6.5–E8.0). Of these seven abnormal embryos, six were shown by PCR to contain the $\Delta Np73\alpha$ transgene (Figure 1c). In contrast, implantation of zygotes injected with the mutant $\Delta Np73\alpha$ (L322P) construct resulted in 26 successful implantation sites, all of which contained viable embryos at day 8.5. Only one of these embryos showed phenotypic abnormality, and 15 embryos were shown by PCR to carry the mutant $\Delta Np73\alpha$ L322P transgene (Figure 1c). Taken together, we conclude that forced overexpression of wild-type $\Delta Np73\alpha$, but not the L322P mutant, in developing mice is embryonically lethal. We,

therefore, attempted to switch to an inducible expression system.

We generated a new set of $\Delta Np73\alpha$ transgenics in the tet-inducible expression vector pTRE2 (Tet-On, Clontech) (Figure 2a). The TRE promoter is designed to be inactive in the absence of a tetracycline-sensitive transactivator and tetracycline. We first verified the inducible expression of $\Delta Np73\alpha$ through transient and stable transfections into HeLa cells stably transfected with the tet-inducible transactivator (Clontech) (Figure 2b). Addition of doxycycline to $\Delta Np73\alpha$ -transfected but not to empty vector-transfected cells resulted in abundant $\Delta Np73\alpha$ protein expression, indicating that this construct was readily tet-inducible. However, we also reproducibly detected low levels of $\Delta Np73\alpha$ protein expression in stably transfected cells in the absence of doxycycline, indicating that tetracycline regulation of transgene expression was leaky.

Since leaky expression was relatively low, we proceeded with pronuclear injections with this construct in an attempt to create tet-inducible $\Delta Np73\alpha$ transgenic mice. However, we encountered an abnormally low success rate (expected to be 30–40%). Only one out of 63 mice generated from these injections contained the transgene (1.5%), as detected by genomic PCR and Southern blotting. This founder was mated with a mouse transgenic for the tetracycline-sensitive transactivator²³ and the transgene was found to be inherited in a Mendelian ratio. However, when RNA samples from the resulting double heterozygous pups were analyzed, we were unable to detect expression of the transgene, either before or after adding doxycycline to the drinking water of the pups (data not shown). We assumed that the TRE $\Delta Np73\alpha$ transgene was also leaky *in vivo*, causing all animals with transgene expression to die *in utero*. The single transgenic founder we obtained survived because the transgene had integrated into a transcriptionally silent locus of the genome.

To test this possibility directly, we injected more pronuclei with our tet-inducible $\Delta Np73\alpha$ construct and harvested the resulting embryos at days E8.5, E9.5 and E10.5. In our hands, micromanipulation of mouse zygotes in general, involving injection of the embryos with a transgene and transfer of injected embryos into a foster mother, normally yields a 30–40% transgenic birth rate. Here, we also found that wild-type embryos consistently showed a 30–40% survival rate at E10.5 (Figure 2c, d). However, as predicted, we found that the survival rate for $\Delta Np73\alpha$ transgenic embryos dropped to 0% from E8.5 to E10.5 (Figure 2c, d). At day E8.5, we obtained only viable embryos (23 in total) and saw no evidence of resorption. In all, 15 of those embryos (65%) were shown by PCR to contain the transgene (data not shown). At day E9.5, we obtained 13 viable embryos and 10 empty uterine sacs, indicating that 10 of 23 embryos had died and undergone resorption. Only four out of the 13 viable embryos (31%) contained the transgene. At day E10.5, we detected only four viable wild-type embryos and 10 additional implantation sites containing resorbing transgenic embryos in the transgenic litter. The kinetics of loss of embryonic viability indicates that $\Delta Np73\alpha$ transgenics are unable to develop past E9.5. Importantly, forced overexpression of the inactive mutant $\Delta Np73\alpha$ (L322P) allele in transgenic animals did not lead to embryonic death (Figure 1c), indicating that this embryonic

Figure 2 Expression of $\Delta Np73\alpha$ from a leaky tet-inducible promoter is embryonically lethal. **(a)** pTRE- $\Delta Np73\alpha$ construct. Flag-tagged wild-type human $\Delta Np73\alpha$ cDNAs was subcloned into the pTRE-pur expression vector (Clontech). The 3' end of the beta-globin gene contains a splice site downstream of the stop codon, so that transgenic DNA can be distinguished from the expressed and spliced transgenic mRNA by PCR. Arrows indicate the location and orientation of PCR primers used for genotyping. The $\Delta Np73\alpha$ -specific primers are the same as in Figure 1a. The vector-specific primers were TRE2 5' (Clontech 5'-gatcgcttgagagacgccatccacgc) and beta globin 3' (5'-ataggcagcctgcacctgagg). Kzk = Kozak sequence. **(b)** Leaky expression of the tet-inducible $\Delta Np73\alpha$ construct. HeLa Tet-on cells (Clontech) were transiently or stably transfected with either the pTRE- $\Delta Np73\alpha$ construct or empty vector, and subsequently left untreated or exposed to 1 μ g/ml doxycycline for 24 h. Cell lysates were prepared and analyzed for expression by Western blotting with anti-Flag. Equal total protein was loaded per lane. The Tet-On/Off inducible expression system was shown to provide relatively tight regulation of protein expression²⁵ and has been successfully used in many different cell culture and *in vivo* applications. However, a recent study comparing four different inducible expression systems (Tet-On/Off, MMTV Promoter, Ecdyson P and T7 Promoter) revealed that the Tet-On system leads to the highest basal, leaky protein expression.²⁶ **(c, d)** Embryonic survival at days 8.5, 9.5 and 10.5 of gestation. **(c)** Genomic DNA was recovered from viable embryos and tested by PCR for the presence of the $\Delta Np73\alpha$ transgene. Total survival = % of total implantation sites with viable embryos; transgenic survival = % of surviving embryos that contain the transgene. **(d)** Survival as a function of genotype. **(e)** Top: Pronuclei were again injected with the Flag-tagged pTRE- $\Delta Np73\alpha$ construct. Day E8.5 embryos were recovered and genotyped as described. Bottom: In addition, protein lysates were prepared and analyzed for transgene expression by Western blot using anti-Flag antibody. * phenotypically abnormal embryo, 'cross' = dead embryo – + cell lysates from vector or CMV-Flag-tagged wt $\Delta Np73\alpha$ transfected H1299 lysates. Actin was used as loading control. **(f)** $\Delta Np73\alpha$ transgenic embryos are histologically abnormal and are deficient in a p53-mediated stress response in the embryonic tissue. Wild-type and CMV- $\Delta Np73\alpha$ transgenic embryos were irradiated with 1.5 Gy IR *in utero* at day E7.5. After 6 h, the pregnant females were sacrificed and the decidua were recovered, formalin-fixed, paraffin-embedded and sectioned. Nonirradiated and nontransgenic E7.5 embryos were also prepared as controls (not shown). The apoptotic response was assessed by TUNEL staining (Roche) and morphology was assessed by H + E staining. Transgene positive embryos were confirmed by immunostaining with a polyclonal Flag antibody, and by PCR amplification of DNA, which had been extracted from serial paraffin sections. Representative embryos are shown. Stars denote amniotic cavity surrounded by embryonic tissue. a, amnion; b, amniotic cavity; c, embryonic ectoderm; d, embryonic mesoderm; e, *exocoelomic* cavity; f, visceral endoderm; g, chorionic ectoderm; h, extraembryonic tissue

lethality is specific for wild-type $\Delta Np73\alpha$ protein expression. To prove that the lethal phenotype of these transgenic embryos was in fact due to leaky expression of $\Delta Np73\alpha$ protein, we repeated these experiments and assayed for transgene expression in day E8.5 embryos, using Flag immunoblotting of whole embryo extracts (Figure 2e). From a litter of seven embryos, three were nontransgenic and phenotypically normal. Of the remaining four, which were all transgenic, one had died and three were phenotypically abnormal. Of note, the three abnormal but still viable embryos also expressed the transgenic $\Delta Np73\alpha$ protein (Figure 2e).

To further support the hypothesis that $\Delta Np73$ might function as a potent pan inhibitor of the p53 gene family in

development, we undertook a histological and functional characterization of $\Delta Np73\alpha$ transgenic embryos. At E7.5, wild-type embryos had undergone gastrulation, generating an amniotic membrane and chorionic ectoderm (Figure 2f top left). In contrast, $\Delta Np73\alpha$ transgenic embryos lacked these developmental landmarks and instead resembled E6–6.5 embryos (Figure 2f, top right). Thus, transgenic embryos are histologically abnormal. Combined with loss of viability past E9.0–9.5 (Figure 2d), this suggests that they fail at gastrulation and subsequently die.

A pronounced p53-dependent apoptotic response in E6.5–E7.5 embryos *in utero*, which is our window of interest, can be triggered by γ IR of pregnant mice (6 h after 1.5 Gy).²⁴ This

Figure 2 Continued.

apoptosis is not observed in $p53^{-/-}$ mice.²⁴ On the other hand, it is essential for survival of wild-type mice, since no $p53^{-/-}$ mice are born that have been subjected to this dose of radiation.²⁴ Therefore, we compared wild-type and transgenic embryos under this condition. As anticipated, wild-type embryos irradiated *in utero* with 1.5 Gy showed a robust apoptotic response limited to the embryonic tissue. In contrast, transgenic embryos either completely lacked this response or showed only a remnant activity compared to wild-type (representative embryos in Figure 2f compare left and right TUNEL panels).

Our finding of embryonic lethality by deregulated $\Delta Np73\alpha$ expression, a potent pan-dominant negative inhibitor of p53, TAp73 and most likely of TAp63, is significant, considering that single gene deficiencies of neither p53, p63 and p73

cause embryonic lethality. We know that both p53 and p73 play an important role in neuronal differentiation through apoptotic regulation of neuronal growth. Thus, we expected exogenous overexpression of $\Delta Np73$ to specifically deregulate neuronal differentiation. However, our results from this study clearly show that deregulated expression of $\Delta Np73$ during embryogenesis completely disables development earlier than day E8.5–E9.5, prior to the neurogenesis of the central nervous system. In summary, deregulated expression of the dominant negative pan p53/TA63/TA73 inhibitor $\Delta Np73\alpha$ causes an earlier and, therefore, broader disruption of development than even the most severe single gene deficiency of the p53 family, that is, that of p63. Thus, this data demonstrates that the gene family members have distinct nonoverlapping functions in development, confirming earlier conclusions from single gene knockout studies, but interestingly, whose combined action appears to be more than the sum of its parts. Moreover, since homozygous compound double or even triple knockout animals have not yet been reported, our study shows that the p53 gene family as a whole is an upstream survival/death checkpoint that functions as an essential rheostat in early embryonic development.

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