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DeltaNp73 transcription factors modulate cell survival and tumor development

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The p73 locus encodes two types of transcription factors: full length pro-apoptotic isoforms (TAp73), and N-terminally truncated anti-apoptotic proteins (DeltaNp73). To study the function of DeltaNp73 in vivo, we generated mutant mice in which *DeltaNp73* is inactivated, but *TAp73* expression is intact. In addition, we knocked in the locus the Cre recombinase, and the enhanced green fluorescent protein (EGFP). Using this allele, we refined the expression of DeltaNp73 during brain development and emphasized the importance of the thalamic eminence, a transient source that contributes neurons to the telencephalon. We showed that *DeltaNp73* inactivation increases apoptosis in neurons.[1] We also investigated the role of DeltaNp73 in carcinogenesis by inducing tumors with methylcholanthrene in mutant and control mice, and found that mutant females, but not males, have decreased propensity to tumor development. Both effects on neuronal apoptosis and tumor development were milder than predicted from in vitro studies.

Key words: cajal retzius cells, p73, TAp73, apoptosis, methycholanthrene, fibrosarcoma, oncogene

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Introduction to p73

p73 proteins are transcription factors transcribed from the *Trp73* locus.[2] They have close similarity to p53 and p63, which play major roles in tumor suppression and in development.[3-6] Several mRNA and proteins are produced from the *Trp73* locus, by usage of two promoters and several alternative splicing and polyadenylation sites. Transcripts from the first promoter encode proteins with transactivation (TA) domain. TAp73 factors bind p73-responsive promoters and activate transcription of p53 target genes such as *p21^{waf1}, 14-3-3, Gadd45, Btg2 or Bax*.[4] Like p53, TAp73 proteins have pro-apoptotic properties and their mechanism of action is subject to intense investigation. Upon stress or DNA damage, activated c-Jun N-terminal kinase JNK phosphorylates 14-3-3 at a site that mediates binding to the c-Abl tyrosine kinase. c-Abl thus released moves to the nucleus where it phosphorylates p73. Furthermore, JNK itself is able to phosphorylate p73, contributing to its stabilization. Phosphorylated p73 associates with Pin1, is acetylated by p300, and activates transcription of pro-apoptotic genes. Another partner of p73 is the Yes-associated protein Yap1, which is also phosphorylated and stabilized by cAbl. Phosphorylated Yap1 binds p73 and serves as a transcriptional co-activator, partly by preventing p73 binding to the E3 ligase Itch, thus protecting it from proteasomal degradation.[7,8]

A second set of transcripts are initiated from an alternative *Trp73* promoter in intron 3. They encode DeltaNp73 proteins that lack the N-terminal TA domain.[9] Although DeltaNp73 proteins have no direct transcriptional activity, they can form dimers with TAp73 proteins, and are thought to behave as dominant negative, anti-apoptotic factors.[10] They are also able to heterodimerize with and inactivate p53 and p63, suggesting that they may have anti-apoptotic, tumor promoting activity.[11] Additionally, it is worth mentioning that isoforms lacking the TA domain may also arise from the first promoter, by alternative splicing of exons 2-3 (DeltaEx2, Delta Ex2/3).[12]

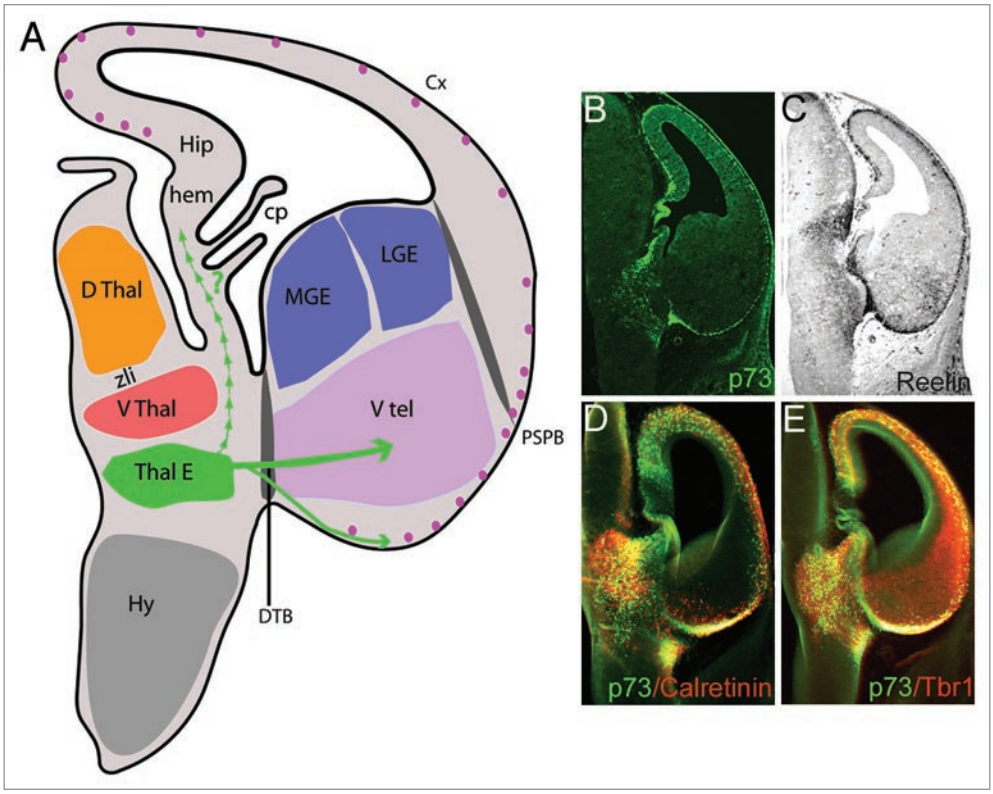

Figure 1. *DeltaNp73* is expressed in the thalamic eminence. (A) Outline of a typical coronal section in the forebrain at E13.5. The thalamic eminence (Thal E) is a transient region that provides neurons to the basal telencephalon (thick arrow), to the marginal zone in basal telencephalon (thinner arrow) and possibly also to the medial cortex (small arrows). Cajal-Retzius cells are schematized as pink dots. Cp, choroid plexus; Cx, cortex; DTB, diencephalon-telencephalon junction; D. Thal, dorsal thalamus; Hip, hippocampus; Hy, hypothalamus; LGE & MGE, lateral and medial ganglionic eminences; PSPB, pallial subpallial boundary; V tel, ventral telencephalon; V Thal, ventral thalamus; zli, zona limitans intrathalami. (B and C) Immunohistochemistry on adjacent cryostat sections shows the expression of DeltaNp73 (B) and Reelin (C) in the thalamic eminence; note that Reelin is also expressed in ventral thalamus. (D and E) vibratome sections showing the expression of DeltaNp73 in migrating cells of the thalamic eminence (EGFP) and colocalization with Calretinin (D, red) and Tbr1 (E, red).

DeltaNp73 and Brain Development

Inactivation of all p73 isoforms in mice generates a striking brain phenotype. In *p73*$^{-/-}$ mice, the number of Reelin producing Cajal-Retzius cells (CRc) is dramatically reduced, the shape of the dentate gyrus is abnormal, with amputation of the lower blade, and the hippocampal fissure fails to form.[13,14] Selective inactivation of *TAp73* isoforms leads to subtle abnormalities of the granule cell layer in the dentate gyrus, but does not affect development of CRc,[15,16] suggesting that inactivation of *DeltaNp73* may underlie a significant part in the p73$^{-/-}$ phenotype. To understand better its role in vivo, we inactivated *DeltaNp73* selectively, while leaving all *TAp73* forms unaffected, and inserted in the locus sequences that encode Cre and EGFP.[1]

Using the EGFP reporter, we identified a zone of strongly DeltaNp73 positive cells in the ventral diencephalon at E11.5-E13.5 (**Fig. 1**). After E13.5, these cells decreased rapidly in number, while they migrated and contributed to basal forebrain, and possibly to the marginal zone where some may give rise to CRc. Diencephalic DeltaNp73 positive neurons were also labeled with reelin, calretinin and Tbr1, and correspond to the thalamic eminence (TE),[17] also referred to as diencephalic perireticular nuclei.[18] The transient TE derived cells might function as guidepost cells for axonal tracts in the internal capsule, such as the thalamocortical tract. Recently, a stream of Dbx1 positive cells, referred to as preoptic area, was shown to originate in a diencephalic zone that is very reminiscent of the TE, and migrate to the amygdala.[19] Altogether, these results indicate that the TE may be a previously unrecognized source of CRc (also personal communication of Fairen A and Pierani A), and the *DeltaNp73* mutant mouse provides a useful tool to further study this population of neurons.

When compared to wildtype littermates, homozygous *DeltaNp73* mutant mice display increased cell death in all p73-expressing cells as predicted from the proposed anti-apoptotic activity of DeltaNp73. The number of CRc, preoptic area cells, GnRH and vomeronasal neurons was markedly reduced, and choroid plexuses were atrophic. CRc appear in telencephalic marginal zones early in development, and most of them disappear after birth in rodents.[20] They originate in multiple regions in the forebrain and migrate tangentially in subpial location.[21-26] It was proposed that the balance between TAp73 and DeltaNp73 proteins may regulate their apoptosis. Consistent with that view, we found that CRc disappear prematurely in *DeltaNp73* mutant mice. However, the cell death phenotype is mild suggesting

that *DeltaNp73* is not as important for neuronal survival as inferred by in vitro studies. In *DeltaNp73*$^{-/-}$ mice, the loss of function of DeltaNp73 could be compensated by DeltaEx2, DeltaEx2/3, DeltaNp53 (p44) or DeltaNp63 isoforms. Finally, the neural phenotypes in *TAp73* and *DeltaNp73* mutant mice are less severe than that in full *p73*$^{-/-}$ animals[1,13,16,27] suggesting that TAp73 and DeltaNp73 may have redundant rather than or in addition to opposite functions.

DeltaNp73 and Cancer

Due to their putative role in tumor progression, p73 proteins generated a keen interest (more than 1,350 entries in PubMed, oct 2009). However, unlike *p53*, *p73* is rarely mutated in human cancers.[9,28] Rather, *DeltaNp73* was found to be overexpressed in several human tumors,[4,9] and overexpression was associated with a poor prognosis and advanced stages of breast and colon carcinomas.[29-31]

The role of p73 in tumorigenesis was examined in different *p73* knock-out mice. Although no increased tumor susceptibility was initially reported in young mice deficient in all p73 isoforms,[9,14] tumors such as lung adenocarcinomas, thymic lymphoma and benign premalignant lesions were subsequently found in aging p73 heterozygous mice. Interestingly, they are reminiscent of those that appear in *p63*$^{+/-}$ mice.[3] 30% of *TAp73*$^{+/-}$ and 73% of *TAp73*$^{-/-}$ mice spontaneously develop malignancies, in particular lung adenocarcinomas, confirming that TAp73 has a tumor suppressor function. In addition, following tumor induction with dimethylbenzanthracene (DMBA), the time of survival of *TAp73*$^{-/-}$ females is shorter than that of *TAp73*$^{+/+}$ females. *TAp73* deletion induces the appearance of multinucleated cells in MEFs cultured in vitro, suggesting that *TAp73* contributes to genome stability.[15,16]

The anti-apoptotic activity of DeltaNp73 predicts that *DeltaNp73*$^{-/-}$ mice should display resistance to carcinogenesis. To test this hypothesis, we performed experimental tumor induction with methylcholanthrene (MCA), a potent carcinogen that induces fibrosarcomas at the inoculation site.[32] Twenty one days after birth, we injected 200 µg MCA subcutaneously in 33 *DeltaNp73*$^{-/-}$ (17 males and 16 females) and 36 *DeltaNp73*$^{+/+}$ littermate controls (19 males and 17 females), and monitored tumor development twice a week for 34 weeks. Mice with a tumor diameter larger than 5 mm were scored positive. Animals were sacrificed when the tumor diameter was larger than 2 cm. Results were analyzed using Kaplan Meier statistics.[33]

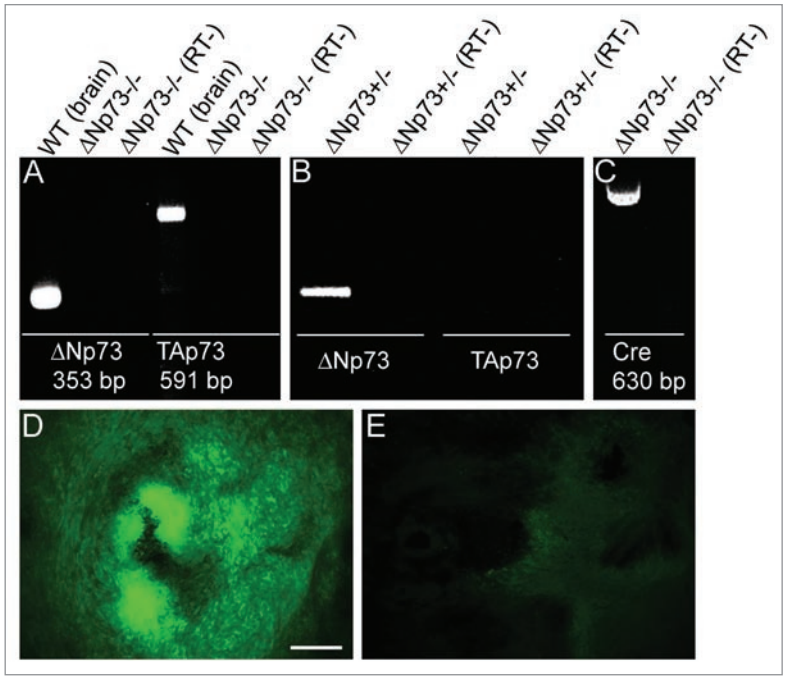

Figure 2. *DeltaNp73* expression in MCA induced tumors. (A–C) RT-PCR analysis. (A) *DeltaNp73* and *TAp73* are both expressed in wildtype (WT) brain tissue, but not in mutant tumor extracts. (B) *DeltaNp73*, but not *TAp73*, is expressed in tumor tissue induced in heterozygous *DeltaNp73* mice. (C) The knocked-in Cre is expressed in mutant tumors, indicating the expression of *DeltaNp73* in tumor tissue. RT: reverse transcriptase negative controls. (D and E) Direct histofluorescence shows expression of EGFP from the modified *DeltaNp73* locus, in a tumor induced in a *DeltaNp73*$^{-/-}$ mouse (D), but not in a tumor induced in wildtype (E). Bar = 100 µm.

In all injected mice, regardless of their genotype, MCA injection induced fibrosarcomas at the inoculation site. Using RT-PCR and histofluorescence, we found that DeltaNp73 was similarly expressed in induced tumor tissue in *DeltaNp73*$^{+/+}$ and *DeltaNp73*$^{+/-}$, and replaced by the knocked-in EGFP in *DeltaNp73*$^{-/-}$ mice (**Fig. 2**), confirming activation of DeltaNp73 expression in tumors. By contrast, TAp73 expression was never detected (**Fig. 2**). No difference was seen between heterozygous and wildtype mice. For further analysis, we only considered DeltaNp73$^{-/-}$ and wildtype mice and found that 50% of mice developed a tumor after 7 to 9 weeks (7 weeks for *DeltaNp73*$^{+/+}$ and 8 weeks for *DeltaNp73*$^{-/-}$ males, 9 weeks for *DeltaNp73*$^{+/+}$ and 9 weeks for *DeltaNp73*$^{-/-}$ females). 34 weeks after MCA injection, 18/19 of *DeltaNp73*$^{+/+}$ males, 15/17 of *DeltaNp73*$^{-/-}$ males, 17/17 of *DeltaNp73*$^{+/+}$ females and 15/16 of *DeltaNp73*$^{-/-}$ females had a tumor. Kaplan Meier statistical analysis with the MedCalc program[34] indicated that all groups developed tumors with similar frequency and kinetics (**Fig. 3A and C**) and no difference in the average latency time of tumor development was detected between *DeltaNp73*$^{+/+}$ and *DeltaNp73*$^{-/-}$ animals using student t-test (**Fig. 3B and D**). However, statistical differences appeared in survival curves when animals were sorted by sex. Although deletion of *DeltaNp73* did not modify life expectancy in males, *DeltaNp73*$^{-/-}$ females had a higher survival probability and a longer mean lifetime than *DeltaNp73*$^{+/+}$ females ($p = 0.0067$). This effect was not related to differences in susceptibility to MCA between normal males and females mice ($p = 0.3932$).

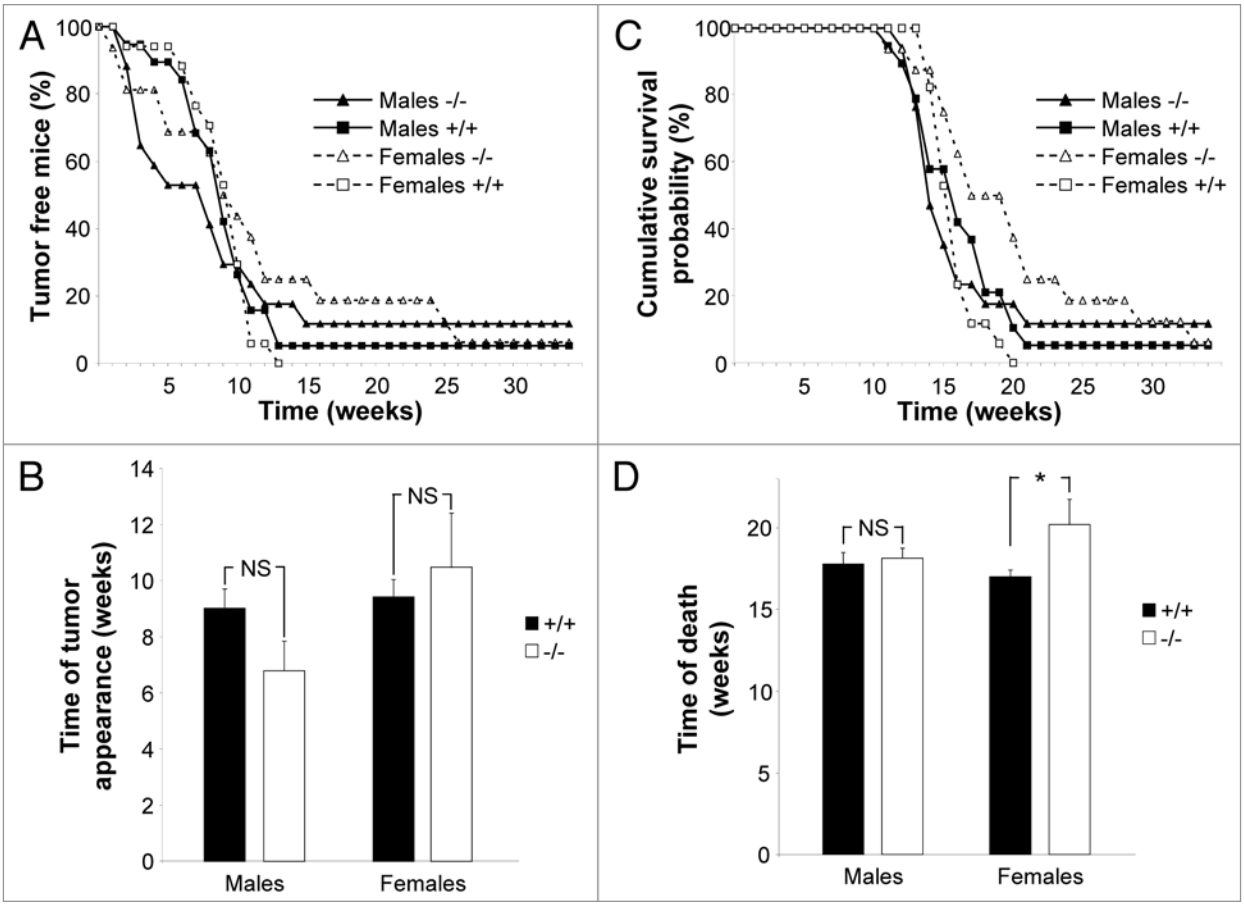

Figure 3. Effects of *DeltaNp73* on tumor progression. Kaplan-Meier analysis showed no difference in tumor latency (A), but a significant increase in survival time was observed in female *DeltaNp73* mutant mice (C), which was confirmed using t-test (B and D). NS: non significant; *significant ($p < 0.05$).

The observation that *DeltaNp73* deletion influenced tumor proliferation only in *DeltaNp73*$^{-/-}$ females is intriguing. However, similar effects have been reported before. Increased susceptibility to DMBA tumorigenesis was described for *TAp73* knock-out females.[16] Although no data was reported for males in that work, the results hint at gender susceptibility differences similar to the ones we found. Mice homozygous for *Chk**1100delC2 mutation are prone to tumor induction, and this is heavily gender sensitive, with females much more susceptible than males. In this case, the mechanism proposed is estrogen hormone sensitivity.[35] In our *DeltaNp73* mutant mice, the loss of GnRH-positive neurons is likely to alter gonadotrophin secretion. Several tumors are known to express GnRH and respond to receptor agonists or antagonists.[36]

In conclusion, germline deletion of *DeltaNp73* increases apoptosis in neurons and decreases susceptibility to tumor growth in a gender-specific manner. Our observations are compatible with the view that DeltaNp73 has anti-apoptotic activity, even though this activity is weaker than that predicted in in vitro cell culture studies.

Methods

RT-PCR. Total RNA was extracted using NucleoSpin® RNA II kit (Macherey-Nagel). Contaminating genomic DNA was removed by treatment with DNAse (Macherey-Nagel). RNA was converted to single-stranded cDNA using the ImProm-II reverse transcriptase (Promega) with random primer (0.5 µg/ml) in 20 µl of 1X ImProm-II buffer containing 6 mM $MgCl_2$, 0.5 mM each dNTP, and 20 U recombinant RNasin ribonuclease inhibitor. The primers pairs used in RT-PCR are as follows:

TAp73: 5'-GGA GAT GGC CCA GAC CTC TTC TTC C-3' and 5'-TTG AAG TCC CTT CCA AGC TCG TGG T-3'.

DeltaNp73: 5'-GGT GTG GAC ACT TTG ATC TGG ATG G-3' and 5'-CTT TAC GTC GGT GAC CCC ATG AGA C-3'.

Cre: 5'-ATC CGA AAA GAA AAC GTT GA-3' and 5'-ATC CAG GTT ACG GAT ATA GT-3'.

PCR parameters consisted of a 3 min denaturation at 94°C, followed by 30 cycles of 94°C for 30 s and 68°C for 90 s. PCR products were analyzed on agarose gel and visualized by ethidium bromide staining.

Immunohistochemistry. E13.5 embryos were fixed by immersion in 4% PFA in PBS. Vibratome or cryostat sections were incubated with the following primary antibodies: rabbit anti-GFP (1:1,000, Chemicon), chicken anti-GFP (1:1,000, Aves labs), rabbit anti-Tbr1 (1:2,500, Chemicon), rabbit anti-calretinin (1:2,000, Swant) and mouse

anti-reelin (G10). Signals were detected by immunofluorescence using goat anti-chicken Alexa Fluor 488 (1:800, Invitrogen), and goat anti-rabbit IgG Alexa Fluor 594 (1:800, Invitrogen). For reelin IHC, the signal was detected by DAB using the ABC kit (Vector Laboratories). Preparations were examined with a Zeiss Axioskop microscope, photographed with an Eclipse system (Nikon), and figures were edited with Photoshop (Adobe).

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