Neuron, Vol. 48, 743–756, December 8, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.neuron.2005.10.027

P63 Is an Essential Proapoptotic Protein during Neural Development

W. Bradley Jacobs,[1,2,3,4] Gregory Govoni,[7,9] Daniel Ho,[1,7] Jasvinder K. Atwal,[7,10] Fanie Barnabe-Heider,[2,7] William M. Keyes,[8] Alea A. Mills,[8] Freda D. Miller,[2,3,5,6,7] and David R. Kaplan[1,3,5,7,*]
[1] Cancer Research and
[2] Developmental Biology Programs
Hospital for Sick Children
[3] Institute of Medical Sciences
[4] Division of Neurosurgery
[5] Department of Molecular and Medical Genetics
[6] Department of Physiology
University of Toronto
Toronto ON
Canada M5G 1X8
[7] Department of Neurology and Neurosurgery
McGill University
Montreal QC
Canada H3A 2B4
[8] Cold Spring Harbor Laboratory
Cold Spring Harbor, New York, 11724

Summary

The p53 family member p63 is required for nonneural development, but has no known role in the nervous system. Here, we define an essential proapoptotic role for p63 during naturally occurring neuronal death. Sympathetic neurons express full-length TAp63 during the developmental death period, and TAp63 levels increase following NGF withdrawal. Overexpression of TAp63 causes neuronal apoptosis in the presence of NGF, while cultured *p63*$^{-/-}$ neurons are resistant to apoptosis following NGF withdrawal. TAp63 is also essential in vivo, since embryonic *p63*$^{-/-}$ mice display a deficit in naturally occurring sympathetic neuron death. While both TAp63 and p53 induce similar apoptotic signaling proteins and require BAX expression and function for their effects, TAp63 induces neuronal death in the absence of p53, but p53 requires coincident p63 expression for its proapoptotic actions. Thus, p63 is essential for developmental neuronal death, likely functioning both on its own, and as an obligate proapoptotic partner for p53.

Introduction

During development, the nervous system is confronted with the problem of establishing appropriate neuronal connectivity. This problem is partially solved by overproducing neurons and then eliminating those cells that do not successfully compete for target territory (Oppenheim, 1991). The process of naturally-occurring cell death is perhaps best understood in sympathetic neurons of the peripheral nervous system, where the ultimate survival of any given neuron is determined by its ability to sequester sufficient amounts of target-derived NGF. NGF then mediates a retrograde survival signal through interactions with the TrkA/NGF receptor on the terminal arbor (Miller and Kaplan, 2001). Interestingly, a second neurotrophin receptor, p75NTR, plays a functionally antagonistic role in this system and actually promotes the rapid elimination of those sympathetic neurons that do not compete successfully for target-derived NGF (Bamji et al., 1998; Majdan et al., 2001). Thus, the ultimate survival of any given neuron during this period is determined by the interaction between TrkA and p75NTR, a functional interaction that is best exemplified by studies in animals that have mutations in one or both of these genes (Bamji et al., 1998; Majdan et al., 2001; Smeyne et al., 1994).

What are the intracellular signals that regulate naturally occurring sympathetic neuron death? Studies in cultured sympathetic neurons indicate that the prosurvival effects of TrkA depend upon PI3-kinase-Akt signaling and that sympathetic neuron apoptosis requires JNK, c-JUN, BIM_{EL}, and BAX (Huang and Reichardt, 2001; Kaplan and Miller, 2000; Palmada et al., 2002). We have also previously demonstrated a key role for the p53 family in developmental sympathetic neuron death. These studies showed that p53 is necessary for efficient neuronal apoptosis in vivo (Aloyz et al., 1998; Slack et al., 1996) and that p73, a second family member, functions as an essential prosurvival protein (Pozniak et al., 2000). p73 was expressed in developing sympathetic neurons as a truncated isoform lacking the N-terminal transactivation domain, ΔNp73, and levels of this isoform decreased dramatically when sympathetic neurons were withdrawn from NGF. Increased expression of ΔNp73 rescued these neurons from apoptosis induced by NGF withdrawal, and the death of developing sympathetic neurons in vivo was greatly enhanced in *p73*$^{-/-}$ mice. ΔNp73 appears to function in part by binding to and inhibiting the activity of p53 (Lee et al., 2004; Pozniak et al., 2000). This prosurvival role for ΔNp73 also generalizes to other populations of neurons, since *p73*$^{-/-}$ mice display ongoing cortical neuron death postnatally (Pozniak et al., 2002), and the loss of a single *p73* allele was sufficient to cause enhanced loss of adult sensory neurons following axonal injury (Walsh et al., 2004).

While these findings support a model in which developmental apoptosis is at least partially dependent upon the antagonistic activities of ΔNp73 and p53, a number of lines of evidence indicate that p53 is not the major proapoptotic protein for sympathetic neurons. First, cultured *p53*$^{-/-}$ sympathetic neurons display only a modest resistance to NGF withdrawal-induced apoptosis (Vogel and Parada, 1998). Second, only a partial in vivo deficit in developmental sympathetic neuron death is observed in *p53*$^{-/-}$ mice (Aloyz et al., 1998), and the coincident absence of p53 only partially rescues the enhanced neuronal apoptosis seen in *p73*$^{-/-}$ mice (Lee et al., 2004). We have therefore hypothesized that at least one other

*Correspondence: dkaplan@sickkids.ca
[9] Present address: Stanford University, San Francisco, California.
[10] Present address: Genentech, South San Francisco, California.

protein functions together with p53 to stimulate apoptosis and that the third p53 family member, p63, is a candidate for this role.

The physiological role of p63 has been primarily examined during epithelial cell development. The *p63*$^{-/-}$ mouse has truncation of the limbs and an absence of epidermis, prostate, breast, and uroepithelial tissues (Mills et al., 1999; Yang et al., 1999). Initially, p63 was thought to be required for the maintenance of the epidermal stem cell population, acting predominantly as a truncated ΔNp63 isoform (Lee and Kimelman, 2002; Yang et al., 1999). More recently, however, others have suggested that p63 is important for epithelial development due to its role in initiating epithelial stratification and maintaining the proliferative ability of basal keratinocytes (Koster et al., 2004), perhaps through regulation of PERP expression, which is essential for epithelial desmosome function (Ihrie et al., 2005). Interestingly, mutations in *p63* are found in a number of dominant human syndromes resulting in abnormal limb development or ectodermal dysplasia and also in variable degrees of mental retardation (Irwin and Kaelin, 2001). However, while p63 can promote apoptosis in cell lines (Moll and Slade, 2004), and has been demonstrated to play a proapoptotic role in the developing CNS following γ irradiation-induced DNA damage (Flores et al., 2002), p63 is not known to play a proapoptotic role in any developmental cell death process.

In this report, we show that p63 is primarily expressed in the nervous system as a TAp63 isoform that is induced by withdrawal of survival factors. TAp63, when overexpressed, is a potent apoptotic protein in neurons, and developing *p63*$^{-/-}$ sympathetic neurons show dramatic deficits in apoptosis both in culture and in vivo. Moreover, we show that while TAp63 alone is sufficient to promote neuronal apoptosis, p53 requires TAp63 to induce neuronal death. Thus, TAp63 is an essential proapoptotic protein during neuronal development, likely functioning both on its own and as an obligate proapoptotic partner for p53.

Results

Developing Sympathetic Neurons Express Only Full-Length TAp63 Isoforms, and Their Levels Increase following NGF Withdrawal

To ask whether p63 might play a role in the developing nervous system, we initially characterized its expression pattern in the central and peripheral nervous system using RT-PCR. Analysis using primers specific to the different *p63* isoforms (Figure 1A and Figure S1A in the Supplemental Data available with this article online) demonstrated that *p63* was expressed as both TA and ΔN variants in the cortex from E13.5 into adulthood and that mRNAs for both α and γ C-terminal variants were present. mRNAs for these isoforms were also expressed in cultured E12.5 multipotent cortical precursors and in postmitotic cortical neurons (Figure 1A). In contrast, analysis of RNA from cultured neonatal sympathetic neurons demonstrated that only the full-length *TAp63*α and γ isoforms were expressed (Figure 1B).

To confirm this expression profile at the protein level, we performed two-dimensional gel electrophoresis and Western blot analysis with an antibody that recognizes all isoforms of p63. This analysis revealed that, in the developing cortex at the protein level, the only detectable p63 isoform was TAp63α (Figure 1C). Confirmation of the identity of this spot was obtained first by comparing the migration pattern of the endogenous protein with that of exogenously expressed recombinant TAp63α (Figure 1D) and second, by demonstrating that the immunoreactive TAp63α was not present in a *p63*$^{-/-}$ cortical lysate (Figure 1C). A similar Western blot analysis of neonatal sympathetic neurons cultured in the presence of NGF demonstrated that p63 was barely detectable in these neurons. Following NGF withdrawal, p63 expression remained low until 22 hr after NGF withdrawal, when a dramatic increase was observed in protein expression of the TAp63γ variant (Figure 1E); this is a time course that coincides with NGF withdrawal-mediated apoptosis. Given the presence of nonspecific bands, two-dimensional gel electrophoresis was used to confirm the identification of this protein as TAp63γ (Figure 1F), since it migrated at the same isoelectric point and mobility as exogenously expressed TAp63γ (Figure 1G). This analysis also confirmed that the levels of TAp63γ increased 24 hr following NGF withdrawal (Figure 1F). Thus, developing sympathetic neurons express only TAp63 isoforms, and levels of TAp63 increase markedly when sympathetic neurons are withdrawn from NGF.

TAp63 Is Sufficient to Cause Apoptosis of Sympathetic Neurons

To test the possibility that the increased TAp63 levels observed following NGF withdrawal were causally related to the ensuing neuronal apoptosis, we expressed exogenous TAp63 in neonatal sympathetic neurons maintained in NGF. To perform these experiments, we generated recombinant bicistronic adenoviruses that expressed green fluorescent protein (GFP) along with TAp63α, β, or γ (Figure S1B). Cultured sympathetic neurons maintained in 10 ng/ml NGF were infected with one of these three TAp63 adenoviruses or with a control adenovirus expressing GFP. Expression of these TAp63 isoforms was confirmed by Western blot analysis (Figure 2E and data not shown), and apoptosis was assessed 48 hr following infection, using terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling (TUNEL). This analysis demonstrated that approximately 55% of sympathetic neurons infected with TAp63γ (Figures 2A–2D and 2F) were TUNEL-positive, confirming that increased TAp63 levels were sufficient to cause these neurons to apoptose. Similar results were also obtained following TAp63α overexpression (data not shown).

To determine if this apoptotic effect was specific for full-length p63 isoforms, we performed a similar analysis examining ΔNp63 isoforms after first generating bicistronic adenoviruses expressing ΔNp63α, β, or γ along with GFP (Figure S1B). Adenovirus-induced expression of any of these three ΔNp63 variants had no effect on the survival of sympathetic neurons maintained in NGF (data not shown), and, in fact, ΔNp63α and β rescued sympathetic neurons from apoptosis following NGF withdrawal (Figures 2G–2L) as we have previously observed for ΔNp73 isoforms (Pozniak et al., 2000). Thus,

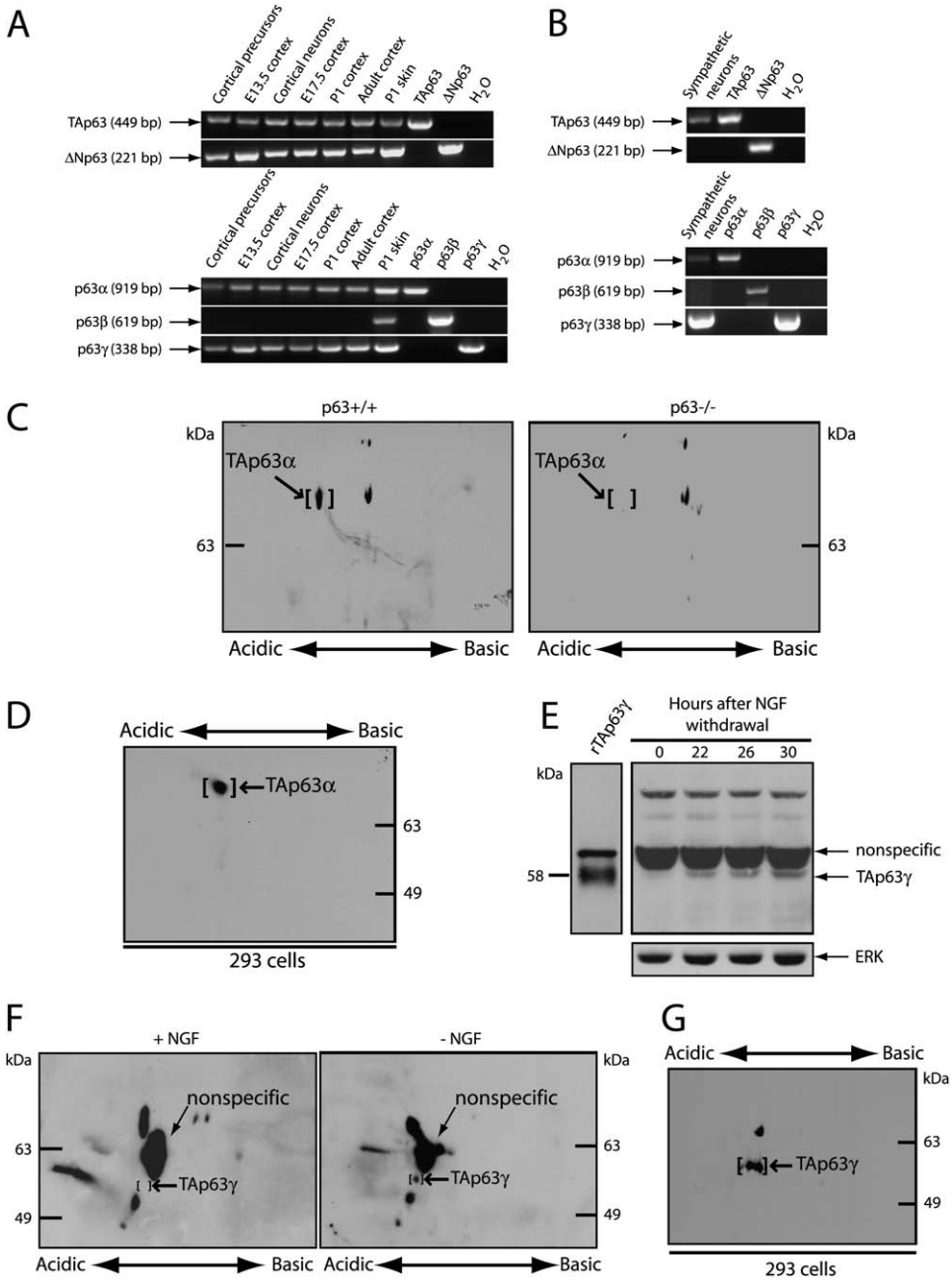

Figure 1. TAp63 Isoforms Are Predominant in the Developing Peripheral and Central Nervous Systems

(A and B) RT-PCR analysis for total *TAp63* versus *ΔNp63* variants and for total α, β, and γ p63 variants in the murine cortex at various time points, in cultured embryonic cortical precursors and postmitotic neurons, and in neonatal murine sympathetic neurons. Postnatal day 1 skin (P1 Skin) and recombinant adenovirus plasmids encoding the various isoforms (Figure S1) were used as positive controls.

(C) Two-dimensional gel electrophoresis and Western blot analysis with a pan-p63 antibody of the cortex from E18.5 $p63^{+/+}$ and $p63^{-/-}$ mice.

(D) Two-dimensional gel electrophoresis and Western blot analysis with a pan-p63 antibody of lysates from HEK 293 cells infected with the TAp63α adenovirus, to define the mobility of the TAp63α isoform. The square brackets in (C) and (D) indicate the position where recombinant TAp63α runs when analyzed in a similar fashion.

(E) Western blot analysis with a pan-p63 antibody of lysates of cultured neonatal sympathetic neurons withdrawn from NGF at time points ranging from 0 to 30 hr. The lane labeled rTAp63γ shows migration of the recombinant protein in sympathetic neurons. The blot was reprobed for total ERK protein as a loading control. A nonspecific band is noted above the TAp63γ band.

(F and G) Two-dimensional gel electrophoresis and Western blot analysis of cultured neonatal sympathetic neurons either maintained in NGF (+ NGF) or withdrawn from NGF for 24 hr (− NGF), using a pan-p63 antibody (F). The square brackets indicate the position where recombinant TAp63γ runs when analyzed in a similar fashion (G). Spots above the TAp63γ spots are nonspecific.

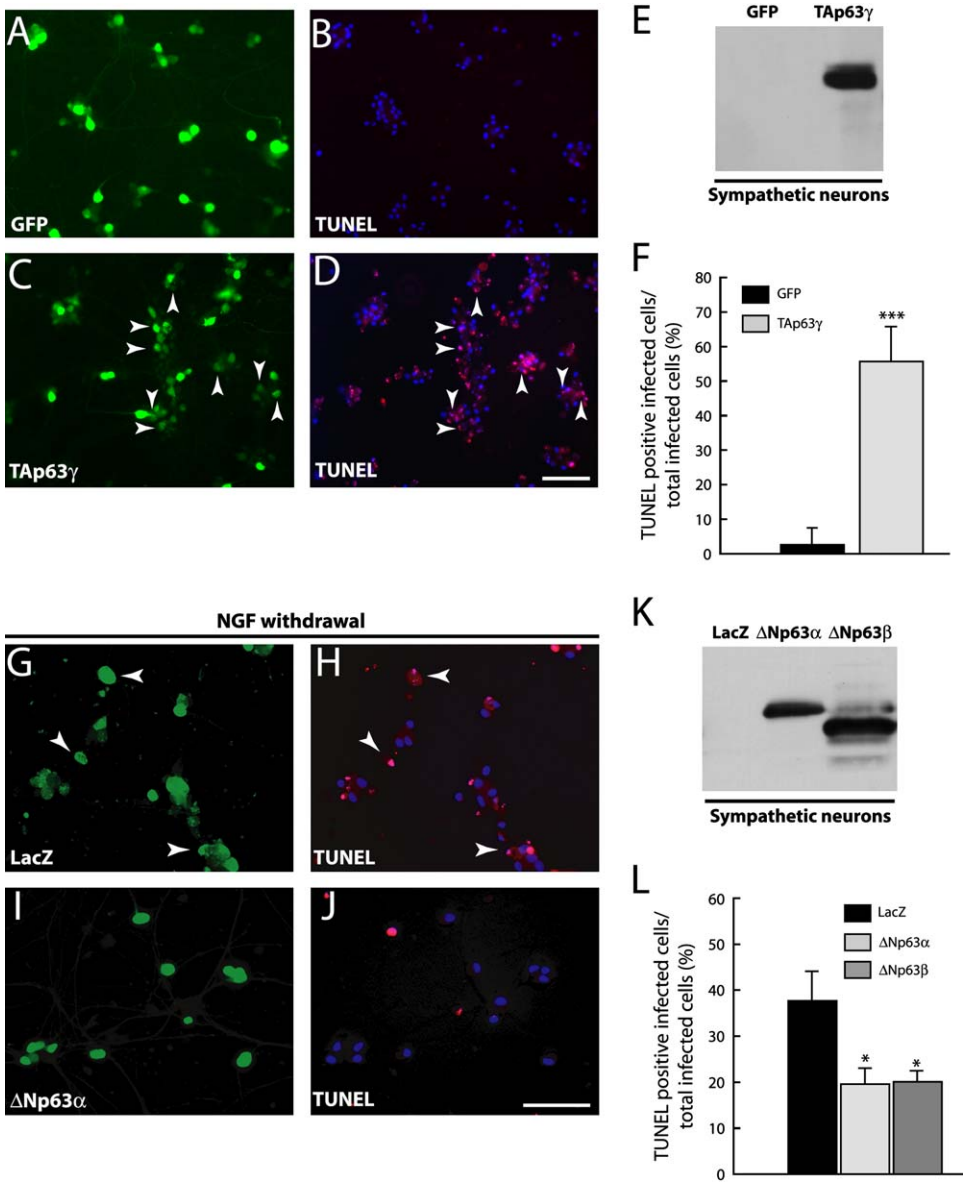

Figure 2. TAp63 Isoforms Cause Sympathetic Neuron Apoptosis, while ΔNp63 Variants Rescue Sympathetic Neurons from Apoptosis following NGF Withdrawal

(A–D) Photomicrographs of sympathetic neurons infected with 100 moi of recombinant adenoviruses expressing either GFP (A) or bicistronic GFP-TAp63γ (C), maintained in 10 ng/ml NGF for 48 hr and then labeled for TUNEL (B and D). Neurons were also stained with Hoechst 33258 to visualize all of the nuclei in the field (blue). Arrowheads indicate TUNEL-positive infected cells. Scale bars, 100 μm. (E) Western blot analysis with a pan-p63 antibody of sympathetic neurons infected with adenoviruses expressing either GFP or TAp63γ. (F) Quantification of pooled results from three separate experiments, demonstrating that expression of TAp63γ, but not GFP, caused sympathetic neurons to apoptose even in the presence of NGF. ***$p < 0.005$, Student's t test. (G–J) Photomicrographs of sympathetic neurons infected with recombinant adenoviruses expressing either β-galactosidase (LacZ) or ΔNp63α, withdrawn from NGF 24 hr postinfection and then analyzed 48 hr later as in (F). Scale bars, 100 μm. (K) Western blot analysis with a pan-p63 antibody in sympathetic neurons infected with adenoviruses expressing ΔNp63α, ΔNp63β, or β-galactosidase. (L) Quantification of pooled results from three separate experiments demonstrating that expression of ΔNp63α or β is sufficient to rescue sympathetic neurons from apoptosis following NGF withdrawal. *$p < 0.05$, Student's t test. Error bars indicate ±SEM.

only full-length TAp63 isoforms promote neuronal apoptosis.

p63$^{-/-}$ Sympathetic Neurons Are Resistant to Developmental Cell Death in Culture and In Vivo

These findings suggest that NGF withdrawal causes increased TAp63, which, in turn, induces neuronal apoptosis. To directly test this possibility, we examined developing *p63*$^{-/-}$ sympathetic neurons. Since *p63*$^{-/-}$ mice die at birth (Mills et al., 1999; Yang et al., 1999), we focused on sympathetic neurons of the superior cervical ganglion (SCG) at E18.5, an early time point during naturally occurring sympathetic neuron death, which appears from approximately E16.5 to P15 (Wright et al., 1983). Initially, we characterized *p63*$^{-/-}$ neurons in culture; isolated neurons were maintained in NGF for 5 days and then were withdrawn from NGF and analyzed 24 to 96 hr later. This analysis revealed that *p63*$^{-/-}$

neurons were largely rescued from cell death following NGF withdrawal. At 24 hr, Hoechst nuclear staining revealed that, on average, only 20% of $p63^{-/-}$ neurons were apoptotic as compared to almost half of the neurons from $p63^{+/+}$ littermates (Figure 3A). Ninety-six hours after NGF withdrawal, virtually all $p63^{+/+}$ neurons were dead, while approximately 80% of neurons from $p63^{-/-}$ littermates were still alive (Figures 3B and 3C). Of note, these sympathetic neurons were significantly smaller than their counterparts that were maintained in 50 ng/ml NGF and had few neurites (Figure 3B). When NGF was restored to these NGF-deprived $p63^{-/-}$ neurons, they elaborated extensive neuritic processes and their cell bodies hypertrophied (Figure 3D), indicating that they remained capable of responding to the differentiative actions of NGF. Thus, p63 is necessary for sympathetic neuron death following trophic factor withdrawal.

To ask whether p63 was also essential for developmental neuron death in vivo, we counted the number of sympathetic neurons in the E18.5 SCG. This analysis indicated that, even at this early time point in the naturally occurring cell death period, the $p63^{-/-}$ SCG contained approximately 20% more sympathetic neurons than did the $p63^{+/+}$ SCG ($p63^{-/-}$: 26457 ± 793 neurons; $p63^{+/+}$: 21216 ± 1239 neurons; n = 6 for each genotype) (Figures 4A and 4B). Sections were immunostained with an antibody to tyrosine hydroxylase to confirm that the cell bodies counted were indeed sympathetic neurons (Figure 4C).

To define whether this difference in neuronal number was secondary to a decrease in cell death, as opposed to an increased proliferation of precursor cells, we quantified the number of dying cells using TUNEL and the number of proliferating cells by immunostaining for Ki67, a marker of cellular proliferation. TUNEL analysis of the E18.5 SCG revealed a decrease of greater than 50% in the number of apoptotic cells in the ganglia of $p63^{-/-}$ animals as compared to their wild-type littermates ($p63^{-/-}$: 196 ± 36 TUNEL cells/ganglion; $p63^{+/+}$: 422 ± 23 TUNEL cells/ganglion; n = 4 and n = 6 for $p63^{-/-}$ and $p63^{+/+}$, respectively) (Figures 5A and 5C). In contrast, Ki67 immunostaining of SCG sections revealed no differences in the number of proliferating cells ($p63^{-/-}$: 357 ± 29 cells/mm^2; $p63^{+/+}$: 325 ±13 cells/mm^2; n = 3 for each genotype) (Figures 5B and 5D). Taken together, these data suggest that the increased number of cells present in the SCG of $p63^{-/-}$ mice is secondary to decreased neuronal death and not to increased cellular proliferation. Thus, p63 is an essential proapoptotic protein during naturally occurring sympathetic neuron death.

TAp63 Functions Downstream of JNK and Upstream of the Mitochondrial Death Checkpoint and Requires BAX Expression to Induce Cell Death

We have previously demonstrated that p53 has a partial, but essential, role in promoting sympathetic neuron developmental death (Aloyz et al., 1998; Lee et al., 2004). We therefore asked whether p63 mediates its apoptotic effects by acting in collaboration with, or in parallel to, p53. Initially, we examined downstream proapoptotic signaling in developing sympathetic neurons infected with recombinant adenoviruses expressing TAp63γ or p53 in the presence of NGF and compared that to such signaling in neurons withdrawn from NGF. Consistent with our TUNEL experiments (Figures 2A–2D and 2F), at 56 hr postinfection, both TAp63 and p53 induced the cleavage of caspase-3 to amounts similar to those seen at 24 hr following NGF withdrawal (Figure 6A). Moreover, expression of TAp63, like p53, led to induction of two known p53 target genes, $p21^{Waf1}$ and *APAF-1*, both of which were also induced by NGF withdrawal (Figures 6B and 6C). Finally, we examined two more upstream events in the apoptotic cascade: activation of JNK1 and induction of BIM_{EL}. Western blot analysis for the phosphorylated, activated form of JNK1 at 20 hr and 30 hr postinfection revealed that TAp63 expression had no effect on JNK1 activation (Figure 6D), results similar to those previously observed for p53 (Aloyz et al., 1998). Similarly, increased expression of TAp63 had no effect on the levels of BIM_{EL} at time points ranging from 28 hr to 56 hr postinfection, results similar to those observed with p53, but distinct from the induction of BIM_{EL} seen following NGF withdrawal (Lee et al., 2004) (Figure 6E).

An apoptotic protein that is required for sympathetic neuron cell death and that functions downstream of JNK is BAX (Deckwerth et al., 1996; Putcha et al., 1999). BAX is a direct transcriptional target of p53 family members and regulates cytochrome c release following translocation to the mitochondrion. To determine whether p53 and TAp63 have differential or similar effects on BAX expression in sympathetic neurons, we undertook a real-time PCR analysis of *Bax* gene expression. To do this, we infected sympathetic neurons with GFP, p53, or TAp63γ in the presence of NGF and examined the relative levels of *Bax* in cDNA made from neuronal lysates at 24 hr and 48 hr after viral infection. At 24 hr following viral infection, *Bax* mRNA levels were 5.8 and 4.1 times higher in p53- and TAp63γ-infected neurons, respectively, relative to sympathetic neurons infected with GFP. At 48 hr, *Bax* levels were 7.8 and 11.8 times higher in p53- and TAp63γ-infected neurons, respectively, compared to GFP-infected neurons. To confirm the significance of this robust activation of *Bax* gene expression in response to p53 or TAp63, we infected sympathetic neurons with GFP, p53, or TAp63γ in the presence of NGF and performed Western blots for BAX protein at 48 hr postinfection. As indicated by the real-time PCR, BAX levels were increased by both p53 and TAp63γ (Figure 7A).

We next asked whether TAp63-mediated apoptosis was dependent upon BAX and subsequent cytochrome c release. Initially, we determined whether TAp63 could induce the death of neurons lacking BAX. TAp63γ was overexpressed in neonatal $Bax^{-/-}$ sympathetic neurons, and apoptosis was assessed by Hoechst staining. This analysis revealed that while TAp63γ expression induced apoptosis in 33% of $Bax^{+/+}$ neurons, apoptosis was only evident in 12% of $Bax^{-/-}$ neurons (Figures 7B and 7C). Thus, BAX is required for most of the neuronal apoptosis induced by TAp63 expression, suggesting that TAp63 likely functions upstream of the BAX-dependent mitochondrial apoptotic transition. To directly test this, we assayed mitochondrial cytochrome c release in $p63^{-/-}$ sympathetic neurons withdrawn from NGF, an apoptotic event that occurs directly downstream of

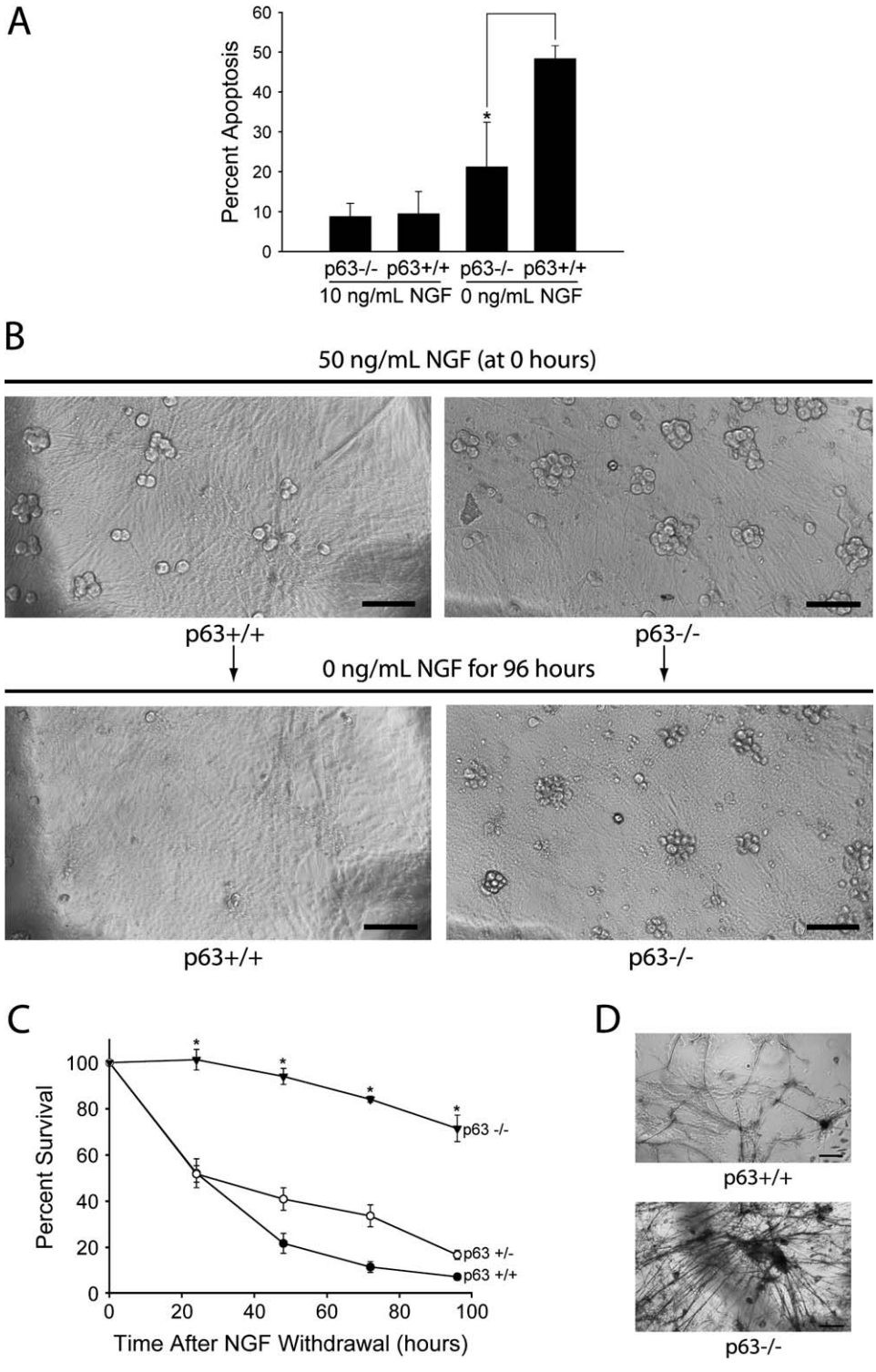

Figure 3. $p63^{-/-}$ Sympathetic Neurons Are Rescued from NGF Withdrawal-Induced Apoptosis in Culture

(A) Quantification of the percentage of apoptotic nuclei in sympathetic neurons cultured from $p63^{+/+}$ or $p63^{-/-}$ mice and withdrawn from NGF for 24 hr (0 ng/ml NGF). Nuclear morphology was defined using Hoechst 33258. Representative data are shown. Similar results were obtained in three separate experiments. Error bars indicate SEM. *$p < 0.05$, Student's t test. (B) Phase photomicrographs of sympathetic neurons cultured from $p63^{+/+}$ or $p63^{-/-}$ SCGs. Neurons were maintained in NGF for 5 days and then withdrawn from NGF for 96 hr. Pictures at 0 hr and 96 hr are from the same field of view. Note that the $p63^{-/-}$ neurons withdrawn from NGF for 96 hr are similar in number to those in the basal experimental condition, although smaller in size. Controls were maintained in 50 ng/ml NGF for the duration of the experiment (data not shown). (C) Quantification of the sympathetic neuron survival experiments, as shown in panel (B). Following withdrawal of NGF, the number of phase-bright $p63^{-/-}$, $p63^{+/-}$, or $p63^{+/+}$ neurons within predefined fields were counted every day for 4 days (96 hr). Data are represented as the percentage of surviving neurons of a given genotype relative to the number of same-genotype neurons surviving in control cultures maintained in 50 ng/ml NGF and are

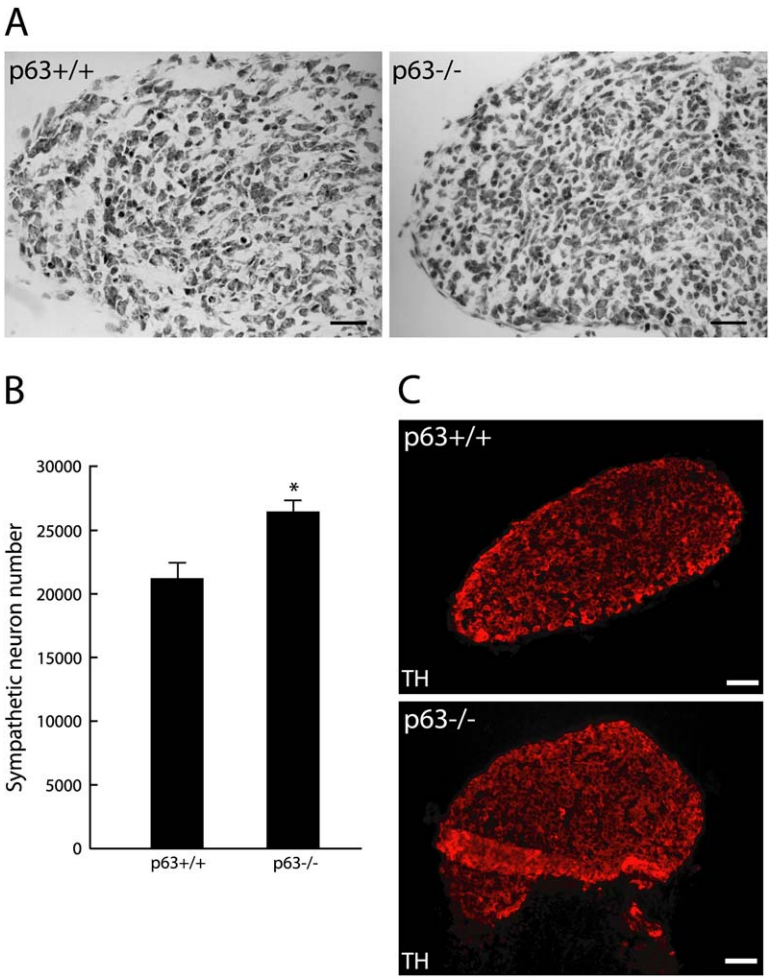

Figure 4. $p63^{-/-}$ Sympathetic Neurons Are Increased in Number during Late Embryogenesis as Compared to $p63^{+/+}$ Neurons

(**A**) Photomicrographs of sections of Nissl-stained $p63^{+/+}$ versus $p63^{-/-}$ E18.5 SCGs. Neuronal morphology was similar between the two genotypes, although $p63^{-/-}$ ganglia were somewhat larger than their $p63^{+/+}$ counterparts. Scale bar, 30 μm.

(B) Quantification of sympathetic neuron number in E18.5 SCGs isolated from $p63^{+/+}$ versus $p63^{-/-}$ littermates. Neuronal numbers were quantified in the ganglia of three animals of each genotype. Error bars represent SEM. *$p < 0.02$, Student's t test.

(C) Fluorescence micrograph of sections of E18.5 $p63^{+/+}$ and $p63^{-/-}$ SCGs immunostained with antibody to tyrosine hydroxylase (TH). Scale bar, 100 μm.

BAX (Kirkland et al., 2002; Lee et al., 2004; Putcha et al., 1999). $p63^{-/-}$ neurons were withdrawn from NGF and cytochrome c release was examined 22 hr later as determined immunocytochemically by an increase in diffuse versus punctate distribution of this protein. While 50% of $p63^{+/+}$ neurons withdrawn from NGF showed cytoplasmic cytochrome c release, only 18% of $p63^{-/-}$ neurons exhibited this release (Figures 7D and 7E). Taken together, these results define a TAp63-BAX-mitochondrial transition pathway that is essential for NGF withdrawal-mediated neuronal apoptosis.

TAp63 Is Dominant to p53 in the Developmental Cell Death Process

While these signaling studies suggest that TAp63γ and p53 are functionally similar, the deficit in apoptosis is much greater in $p63^{-/-}$ than in $p53^{-/-}$ sympathetic neurons (Figure 3) (Lee et al., 2004). We therefore asked whether TAp63 and p53 were functionally interchangeable by expressing TAp63γ in $p53^{-/-}$ neurons and p53 in $p63^{-/-}$ neurons. Initially, we cultured sympathetic neurons from the SCG of E18.5 $p53^{-/-}$ and $p53^{+/+}$ mice, established them for 5 days in NGF, and then infected them with an adenovirus expressing TAp63γ. TUNEL analysis 48 hr later revealed that TAp63γ could effectively induce the apoptosis of both $p53^{-/-}$ and $p53^{+/+}$ neurons (Figures 8A and 8C). Similar results were obtained when TAp63 was expressed in $p53^{-/-}$ sympathetic neurons cultured from P1 and P7 SCGs (data not shown). Thus, TAp63 is sufficient to induce the death of $p53^{-/-}$ neurons. We then performed the converse experiment, culturing E18.5 sympathetic neurons from $p63^{-/-}$ or $p63^{+/+}$ littermates, establishing them for 5 days in NGF, and infecting them with p53 or TAp63γ adenoviruses. TUNEL analysis revealed that while p53 could induce apoptosis of wild-type neurons, it was unable to efficiently induce the apoptosis of $p63^{-/-}$ neurons (Figures 8B and 8D). In contrast, TAp63γ was able to induce apoptosis of either $p63^{-/-}$ or $p63^{+/+}$ neurons (Figures 8B and 8D). Thus, while p63 can induce neuronal apoptosis on its own, p53 requires p63, indicating that p63 is dominant to p53 during developmental sympathetic neuron death.

pooled from three individual experiments. *$p < 0.01$, Student's t test, relative to the $p63^{+/+}$ neurons at the same time point. Error bars indicate ±SEM. (D) Bright-field photomicrographs of $p63^{+/+}$ versus $p63^{-/-}$ sympathetic neurons withdrawn from NGF for 96 hr and then cultured for an additional 96 hr in the presence of 50 ng/ml NGF. Cultures were stained with the mitochondrial dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) to visualize live cell bodies and neurites. Scale bars, 100 μm in (B) and (D).

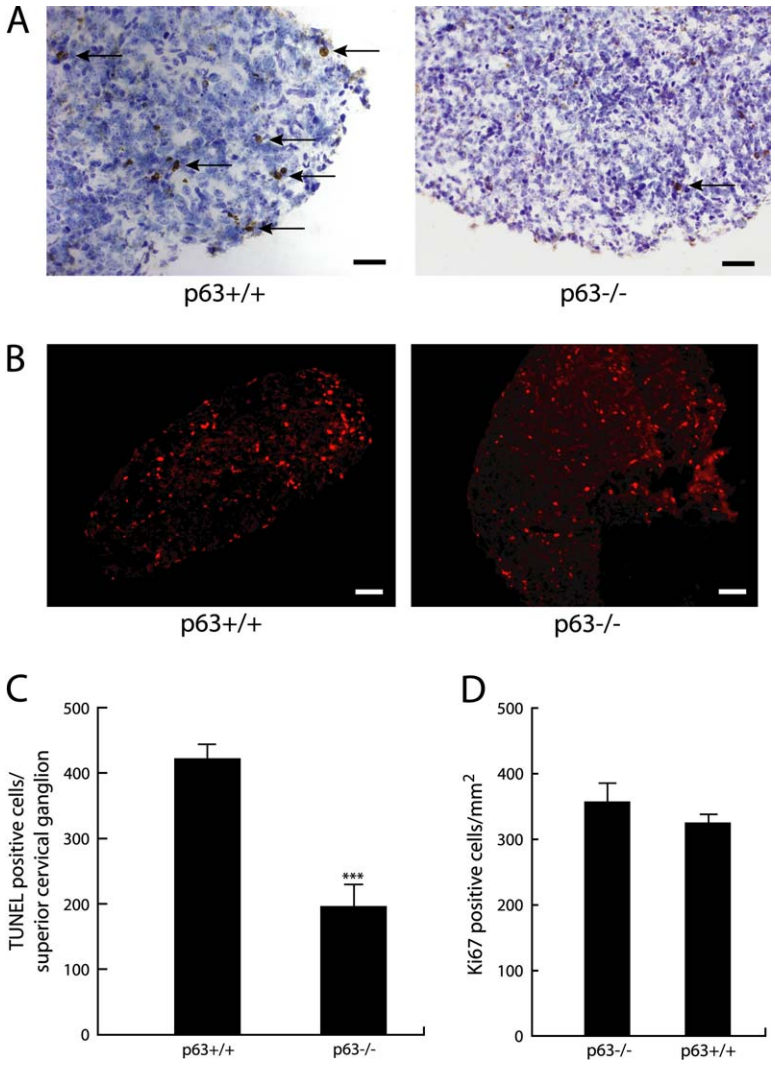

Figure 5. $p63^{-/-}$ Sympathetic Neurons Are Rescued from Developmental Neuron Death In Vivo

(A) Bright-field photomicrograph of sections of $p63^{+/+}$ or $p63^{-/-}$ E18.5 SCG analyzed for apoptosis using TUNEL. Positive cells were visualized with DAB, and sections were counterstained with Nissl. Arrows indicate TUNEL-positive cells. Scale bar, 30 μm.

(B) Fluorescence photomicrographs of sections of E18.5 $p63^{+/+}$ versus $p63^{-/-}$ SCGs immunostained for the proliferation marker Ki67. Scale bar, 100 μm.

(C) Quantification of TUNEL-positive cells in SCGs isolated from $p63^{+/+}$ and $p63^{-/-}$ E18.5 mice. Error bars represent SEM. ***$p < 0.001$, Student's t test.

(D) Quantification of Ki67 immunofloures-cence in E18.5 $p63^{+/+}$ and $p63^{-/-}$ SCGs. $p = 0.3$. Error bars indicate ±SEM.

Discussion

The findings presented here support four major conclusions. First, p63 is predominantly expressed in the nervous system as full-length TAp63 variants and that the levels of TAp63 in sympathetic neurons increase dramatically, coincident with apoptosis. Second, our studies in cultured developing sympathetic neurons demonstrate that TAp63 is both necessary and sufficient for apoptosis following NGF withdrawal. Third, analysis of the embryonic superior cervical ganglion indicates that TAp63 is also essential for naturally occurring sympathetic neuron death in vivo. Finally, although TAp63 and p53 activate similar apoptotic signaling proteins and require BAX activity in sympathetic neurons, TAp63 can induce apoptosis on its own, but p53 requires TAp63 to promote neuronal apoptosis. Thus, TAp63 is an essential proapoptotic molecule during naturally occurring neuronal death, a role that may well generalize to the intact or injured mature nervous system.

Data presented here indicate that TAp63 is an essential component of the apoptotic pathways induced during naturally occurring sympathetic neuron death. During development, sympathetic neuron survival is largely regulated by proapoptotic signals deriving from p75NTR and prosurvival signals deriving from the NGF/TrkA receptor (Kaplan and Miller, 2000). The apoptotic signaling pathways induced by activation of p75NTR and withdrawal of NGF are similar, and a large component of cell death mediated by NGF withdrawal is regulated by p75NTR (Bamji et al., 1998). While a significant amount of work in cultured sympathetic neurons has elucidated a downstream proapoptotic cascade involving JNK, c-JUN (Eilers et al., 1998; Estus et al., 1994; Ham et al., 1995; Palmada et al., 2002), BIM_{EL} (Putcha et al., 2001; Whitfield et al., 2001), BAX (Putcha et al., 1999), and cytochrome c (Deshmukh et al., 2000; Neame et al., 1998), of these only BAX has been shown to be essential for sympathetic neuron survival in vivo (Deckwerth et al., 1996). BAX is known to be a direct transcriptional target of the p53 family (Miyashita and Reed, 1995), and p53 regulates the translocation of BAX into the mitochondrion (Mihara et al., 2003; Schuler et al., 2000). We show here that p53 and TAp63 increase *Bax* gene and protein expression in a similar manner, that BAX activity is essential for TAp63-induced neuronal apoptosis, and that following NGF withdrawal, apoptosis and BAX-dependent cytochrome c release do not occur

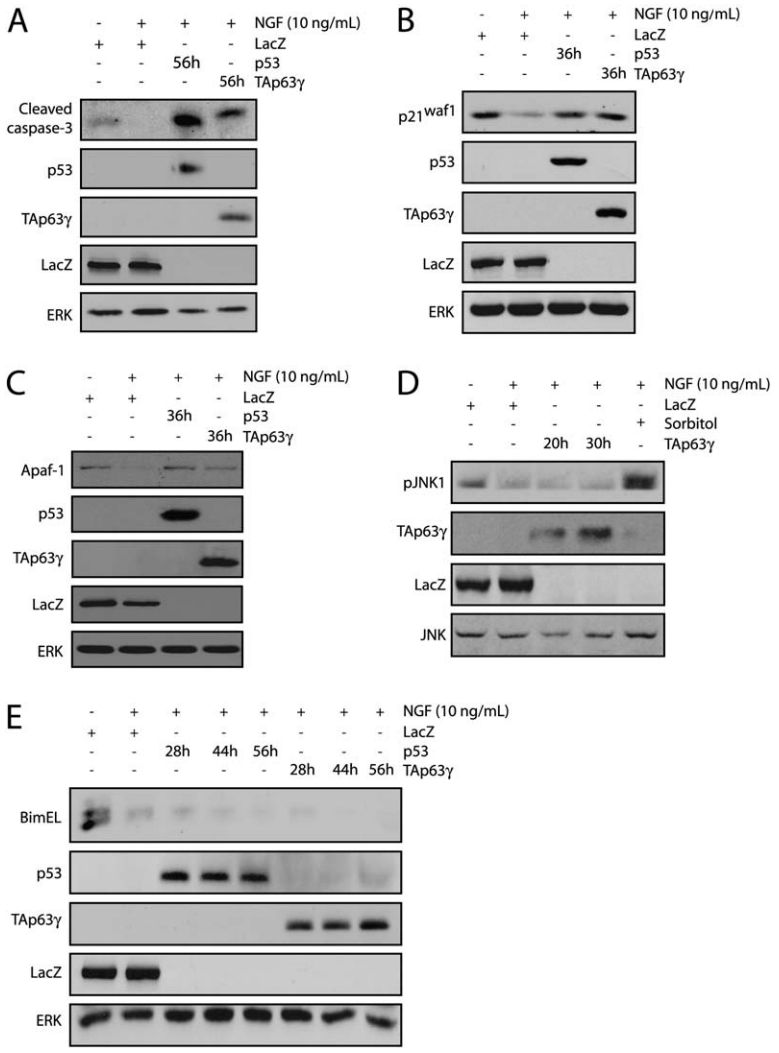

Figure 6. TAp63γ Induces Sympathetic Neuron Apoptosis via a Mechanism Similar to p53

(A–C) Western blot analysis for cleaved caspase-3 (A), $p21^{WAF1}$ (B), and APAF-1 (C) in sympathetic neurons infected with recombinant adenoviruses expressing TAp63γ, p53, or β-galactosidase (LacZ), maintained in 10 ng/ml NGF, and analyzed at the time points indicated. For comparison, LacZ-infected neurons were also withdrawn from NGF for 24 hr. ERK is analyzed as a loading control.

(D) Western blot analysis of activated JNK1 in sympathetic neurons infected with recombinant adenoviruses expressing TAp63γ or LacZ, maintained in 10 ng/ml NGF, and analyzed at the time points indicated. For comparison, LacZ-infected neurons were also withdrawn from NGF for 8 hr. Total JNK protein was analyzed as a loading control, and sorbitol-treated sympathetic neurons were used as a positive control.

(E) Western blot analysis for BIM_{EL} in sympathetic neurons infected with recombinant adenoviruses expressing TAp63γ, p53, or β-galactosidase (LacZ), maintained in 10 ng/ml NGF, and analyzed at the time points indicated. For comparison, LacZ-infected neurons were also withdrawn from NGF for 24 hr. ERK levels were assessed with anti-ERK1/2 as a loading control.

in neurons lacking TAp63. We therefore propose that NGF withdrawal and p75NTR activation converge on TAp63, which then activates BAX to induce the mitochondrial apoptotic transition and downstream events such as caspase-3 activation.

One of the unexpected findings presented here is that TAp63 can induce neuronal apoptosis in the absence of p53 but the converse is not true, indicating that p63 is dominant to p53 during sympathetic neuron death. The requirement of p63 for p53-induced cell death was previously noted in transformed mouse embryonic fibroblasts and in the CNS following γ irradiation-induced DNA damage (Flores et al., 2002), although this requirement was not observed during the apoptosis of immune cells (Senoo et al., 2004). Why would p53 require p63 to mediate neuronal apoptosis, but not the converse? One potential explanation is that TAp63 has transcriptional targets that are not shared with p53 (Harms et al., 2004) and are necessary for neuronal apoptosis. A second explanation is that TAp63 is more efficient than p53 at directly promoting the mitochondrial apoptotic transition independent of any transcriptional effects. Finally, TAp63 and p53 may differ in their interactions with ΔNp73 so that ΔNp73 can efficiently antagonize p53, but not TAp63. However, regardless of the underlying mechanism(s), our findings predict that loss of p63 will also functionally ablate p53, at least with regard to apoptosis.

How widespread is the requirement for p63 during neuronal apoptosis? While we have focused here upon developmental sympathetic neuron death, a number of considerations suggest that it may well have widespread importance in the nervous system. First, as we show here, TAp63 is the predominant p63 isoform in the developing cortex, and we have recently shown that TAp63 can induce apoptosis of developing cortical neurons (F.B.-H., G.G., F.D.M., and D.R.K.; unpublished data). Since ΔNp73 is essential for the maintenance of postnatal cortical neurons (Pozniak et al., 2002) and since there is no apparent phenotype in the postnatal $p53^{-/-}$ cortex, then these findings suggest that the proapoptotic partner for ΔNp73 in this context may well be TAp63. In this regard, it is also interesting that ΔNp63 mRNA is detectable in the developing cortex, suggesting that in some cases this prosurvival isoform may play a role analogous to that of ΔNp73 (Pozniak et al., 2000, 2002; Walsh et al., 2004). Secondly, we show here that p63 is required for p53 to induce apoptosis of developing sympathetic neurons. Since p53

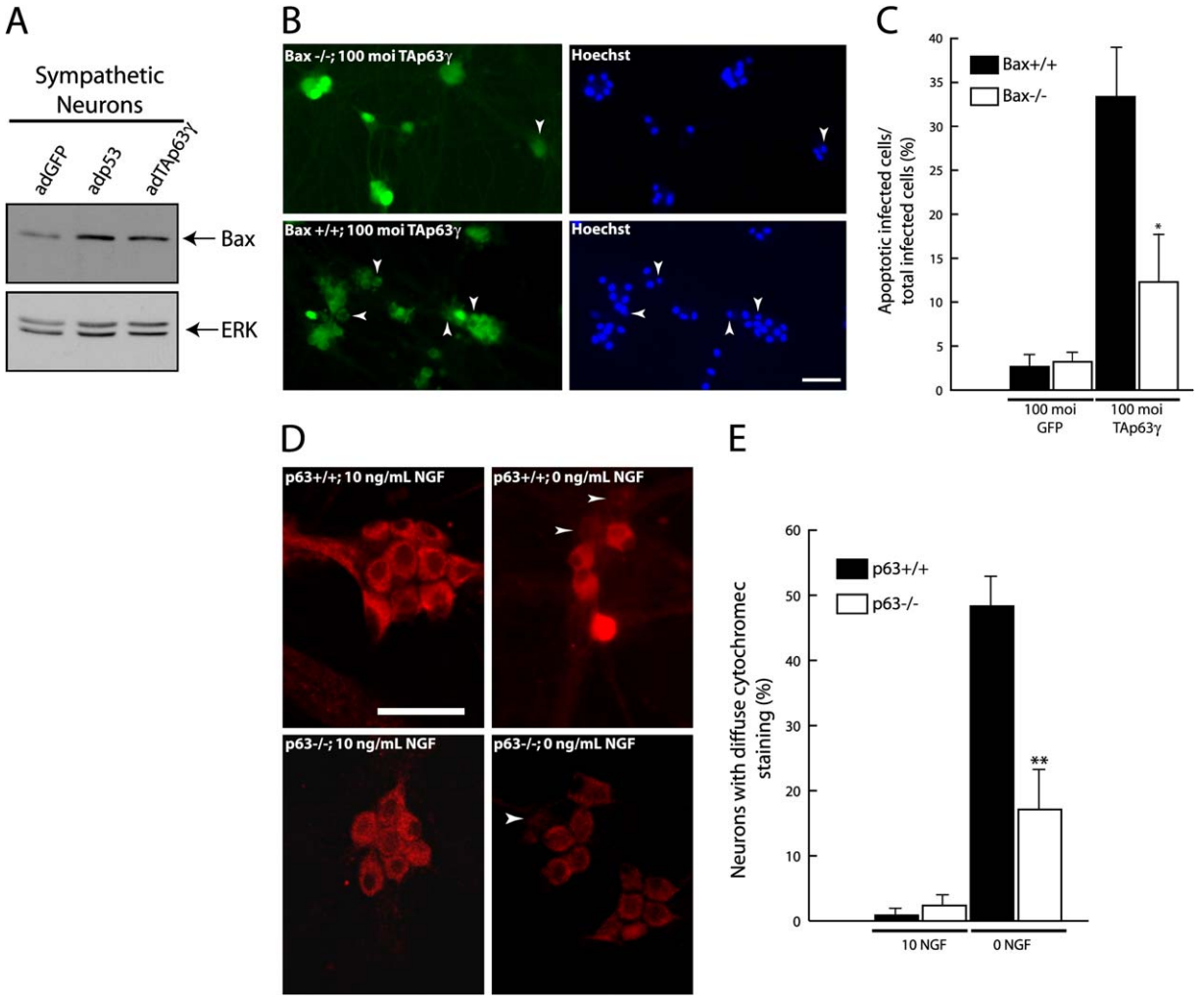

Figure 7. TAp63 Requires BAX to Induce Apoptosis in Sympathetic Neurons, and BAX-Induced Cytochrome c Release Requires p63

(A) Western blot analysis of BAX in sympathetic neurons maintained in 10 ng/ml NGF 48 hr after infection with adenoviruses to GFP, p53, or TAp63γ. The same blot was reprobed for total ERK levels as a control for protein loading.

(B) Requirement of BAX expression for TAp63-induced apoptosis. P2 $Bax^{-/-}$ and $Bax^{+/+}$ sympathetic neurons were infected with a GFP-TAp63γ expressing adenovirus and then maintained in 10 ng/ml NGF for 48 hr postinfection. Infected neurons were analyzed for apoptosis using Hoechst 33258. Arrowheads denote infected neurons that display apoptotic bodies. Scale bar, 50 μm. As a control, $Bax^{-/-}$ and $Bax^{+/+}$ neurons were also infected with a GFP-expressing adenovirus (data not shown).

(C) Quantification of the experiment shown in (A). Error bars represent SEM. *p < 0.5.

(D) Cytochrome c release following NGF withdrawal requires p63 expression. Confocal microscopy images of $p63^{-/-}$ and $p63^{+/+}$ sympathetic neurons withdrawn or not withdrawn from NGF for 22 hr and then immunostained with an antibody to cytochrome c. Arrowheads denote neurons with diffuse staining, consistent with release of cytochrome c from mitochondria. Scale bar, 50 μm.

(E) Quantification of three separate experiments including that in (C). Error bars represent SEM. **p < 0.05.

is essential for neuronal apoptosis in response to excitotoxicity (Miller et al., 2000; Morrison and Kinoshita, 2000) or even axotomy (Martin and Liu, 2002), then our findings predict that TAp63 may also be essential, functioning as an obligate partner for p53. Thus, we propose that TAp63, ΔNp73, and p53 together play a general role in regulating selection of neurons during development and also in both the intact and injured mature nervous system.

In summary, the studies reported here identify TAp63 as a proapoptotic protein that is essential for naturally occurring neuronal death. These findings support a model in which proapoptotic (TAp63, p53) versus prosurvival (ΔNp73) members of the p53 family converge downstream of environmental cues and upstream of the mitochondrial apoptotic transition to determine neuronal life versus death. Moreover, these findings provide an unexpected insight into the mechanisms regulating this convergence, with TAp63 being dominant to p53 during neuronal apoptosis. We suggest that such a model may well hold true for the mature nervous system and, potentially, for the other developing tissues in which p63 plays such a critical role.

Experimental Procedures

Animals

p63 mutant mice harboring the *Brdm2* mutant allele (Mills et al., 1999) were maintained through heterozygote breeding pairs in a C57Bl6 background. Animals heterozygous for the mutant allele

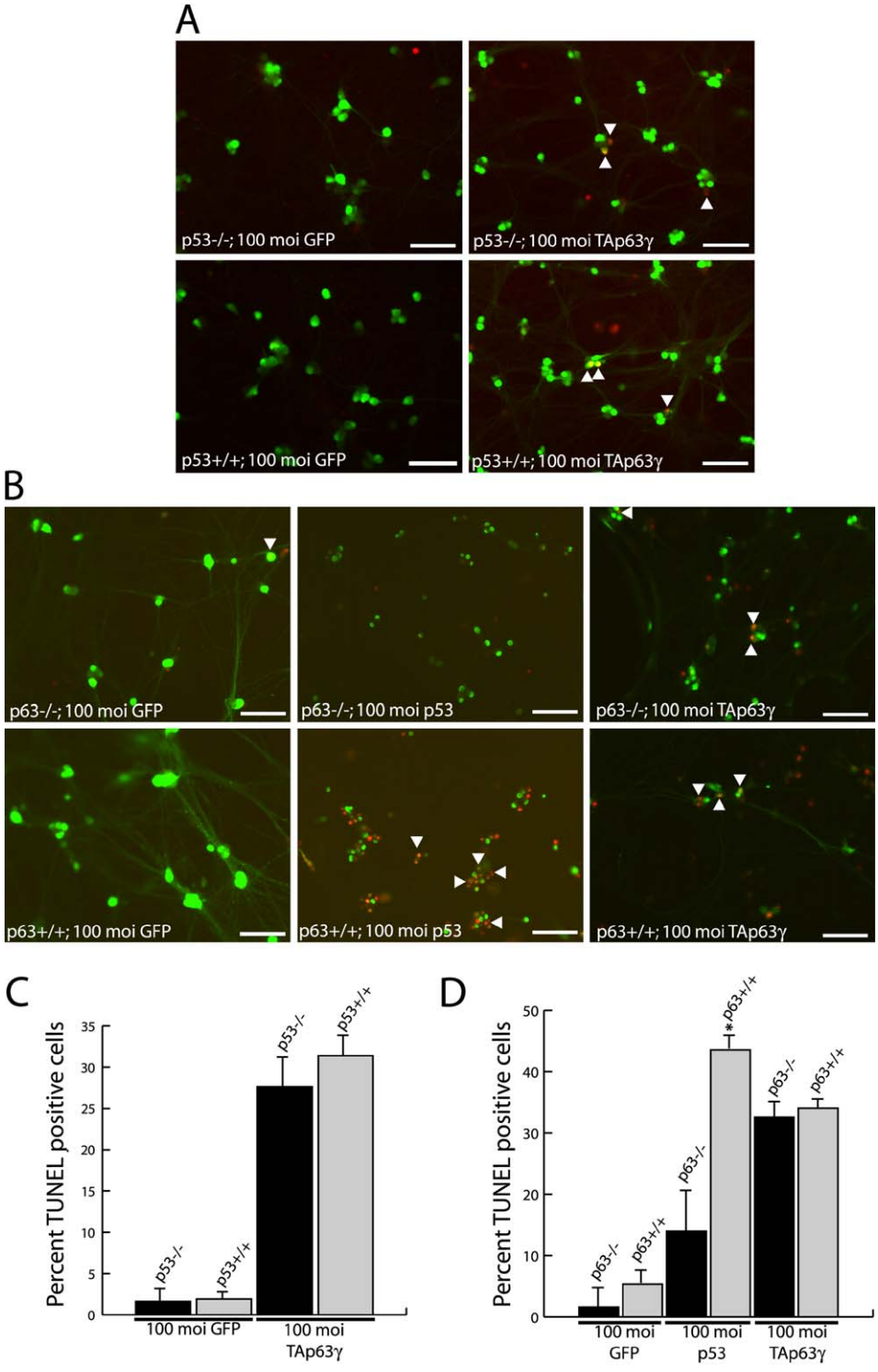

Figure 8. $p63^{-/-}$ Sympathetic Neurons Are Resistant to p53-Mediated Apoptosis

(A) Photomicrographs of E18.5 $p53^{-/-}$ and $p53^{+/+}$ sympathetic neurons infected with adenoviruses expressing either GFP or bicistronic GFP-TAp63γ and maintained in 10 ng/ml NGF for 48 hr after infection. Infected neurons (green) were then analyzed by TUNEL to detect apoptosis (red). Arrowheads represent TUNEL-positive infected neurons. (B) Photomicrographs of $p63^{-/-}$ and $p63^{+/+}$ sympathetic neurons infected with adenoviruses expressing GFP, w-t p53, or bicistronic GFP-TAp63γ and maintained in 10 ng/ml NGF for 48 hr after infection. Neurons infected with w-t p53 were immunolabeled with anti-p53 to detect infected neurons (green), while GFP- and GFP-TAp63γ-infected neurons were identified on the basis of GFP expression. All neurons were then analyzed by TUNEL (red). (C) Quantification of pooled results from three separate experiments as in (A), demonstrating that TAp63γ causes the death of both $p53^{+/+}$ and $p53^{-/-}$ neurons. The y axis represents the percentage of GFP-positive cells that were also TUNEL-positive. Error bars represent SEM. (D) Quantification of pooled results from three separate experiments as in (B), demonstrating that $p63^{-/-}$ neurons are relatively resistant to p53-mediated apoptosis, but die in response to TAp63γ expression. The y axis represents the percentage of infected neurons that were also TUNEL-positive. Error bars represent SEM. *$p < 0.05$ relative to $p63^{-/-}$ neurons infected with p53 adenovirus. Scale bars, 100 μm in (A) and (B).

were identified by PCR using genomic DNA and primers to the mutant allele (5′GTGTTGGCAAGGATTCTGAGACC3′, 5′GGAAGACAAT AGCAGGCATGCTG3′). Animals homozygous for the mutant allele were identified by phenotype. *p53* mutant mice (Donehower et al., 1992) were maintained through heterozygote breeding pairs on a C57Bl6 background. Mice harboring the *p53* mutant allele were identified by PCR using genomic DNA and the primers 5′TACTCTCC TCCCCTCAATAA3′, 5′CTGTCTTCCAGATACTCGGG3′, and 5′CTTG GGTGGAGAGGCTATTC3′. *Bax* mutant mice (Knudson et al., 1995) were maintained through heterozygote breeding pairs on a C57Bl6 background. Mice harboring the *Bax* mutant allele were identified by PCR using genomic DNA and the primers 5′GTTGACCAGAGTGG CGTAGG3′, 5′CCGCTTCCATTGCTCAGCGG3′, and 5′GAGCTGATC AGAACCATCATG3′. CD1 mice (Charles River) and Sprague-Dawley rats (Charles River) were also used for sympathetic neuron cultures. The Animal Care Committee of the Hospital for Sick Children approved all animal use in accordance with the policies established by the "Guide to the Care and Use of Experimental Animals" of the Canadian Council of Animal Care.

Adenoviral Vector Construction

Bicistronic adenoviruses expressing GFP along with TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ were generated, purified over CsCl, and titered according to our previously published protocols (Mazzoni et al., 1999). The recombinant adenoviruses expressing Adwtp53, Ad5LacZ (Slack et al., 1996), and AdGFP (Pozniak et al., 2000) were also used, as we have previously described (Pozniak et al., 2000).

RT-PCR Analysis

CD1 murine cortices were dissected at time points ranging from E13.5 to adulthood. Trizol (Invitrogen) was used to isolate RNA according to the manufacturer's protocol. RNA was obtained from cortical precursor cultures (Barnabe-Heider and Miller, 2003), cortical neuron cultures (Pozniak et al., 2002), and sympathetic neuron cultures, using the RNAqueous-Micro Kit (Ambion) according to the manufacturer's protocol. RNA was treated with DNase (Promega) to avoid contamination with genomic DNA. A sample of RNA was subjected to PCR with *GAPDH* primers (5′ACGGCAAGTTCAATG GCACAGTCA3′, 5′GCTTTCCAGAGGGGCCATCCACAG3′) to ensure that no genomic DNA was present. Reverse transcription was performed using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) primed with random hexamers, according to the manufacturer's instructions. *p63* N-terminal and C-terminal isoform expression patterns were obtained by performing PCR with primers specific for either the TA N-terminal isoform (5′AACCCCAGCTCATT TCTCTG3′) or the ΔN isoform (5′CAATGCCCAGACTCAATTTAGT GA3′), combined with a common N-terminal primer (5′GGCCCGGGT AATCTGTGTTGG3′). Similarly, C-terminal isoforms were obtained by combining a common C-terminal primer (5′AAGAGACCGGAAGG CAGA TGAAG3′) with primers specific to α (5′ACGGGGTGGAAAAG AGATGGTC3′), β (5′GACTTGCCAAATCCTGACA3′), or γ (5′CTCCCC GGGGCTCCACAAG3′) isoforms. PCR products were run on a 1.5% agarose gel in Tris/acetate/EDTA (TAE) buffer. Primer sets yielded products of 449 bp (TAp63), 221 bp (ΔNp63), 919 bp (p63α), 619 bp (p63β), and 338 bp (p63γ).

Real-Time PCR Analysis

cDNA was obtained from rat sympathetic neuron cultures as described above. Real-time PCR was performed using the PTC-200 DNA Engine Thermal Cycler with a Chromo-4 optical module (Bio-Rad) and with SYBR Green as the fluorophore. *Bax* gene expression was determined using the primers 5′CGAGCTGATCAGAACCAT CA3′ and 5′CTCAGCCCATCTTCTTCCAG3′. *β-actin*, using the primers 5′CCTTTTCCAGCCTTCCTTC3′ and 5′TACTCCTGCTTGCTGA TCC3′, served as an internal control. The relative expression of target genes was recorded using Opticon Monitor 3.0 software and analyzed according to the Pfaffl method.

Two-Dimensional Gel Electrophoresis and Western Blot Analysis

For two-dimensional (2D) electrophoresis, E18.5 p63 +/+ and p63 −/− cortices or rat sympathetic neurons were lysed at room temperature in 2D extraction buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 0.5% IPG buffer (pH, 3–10) (Amersham Biosciences), and 100 mM DTT. Protein concentration was quantified using a Bradford Protein Assay (BioRad). Equal amounts of protein (100–500 μg) were used, and the samples were topped off to 200 μl in 2D extraction buffer and bromophenol blue. Each sample was then loaded on separate strip holders (Amersham Biosciences) and covered by one 10 cm drystrip with an immobilized pH gradient (3–10) (Amersham Biosciences). First dimensional isoelectric focusing was performed using an Ettan IPGphor system (Amersham Biosciences). Upon completion, DryStrips were rehydrated twice for 10 min and then with 2D equilibration buffer containing 6M urea, 2% SDS, 50 mM Tris (pH, 8.8), 30% glycerol, 10 mg/ml DTT, and bromophenol blue, prior to SDS-PAGE for separation of proteins by molecular weight.

To detect endogenous p63 proteins in rat sympathetic neurons either maintained in NGF (20 ng/ml) or withdrawn from NGF for 24 hr, mouse monoclonal anti-pan-p63 4A4 antibody was used (1:500; Santa Cruz). A rabbit polyclonal antibody, raised against p73β-GST (1:500; Zymed) that recognizes both p63 and p73, was used to detect p63 protein in p63 +/+ and p63 −/− murine cortices.

For one-dimensional Western blot analysis of cultured sympathetic neurons, lysis and analysis were performed as previously described (Majdan et al., 2001; Vaillant et al., 1999). The antibodies used were mouse monoclonal anti-CIP1/WAF-1/p21 (1:1000; Upstate Biotechnology), rat monoclonal anti-APAF-1 (1:1000; Chemicon), rabbit polyclonal anti-cleaved caspase-3 (1:500; Cell Signaling), rabbit anti-BIM_{EL} (1:500; StressGen), rabbit anti-phospho-JNK (1:1000; Biosource), rabbit anti-JNK1 (1:1000; Santa Cruz), rabbit ERK-1 (1:10,000; Santa Cruz), mouse anti-β-galactosidase (1:10,000; ICN), mouse monoclonal anti-p63 4A4 (1:500; Santa Cruz), rabbit polyclonal p73β-GST antibody (1:500; Zymed), mouse anti-p53 Ab-1 (1:500; Oncogene), and rabbit anti-BAX (1:750; Upstate Biotechnology). The secondary antibodies used were HRP-conjugated goat anti-mouse (BioRad), goat anti-rabbit (Chemicon), and goat anti-rat (Jackson ImmunoResearch).

Sympathetic Neuron Cultures

Mass cultures of sympathetic neurons from the SCG of P1 rats and P1 CD1 mice, E18.5 *p63* mutant mice, E18.5 *p53* mutant mice, and P1 *Bax* mutant mice were prepared as previously described (Majdan et al., 2001; Vaillant et al., 1999).

For adenoviral experiments, sympathetic neurons were cultured for 5 days in Ultraculture (Cambrex) containing 3% rat serum (Wisent), 0.7% cytosine arabinoside (Sigma), and 50 ng/ml NGF and then infected with recombinant adenoviruses in Ultraculture (Cambrex) containing 3% rat serum (Wisent), 50 mM KCl, and 50 ng/ml NGF. Twenty-four hours later, the medium was replaced with fresh Ultraculture containing 10 ng/ml NGF.

The effect of TAp63 or ΔNp63 expression on sympathetic neuron survival was determined using chamber slide (Nalge Nunc International) cultures. Rat sympathetic neurons were cultured for 5 days, as described above, and infected with TAp63 or ΔNp63 adenoviruses. Following medium exchange 24 hr after TAp63 adenoviral infection, sympathetic neurons were maintained in 10 ng/ml NGF for an additional 24 hr, while neurons for the ΔNp63 experiments were washed three times with NGF-free media over 3 hr and then maintained in the absence of NGF for an additional 24 hr. TUNEL analysis was performed as previously described (Aloyz et al., 1998; Pozniak et al., 2000), using an in situ cell death detection kit (Chemicon). β-galactosidase was visualized as previously described (Slack et al., 1996). The number of TUNEL-positive cells was quantified in three random fields per chamber.

Survival experiments using sympathetic neurons from E18.5 *p63* mutant mice were performed in 96-well culture plates (Falcon). To determine neuronal survival, cells were washed with NGF-free media (as above) and then cultured for an additional 24 hr in Ultraculture with or without 10 ng/ml NGF, along with Hoechst 33258 (1:3000). Three hours later, baseline (0 hr) cell counts were performed by quantifying the total number of neurons in three predefined random fields per culture well. Twenty-four hours later, the same fields of view were examined and the number of apoptotic nuclei were counted and expressed as a percentage of the total cells at baseline. Ninety-six-hour survival analysis was done similarly, except that Hoechst 33258 was not used. Instead, following NGF withdrawal, phase-bright neurons were counted in three predefined random

fields per culture well. The same fields were examined daily for 4 days, with the number of phase-bright neurons at each time point expressed as a percentage of the total number of neurons at baseline. To show that phase-bright neurons remained viable after 96 hr without NGF, all sympathetic neurons were then returned to 50 ng/ml NGF for another 4 days. To visualize live cell bodies and neurites, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) was added (1:10) to the media, and cultures were incubated for an additional 60 min.

The effect of w-t p53 or GFP-TAp63 overexpression on $p63^{-/-}$, $p53^{-/-}$, and $Bax^{-/-}$ sympathetic neurons was determined in a similar manner. Adenoviral p53 expression was confirmed using anti-p53 DO-1 antibody (1:100; Santa Cruz) with FITC as a secondary fluorophore. Bicistronic adenoviral GFP-TAp3γ was identified on the basis of GFP expression.

Cytochrome c distribution in $p63^{-/-}$ sympathetic neurons was performed by culturing sympathetic neurons as described above, then washing neurons with NGF-free medium three times over a period of three hours. Twenty-two hours later, cells were fixed with 4% paraformaldehyde and immunostained with anti-cytochrome c 6H2.B4 antibody (1:750; Pharmingen). Alexa-555 was used as the secondary fluorophore, and neurons were visualized using confocal microscopy (Zeiss LSM5 PASCAL) and a 40× oil immersion lens.

Analysis of $p63^{-/-}$ Mice

E18.5 SCGs from *p63* heterozygote progeny were prepared for morphometric analysis as previously described (Majdan et al., 2001). Following Nissl staining, ImageJ image analysis software (NIH) was used to count all neuronal profiles containing nucleoli on every third section and multiply the obtained number by three, following the method of Coggeshall (Coggeshall et al., 1984). Alternate sections were immunostained with tyrosine hydroxylase (1:1000; Chemicon) as described elsewhere (Majdan et al., 2001). TUNEL staining was performed using an in situ cell death detection kit (Chemicon) according to the manufacturer's protocol, peroxidase binding was visualized using diaminobenzidine (DAB; Vector Laboratories), and sections were then counterstained with Nissl. ImageJ image analysis software was used to count all TUNEL-positive neurons in every third section, and this number was then multiplied by three, according to the method of Coggeshall et al. (1984) to obtain the total number of apoptotic cells/ganglion. Ki67 (1:200; Pharmingen) immunostaining was also performed in conjunction with the MOM kit (Vector Laboratories) according to the manufacturer's protocol. The number of Ki67-positive cells was counted in random SCG sections, and the corresponding section surface area was calculated using Northern Eclipse imaging analysis software (Empix Imaging).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.neuron.org/cgi/content/full/48/5/743/DC1/.

Acknowledgments

The authors wish to thank Dr. Wei Cheng, for expert assistance with Bax real-time PCR experiments. W.B.J. was cosupervised by F.D.M. and D.R.K. W.B.J. was supported during the course of these studies by the Canadian Institutes of Health Research (CIHR); D.H., by the Natural Science and Engineering Research Council of Canada (NSERC); J.A., by a McGill Major studentship; and F.B-H, by CIHR and McGill Tomlinson fellowships. F.D.M. and D.R.K are recipients of Canada Research Chairs. This work was supported by CIHR and Brain Repair Program of NeuroScience Canada grants to F.D.M and D.R.K.

Received: April 13, 2005
Revised: August 12, 2005
Accepted: October 20, 2005
Published: December 7, 2005

References

Aloyz, R.S., Bamji, S.X., Pozniak, C.D., Toma, J.G., Atwal, J., Kaplan, D.R., and Miller, F.D. (1998). p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J. Cell Biol. *143*, 1691–1703.

Bamji, S.X., Majdan, M., Pozniak, C.D., Belliveau, D.J., Aloyz, R., Kohn, J., Causing, C.G., and Miller, F.D. (1998). The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J. Cell Biol. *140*, 911–923.

Barnabe-Heider, F., and Miller, F.D. (2003). Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J. Neurosci. *23*, 5149–5160.

Coggeshall, R.E., Chung, K., Greenwood, D., and Hulsebosch, C.E. (1984). An empirical method for converting nucleolar counts to neuronal numbers. J. Neurosci. Methods *12*, 125–132.

Deckwerth, T.L., Elliott, J.L., Knudson, C.M., Johnson, E.M., Jr., Snider, W.D., and Korsmeyer, S.J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron *17*, 401–411.

Deshmukh, M., Kuida, K., and Johnson, E.M., Jr. (2000). Caspase inhibition extends the commitment to neuronal death beyond cytochrome c release to the point of mitochondrial depolarization. J. Cell Biol. *150*, 131–143.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Eilers, A., Whitfield, J., Babij, C., Rubin, L.L., and Ham, J. (1998). Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. J. Neurosci. *18*, 1713–1724.

Estus, S., Zaks, W.J., Freeman, R.S., Gruda, M., Bravo, R., and Johnson, E.M., Jr. (1994). Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J. Cell Biol. *127*, 1717–1727.

Flores, E.R., Tsai, K.Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., and Jacks, T. (2002). p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature *416*, 560–564.

Ham, J., Babij, C., Whitfield, J., Pfarr, C.M., Lallemand, D., Yaniv, M., and Rubin, L.L. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron *14*, 927–939.

Harms, K., Nozell, S., and Chen, X. (2004). The common and distinct target genes of the p53 family transcription factors. Cell. Mol. Life Sci. *61*, 822–842.

Huang, E.J., and Reichardt, L.F. (2001). Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. *24*, 677–736.

Ihrie, R.A., Marques, M.R., Nguyen, B.T., Horner, J.S., Papazoglu, C., Bronson, R.T., Mills, A.A., and Attardi, L.D. (2005). Perp Is a p63-Regulated Gene Essential for Epithelial Integrity. Cell *120*, 843–856.

Irwin, M.S., and Kaelin, W.G. (2001). p53 family update: p73 and p63 develop their own identities. Cell Growth Differ. *12*, 337–349.

Kaplan, D.R., and Miller, F.D. (2000). Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. *10*, 381–391.

Kirkland, R.A., Windelborn, J.A., Kasprzak, J.M., and Franklin, J.L. (2002). A Bax-induced pro-oxidant state is critical for cytochrome c release during programmed neuronal death. J. Neurosci. *22*, 6480–6490.

Knudson, C.M., Tung, K.S., Tourtellotte, W.G., Brown, G.A., and Korsmeyer, S.J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science *270*, 96–99.

Koster, M.I., Kim, S., Mills, A.A., DeMayo, F.J., and Roop, D.R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev. *18*, 126–131.

Lee, A.F., Ho, D.K., Zanassi, P., Walsh, G.S., Kaplan, D.R., and Miller, F.D. (2004). Evidence that DeltaNp73 promotes neuronal survival by p53-dependent and p53-independent mechanisms. J. Neurosci. *24*, 9174–9184.

Lee, H., and Kimelman, D. (2002). A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev. Cell *2*, 607–616.

Majdan, M., Walsh, G.S., Aloyz, R., and Miller, F.D. (2001). TrkA mediates developmental sympathetic neuron survival in vivo by

silencing an ongoing p75NTR-mediated death signal. J. Cell Biol. *155*, 1275–1285.

Martin, L.J., and Liu, Z. (2002). Injury-induced spinal motor neuron apoptosis is preceded by DNA single-strand breaks and is p53- and Bax-dependent. J. Neurobiol. *50*, 181–197.

Mazzoni, I.E., Said, F.A., Aloyz, R., Miller, F.D., and Kaplan, D. (1999). Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J. Neurosci. *19*, 9716–9727.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. Mol. Cell *11*, 577–590.

Miller, F.D., and Kaplan, D.R. (2001). On Trk for retrograde signaling. Neuron *32*, 767–770.

Miller, F.D., Pozniak, C.D., and Walsh, G.S. (2000). Neuronal life and death: an essential role for the p53 family. Cell Death Differ. *7*, 880–888.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R., and Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature *398*, 708–713.

Miyashita, T., and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293–299.

Moll, U.M., and Slade, N. (2004). p63 and p73: roles in development and tumor formation. Mol. Cancer Res. *2*, 371–386.

Morrison, R.S., and Kinoshita, Y. (2000). The role of p53 in neuronal cell death. Cell Death Differ. *7*, 868–879.

Neame, S.J., Rubin, L.L., and Philpott, K.L. (1998). Blocking cytochrome c activity within intact neurons inhibits apoptosis. J. Cell Biol. *142*, 1583–1593.

Oppenheim, R.W. (1991). Cell death during development of the nervous system. Annu. Rev. Neurosci. *14*, 453–501.

Palmada, M., Kanwal, S., Rutkoski, N.J., Gustafson-Brown, C., Johnson, R.S., Wisdom, R., and Carter, B.D. (2002). c-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation. J. Cell Biol. *158*, 453–461.

Pozniak, C.D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D.R., and Miller, F.D. (2000). An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science *289*, 304–306.

Pozniak, C.D., Barnabe-Heider, F., Rymar, V.V., Lee, A.F., Sadikot, A.F., and Miller, F.D. (2002). p73 is required for survival and maintenance of CNS neurons. J. Neurosci. *22*, 9800–9809.

Putcha, G.V., Deshmukh, M., and Johnson, E.M., Jr. (1999). BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. J. Neurosci. *19*, 7476–7485.

Putcha, G.V., Moulder, K.L., Golden, J.P., Bouillet, P., Adams, J.A., Strasser, A., and Johnson, E.M. (2001). Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. Neuron *29*, 615–628.

Schuler, M., Bossy-Wetzel, E., Goldstein, J.C., Fitzgerald, P., and Green, D.R. (2000). p53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. J. Biol. Chem. *275*, 7337–7342.

Senoo, M., Manis, J.P., Alt, F.W., and McKeon, F. (2004). p63 and p73 are not required for the development and p53-dependent apoptosis of T cells. Cancer Cell *6*, 85–89.

Slack, R.S., Belliveau, D.J., Rosenberg, M., Atwal, J., Lochmuller, H., Aloyz, R., Haghighi, A., Lach, B., Seth, P., Cooper, E., and Miller, F.D. (1996). Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. J. Cell Biol. *135*, 1085–1096.

Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature *368*, 246–249.

Vaillant, A.R., Mazzoni, I., Tudan, C., Boudreau, M., Kaplan, D.R., and Miller, F.D. (1999). Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J. Cell Biol. *146*, 955–966.

Vogel, K.S., and Parada, L.F. (1998). Sympathetic neuron survival and proliferation are prolonged by loss of p53 and neurofibromin. Mol. Cell. Neurosci. *11*, 19–28.

Walsh, G.S., Orike, N., Kaplan, D.R., and Miller, F.D. (2004). The invulnerability of adult neurons: a critical role for p73. J. Neurosci. *24*, 9638–9647.

Whitfield, J., Neame, S.J., Paquet, L., Bernard, O., and Ham, J. (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron *29*, 629–643.

Wright, L.L., Cunningham, T.J., and Smolen, A.J. (1983). Developmental neuron death in the rat superior cervical sympathetic ganglion: cell counts and ultrastructure. J. Neurocytol. *12*, 727–738.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R.T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature *398*, 714–718.