An Anti-Apoptotic Role for the p53 Family Member, p73, During Developmental Neuron Death

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p53 plays an essential pro-apoptotic role, a function thought to be shared with its family members p73 and p63. Here, we show that p73 is primarily present in developing neurons as a truncated isoform whose levels are dramatically decreased when sympathetic neurons apoptose after nerve growth factor (NGF) withdrawal. Increased expression of truncated p73 rescues these neurons from apoptosis induced by NGF withdrawal or p53 overexpression. In p73–/– mice, all isoforms of p73 are deleted and the apoptosis of developing sympathetic neurons is greatly enhanced. Thus, truncated p73 is an essential anti-apoptotic protein in neurons, serving to counteract the pro-apoptotic function of p53.

The p53 tumor suppressor protein mediates apoptosis (*1*), particularly in the nervous system, where it is essential for neuronal apoptosis following injury (*2*) and during some types of developmental death (*3*, *4*). The discovery of a related protein, p73 (*5*, *6*), that is enriched in the nervous system (*7*) raised the possibility that it might also play an important role in regulating neuronal survival, particularly because overexpression of full-length p73 causes apoptosis of non-neuronal cells (*5*, *6*, *8*). To elucidate the biological role of p73, we have focused on sympathetic neurons, in which p53 plays a critical apoptotic role (*3*, *4*). Here, we show that p73 is predominantly expressed in these sympathetic neurons as a truncated isoform lacking the transactivation domain, and that this isoform plays an essential anti-apoptotic role, counteracting the pro-apoptotic function of p53.

To determine the function of p73 in developing neurons, we first characterized p73 isoform expression in the neonatal brain and sympathetic superior cervical ganglion (SCG). p73 exists as a group of full-length isoforms (including p73α and -β) and as truncated isoforms that lack the transactivation domain (ΔN-p73) (*5*–*9*). Similar truncated isoforms of the related p63 inhibit p53-dependent transcription (*10*, *11*). Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis with specific primers (*12*) demonstrated that mRNAs encoding both truncated and full-length isoforms of p73 were expressed in the developing brain and SCG (Fig. 1A) (*13*).

We then determined the relative ratio of full-length versus truncated p73 using one- and two-dimensional (1D and 2D) Western blot analysis (*14*). Exogenously expressed full-length p73α, ΔN-p73α, and ΔN-p73β migrated at 80, 62, and 52 kD, respectively (*13*) (Figs. 1B and 2A). On 2D gels, the ΔN-p73 isoforms migrated as a series of spots differing in isoelectric point, potentially reflecting posttranslational modifications such as phosphorylation (Fig. 1B). We then performed a similar analysis on postnatal day 10 (P10) brain tissue derived from the progeny of crosses of p73+/– heterozygote mice (*7*, *15*). The only detectable p73 isoform in p73+/+ mouse brain was ΔN-p73β, consistent with previous mRNA studies (*7*). This protein was absent in the p73–/– brain (Fig. 1C). ΔN-p73β was also the predominant p73 isoform in purified, cultured neonatal sympathetic neurons (Fig. 1D, top panel).

When cultured sympathetic neurons of the SCG are withdrawn from their obligate survival factor, nerve growth factor (NGF), they undergo apoptosis in a p53-dependent fashion (*3*). To determine whether ΔN-p73β might also play a role in regulating neuronal apoptosis, cultured sympathetic neurons were withdrawn from NGF (*16*). Two-dimensional Western blots showed that levels of ΔN-p73β were decreased 24 hours after NGF withdrawal (Fig. 1D), a time point at which sympathetic neurons are committed to apoptosis, and p53 levels are increased (*3*).

To determine whether the decrease in ΔN-p73β levels was important for neuronal apoptosis after NGF withdrawal, we generated recombinant adenoviruses expressing ΔN-p73α or -β and green fluorescent protein (GFP) (Fig. 2, A and B) (*17*). Cultured sympathetic neurons were infected with one of these two adenoviruses or with a control, GFP adenovirus, and were withdrawn from NGF. Two days later, survival was deter-

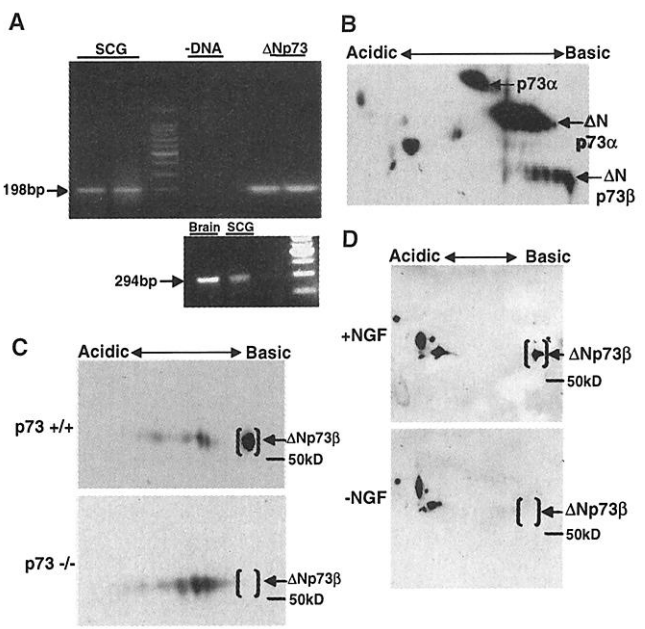

Fig. 1. p73 is predominantly present in developing neurons as a truncated isoform whose levels are regulated by NGF. (**A**) RT-PCR analysis of p73 mRNA transcripts in total RNA prepared from neonatal SCG or brain using primers specific for ΔN-p73 (top), or full-length p73 (bottom) transcripts. Controls include p73 plasmid DNA, and reactions containing no cDNA (–DNA). Predicted size of the PCR products is indicated to the left. (**B**) Two-dimensional Western blot analysis of recombinant full-length p73α, ΔN-p73α, and ΔN-p73β. Recombinant proteins expressed in sympathetic neurons were separated on the basis of charge in the first dimension (i.e., acidic, basic) and molecular weight in the second, and were probed with a pan-p73 antibody. (**C** and **D**) Two-dimensional Western blot analysis of p73 in lysates of P10 brain from p73–/– versus p73+/+ littermates (C) or of sympathetic neurons either maintained in 10 ng/ml NGF (+NGF) or withdrawn from NGF (-NGF) for 24 hours (D). In both panels, brackets denote the location of ΔN-p73β. Spots on the left of the blots are invariant, nonspecific proteins. For (D), similar results were obtained in four independent experiments.

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mined with MTT assays 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (*18*). Although all control neurons were dead 2 days after NGF withdrawal (Fig. 2C), ectopic expression of ΔN-p73α or -β rescued sympathetic neurons from death in a dose-dependent manner (Fig. 2C). In these experiments, 500 multiplicity of infection (MOI) of ΔN-p73β adenovirus rescued, on average, 59% of sympathetic neurons ($n = 4$), whereas 500 MOI of ΔN-p73α adenovirus rescued, on average, 62% ($n = 5$). TUNEL confirmed that ΔN-p73α or -β, but not GFP, rescued sympathetic neurons from apoptosis and that, for the infected cells, this rescue was as efficient as 10 ng/ml NGF (Fig. 2, D and E).

To determine whether p73 also functions as a neuronal anti-apoptotic protein in vivo, we analyzed sympathetic neurons in neonatal p73–/– mice. The SCG contains 20,000 to 25,000 neurons at birth and over the ensuing 3 weeks, about half of these neurons undergo apoptosis (*19*, *20*). Analysis of the SCG from progeny of p73+/– crosses (*21*) revealed that sympathetic neuron numbers were similar in the newborn p73+/+ and p73–/– SCG (Fig. 3A) [p73+/+, 20,148 ± 1220; p73−/−, 21,313 ± 1757 ($n = 4$ mice each)]. By P10, neuronal numbers were greatly decreased in the SCG of p73−/− animals relative to their p73+/+ littermates (Fig. 3A) [p73+/+, 21,044 ± 1463 ($n = 4$ mice); p73−/−, 9973 ± 1074 ($n = 3$ mice)]. This difference was obvious morphologically; the SCG of p73–/– mice at P10 were reduced in size and their neuronal density was decreased (Fig. 3A). Thus, in p73–/– mice, where all isoforms of p73 are deleted (*7*), neuronal apoptosis is greatly increased. Moreover, because full-length p73 is also absent, then ΔN-p73 must inhibit apoptosis through mechanism(s) that do not involve full-length p73. This is in contrast to our findings in p53–/– mice, where sympathetic neuron apoptosis was decreased over the same time period (*3*).

One explanation for these findings is that ΔN-p73 directly interacts with p53 and inhibits its apoptotic function. To test this hypothesis, lysates of 293 cells infected with p53 adenovirus were incubated with glutathione *S*-transferase (GST)–linked ΔN-p73α or ΔN-p73β. GST-pulldown assays (*22*) revealed that both of these truncated p73 isoforms interacted with p53 (Fig. 3B). We then determined whether ΔN-p73β could inhibit p53-mediated neuronal apoptosis. Infection of sympathetic neurons with a p53 adenovirus caused neuronal apoptosis in a dose-dependent fashion (Fig. 3C), as previously reported (*4*), and coinfection with the ΔN-p73β adenovirus

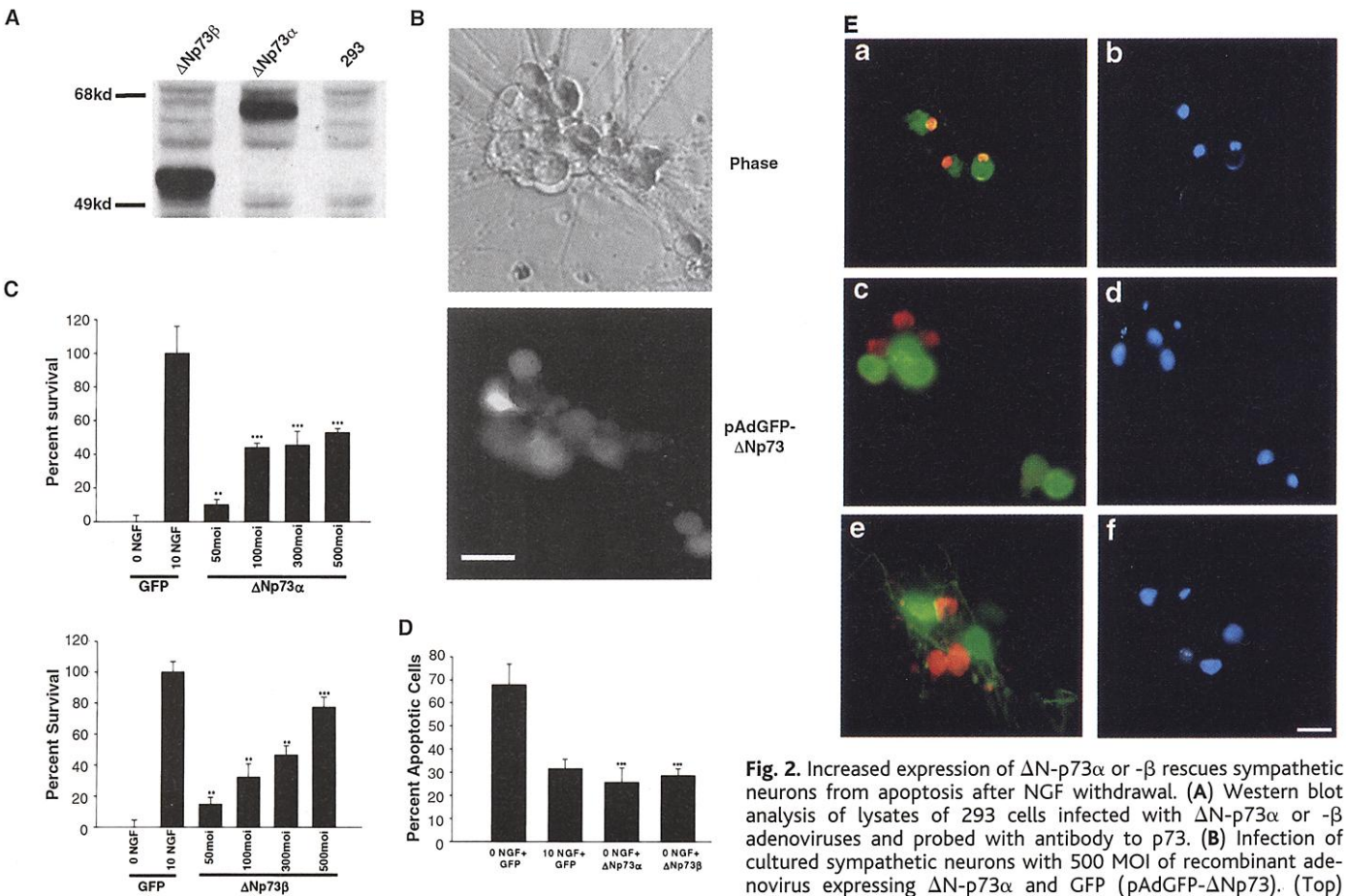

Fig. 2. Increased expression of ΔN-p73α or -β rescues sympathetic neurons from apoptosis after NGF withdrawal. (**A**) Western blot analysis of lysates of 293 cells infected with ΔN-p73α or -β adenoviruses and probed with antibody to p73. (**B**) Infection of cultured sympathetic neurons with 500 MOI of recombinant adenovirus expressing ΔN-p73α and GFP (pAdGFP-ΔNp73). (Top) phase micrograph; (bottom) fluorescence micrograph. Bar, 20 μm. (**C**) MTT survival assays of sympathetic neurons infected with various MOIs of ΔN-p73α (top) or -β (bottom) adenovirus and withdrawn from NGF for 48 hours. Values were normalized to sister cultures infected with 300 (top) or 500 (bottom) MOI GFP adenovirus and withdrawn from NGF (0% survival) or maintained in 10 ng/ml NGF (100% survival). Each point was performed in triplicate or quadruplicate. Values are mean ± standard error. $^{**}P < 0.05$ and $^{***}P < 0.005$, relative to 0NGF + GFP (Student's *t* test). Similar results were obtained in five (ΔN-p73α) or four (ΔN-p73β) different independent experiments. (**D** and **E**) TUNEL of sympathetic neurons infected with 500 MOI of recombinant adenovirus expressing ΔN-p73α, ΔN-p73β, or GFP alone and withdrawn from NGF for 48 hours. Representative data are shown in (E). Left panels, TUNEL (red) and GFP (green) labeling; right panels, Hoechst labeling (blue) of the same fields. (a and b) GFP adenovirus; (c and d) ΔN-p73α; (e and f) ΔN-p73β. To quantitate, we counted labeled (GFP and TUNEL) cells in at least three separate fields; the number of double-labeled cells was expressed as a percentage of the total number of GFP-expressing cells in (D) ($^{***}P<0.005$). Similar results were obtained in five independent experiments. Bar in (E), 20 μm.

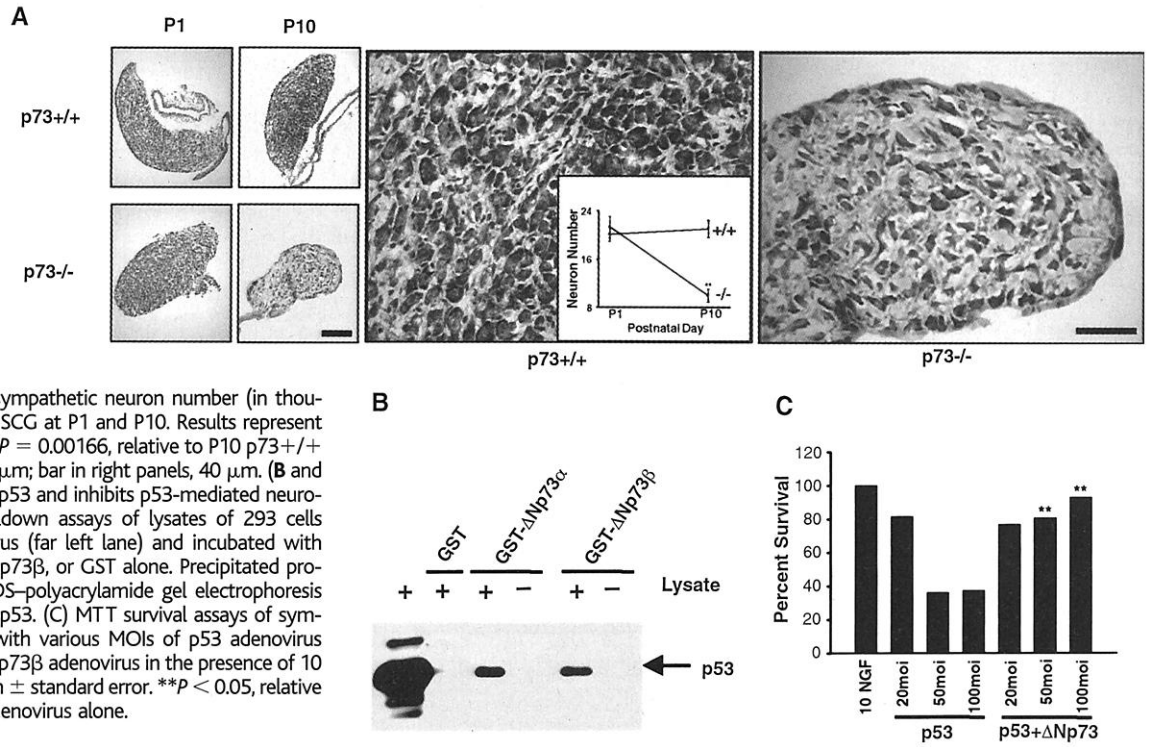

Fig. 3. (**A**) Deletion of p73 leads to increased death of sympathetic neurons in the developing SCG. Photomicrographs of cresyl violet–stained SCGs from p73+/+ and p73−/− littermates at postnatal days 1 and 10 (P1 and P10). The right panels are higher-magnification micrographs illustrating decreased neuronal density in the P10 p73−/− SCG. Inset shows quantitation of sympathetic neuron number (in thousands) in p73+/+ and –/– SCG at P1 and P10. Results represent mean ± standard error. ***$P = 0.00166$, relative to P10 p73+/+ SCG. Bar in left panels, 500 μm; bar in right panels, 40 μm. (**B** and **C**) ΔN-p73β interacts with p53 and inhibits p53-mediated neuronal apoptosis. (B) GST pulldown assays of lysates of 293 cells infected with p53 adenovirus (far left lane) and incubated with GST-tagged ΔN-p73α, ΔN-p73β, or GST alone. Precipitated proteins were separated by SDS–polyacrylamide gel electrophoresis and were probed with anti-p53. (C) MTT survival assays of sympathetic neurons infected with various MOIs of p53 adenovirus plus or minus 100 MOI ΔN-p73β adenovirus in the presence of 10 ng/ml NGF. Values are mean ± standard error. **$P < 0.05$, relative to the same MOI of p53 adenovirus alone.

reversed this effect (Fig. 3C). Thus, ΔN-p73 isoforms bind to p53, and can rescue p53-mediated neuronal apoptosis.

Together, three lines of evidence indicate an essential role for ΔN-p73 as an anti-apoptotic protein in neurons. First, the predominant form of p73 in the developing brain and sympathetic neurons is a truncated isoform lacking the transactivation domain. Second, when NGF is withdrawn, levels of ΔN-p73β dramatically decrease, and prevention of this decrease is sufficient to inhibit neuronal apoptosis. Finally, the absence of p73 in mice causes enhanced death of developing sympathetic neurons. Because ΔN-p73β interacts with p53 and inhibits p53-dependent neuronal apoptosis (Fig. 3, B and C) and because sympathetic neuron death following NGF withdrawal is p53-dependent (*3*), the data presented here suggest that one mechanism whereby ΔN-p73 inhibits neuronal apoptosis is by acting as a direct antagonist to p53. Whether the anti-apoptotic function of ΔN-p73 explains some aspects of the p73–/– central nervous system (CNS) phenotype (*7*) remains to be seen.

The finding of an inhibitory form of p73 that is capable of opposing the apoptotic functions of p53 has wide-ranging implications for many cell types. We predict that in cell types and/or situations where the truncated variant is most abundant, p73 will act in opposition to p53. Conversely, in cells in which the full-length isoform is predominant, p73 would collaborate with p53 based on its ability to induce apoptosis in a variety of cell types (*8*), including sympathetic neurons (*13*). Because the anti-apoptotic ΔN-p73 isoform is generated from the same gene as full-length p73 via alternative promoter usage (*7*), this opposition versus collaboration could be very quickly regulated at the transcriptional level. Whether ΔN-p73 antagonizes other essential functions of p53 is a possibility that remains to be determined.

References and Notes

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12. Total RNA was prepared and RT-PCR performed as described in [X.-M. Yang *et al.*, *J. Neurosci.* **18**, 8369 (1998)]. Primers specific to truncated p73 were 5′ATGGGCCCTGTGTATGAATCCTTG3′ and 5′GGTATTGGAAGGGATGACAGGCG3′. Primers specific to full-length p73 were 5′GAGCACCTGTGGAGTTCTCTAGAG3′ and 5′GGTATTGGAAGGGATGACAGGCG3′.
13. C. D. Pozniak *et al.*, data not shown.
14. Whole brain and sympathetic neurons were lysed and 1D Western blot analysis performed as described using antibody to p73 (1:200) (ER-15; Neomarkers, Union City, CA) (*3*). Two-dimensional gels were run as per manufacturer's instructions (Amersham Pharmacia Biotech) using lysates of sympathetic neurons infected with adenoviruses expressing full-length human p73α, and mouse ΔN-p73α and ΔN-p73β to standardize the system.
15. Mice heterozygous for a targeted mutation in the p73 gene (*7*) were maintained in a c129-Balb/c background. Progeny from p73 heterozygote crosses were screened for the mutant and wild-type alleles with PCR.
16. Purified cultures of sympathetic neurons were cultured and then withdrawn from NGF as described (*3*, *19*).
17. Recombinant adenoviruses expressing ΔN-p73α or -β were generated, purified over CsCl, and titered as described (*23*, *24*).
18. Sympathetic neurons were cultured for 4 to 5 days in 50 ng/ml NGF and then infected with recombinant adenovirus as previously described (*3*). Three days later, neurons were withdrawn from NGF or were maintained in 10 ng/ml NGF, and MTT survival assays and TUNEL were performed after 2 days as described (*25*).
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21. For morphometric analysis, the SCGs were prepared and analyzed as previously (*3*, *19*). Sections were stained with toluidine blue or cresyl violet and were analyzed with a computer-based image analysis system that counted every third section.
22. Constructs were made by fusing the GST domain to the NH_2-terminus of ΔN-p73α or ΔN-p73β, and proteins were produced in *Escherichia coli*. To assess interactions, lysates of 293 cells infected with p53 adenovirus (*4*) were incubated with the GST fusion proteins for 4 hours at 4°C and were precipitated with glutathione-agarose. Precipitated proteins were analyzed by Western blot analysis with antibody to p53 (DO-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
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