Conditional ablation of p63 indicates that it is essential for embryonic development of the central nervous system

Gonzalo I Cancino¹, Michael P Fatt¹,², Freda D Miller¹,³,⁴, and David R Kaplan¹,²,⁴,*

¹Program in Neurosciences and Mental Health; Hospital for Sick Children; Toronto, ON Canada; ²Institute of Medical Science; University of Toronto; Toronto, ON Canada; ³Departments of Physiology; University of Toronto; Toronto, ON Canada; ⁴Molecular Genetics; University of Toronto; Toronto, ON Canada

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Introduction

The p53 family consists of p53 and the closely-related proteins p63 and p73. While p53 is best-known for its role as a tumor suppressor, p63 and p73 play prominent roles during development of organisms ranging from C. elegans to mammals. This is best exemplified by studies of mice lacking the different family members. Mice lacking p53 are predisposed to tumors, but are largely viable,¹,² although a subset of p53 knockouts do exhibit exencephaly.³ In contrast, p63⁻/⁻ mice are not viable postnatally, and display profound deficits in limb and skin morphogenesis.⁴,⁵ Some p73⁻/⁻ mice survive to adulthood, but most die within the first few postnatal weeks, due to deficits in the immune and nervous systems.⁶ Analyses of mice carrying floxed and isoform-specific alleles of p63 and p73 have demonstrated that at least some of these deficits are due to important roles for these 2 proteins in tissue-specific stem cell populations.⁷⁻¹⁷ Thus, the highly homologous p53 family members play important but distinct roles during development.

p63 and p73 mediate their biological effects as 2 major classes of isoforms generated by alternative promoter usage. The full-length isoforms contain an N-terminal transactivation (TA) domain that is necessary for transcription, while the truncated ΔN isoforms lack the TA domain and function, at least in part, as dominant-negative proteins that inhibit their full-length counterparts either by forming inactive tetramers or by competing for promoter binding sites.¹⁸ These 2 classes of isoforms are thought to play different roles in development, with the TA isoforms acting like p53 to regulate proliferation, maintain genome integrity, regulate cell metabolism and induce apoptosis, and the ΔN isoforms acting to promote cell survival and suppress senescence.¹⁸,¹⁹

Numerous studies indicate that p73 is a key protein in both the developing and adult nervous systems. The ΔNp73 isoforms are essential for survival of peripheral sympathetic and sensory neurons²⁰⁻²² and cortical neurons.¹⁹,²³ The full-length TAp73 isoforms regulate hippocampal development and self-renewal of neural stem cells, and differentiation and synaptogenesis of PNS and CNS neurons.⁶,²⁴⁻³⁰ Analysis of mice haploinsufficient for...
all isoforms of p73 have also implicated this protein in neural precursor senescence\(^ {31}\) and neurodegeneration.\(^ {32-34}\)

Like p73, p63 is an important regulator of cell survival in the postnatal nervous system, where it regulates survival of peripheral neurons.\(^ {35}\) Moreover, we recently showed that haploinsufficiency or conditional acute ablation of p63 in the adult nervous system caused the death of adult neural precursors by activation of a pro-apoptotic p53-PUMA pathway, thereby causing deficits in hippocampal-dependent memory formation.\(^ {36}\) However, it is still unclear whether p63 plays a role in the developing brain, since different approaches have led to different conclusions. In our own work, we showed that acute knockdown or knockout of p63 in embryonic cortical precursors using \textit{in utero} electroporation caused precursor death via a p53-dependent pathway,\(^ {37}\) results very similar to what we reported for adult neural precursors.\(^ {31,36}\)

In contrast, a second study examined the embryonic forebrain of p63\(^ {−/−}\) mice and concluded that there were no deficits in neural development when p63 was constitutively ablated.\(^ {38}\)

Here, we have tested the hypothesis that p63 is important for embryonic brain development using a more definitive approach. Specifically, we inducibly ablated p63 in embryonic neural precursors of p63\(^ {fl/fl}\) mice with a tamoxifen-dependent nestin-CreERT2 driver line. Using this approach, we report that acute ablation of p63 leads to a robust increase in the death of neural precursors of the embryonic cortex, thereby causing cortical thinning, ventricular enlargement, and decreased numbers of embryonic cortical precursors and neurons. Moreover, we confirm, as previously published,\(^ {38}\) that there is no difference in the number of neural precursors in cortices of p63\(^ {−/−}\) and p63\(^ {+/+}\) embryos. However, we also show that \(ΔN\)p73 mRNA is specifically upregulated in p63\(^ {−/−}\) cortical precursors, suggesting that the lack of an embryonic neural phenotype in p63\(^ {−/−}\) mice is likely due to compensatory survival-promoting mechanisms that occur within a constitutive knockout background. Thus, as we previously concluded using \textit{in utero} electroporation,\(^ {37}\) p63 is an important prosurvival protein during embryonic neural development.

**Results**

Acute conditional deletion of p63 in embryonic neural precursors perturbs forebrain morphogenesis

To ask whether p63 plays a role during embryonic murine brain development, we crossed p63\(^ {fl/fl}\) mice to mice carrying a nestin-CreERT2 driver that efficiently promotes recombination of floxed genes in embryonic forebrain neural precursors following tamoxifen treatment.\(^ {39}\) These p63\(^ {fl/fl}\);nestin-CreERT2\(^ {i/o}\) mice were also crossed to mice carrying a floxed YFP reporter gene in the Rosa26 locus (R26YFP\(^ {m/m}\)), thereby allowing us to monitor tamoxifen-induced recombination by expression of YFP. We previously used the resultant p63\(^ {fl/fl}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {i/o}\) mouse to inducibly ablate p63 postnatally, and showed that it was essential for the survival of adult neural precursors and adult-born neurons.\(^ {36}\)

To ask about p63’s developmental role, we focused on the embryonic cortex, where neurogenesis is ongoing from approximately embryonic day 12 (E12) to E18. Specifically, we treated pregnant mothers with a single intraperitoneal tamoxifen injection at E12 and analyzed coronal sections through the cortex 3 d

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**Figure 1.** Acute inducible ablation of p63 in developing neural precursors alters embryonic forebrain morphology. (A) Schematic showing the experimental approach. p63\(^ {wt/wt}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {+/−}\) (p63WT) or p63\(^ {fl/fl}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {i/o}\) (p63FLOX) embryos were exposed to tamoxifen (TMX, injected into their mothers) at E12, and then their brains were analyzed 3 d later at E15. (B) Representative images of coronal sections through the forebrain of tamoxifen-treated p63\(^ {wt/wt}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {+/−}\) (p63WT, left panels) or p63\(^ {fl/fl}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {i/o}\) (p63FLOX, right panel) embryos immunostained for YFP to detect expression of the recombinated reporter gene. The boxed areas are shown at higher magnification in the insets, and arrows denote positive cells in the ventricular zone. The cortex (Ctx), ganglionic eminences (GE) and lateral ventricles (LV) are all denoted. Note that the YFP is present in the processes of precursors and neurons and thus the cortical layers are not easily distinguished. Scale bar = 200 μm. (C-E) Representative images of coronal sections through the forebrain of p63\(^ {wt/wt}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {+/−}\) (p63WT, left panels) or p63\(^ {fl/fl}\);R26YFP\(^ {m/m}\);nestin-CreERT2\(^ {i/o}\) (p63FLOX, right panels) embryos at rostral (C), medial (D), and caudal (E) levels. Sections were stained with Hoechst 33258 to visualize cell nuclei. The cortex (Ctx), ganglionic eminences (GE) and lateral ventricles (LV) are all denoted. White lines illustrate where cortices were analyzed at dorsal (D), lateral (L) and ventral (V) levels. Scale bar = 200 μm.
later, on E15 (Fig. 1A). Immunostaining for YFP showed that Cre-mediated recombination was extensive; most cells in the ventricular and subventricular precursor zones (VZ/SVZ) were positive for YFP, as were many of the newborn neurons in the intermediate zone (IZ) and cortical plate (CP) (Fig. 1B). To ask about morphological alterations that occurred following recombination, we counterstained these sections with Hoechst 33258 to highlight nuclei. This analysis showed that the forebrain of p63fl/fl;R26YFPfl/fl;nestin-CreERT2C/Ø embryos was perturbed relative to control p63wt/wt;R26YFPfl/fl;nestin-CreERT2C/Ø embryos, with enlarged lateral ventricles, reduced cortical thickness and decreased ganglionic eminence size (Fig. 1C–E).

To quantify these perturbations, we focused on the cortex. Initially, we measured cortical thickness at 3 different rostral to caudal levels, making dorsal, lateral and ventral measurements at each level (Fig. 1C–E). This analysis showed that when p63 was acutely ablated, cortical thickness was significantly reduced throughout the rostrocaudal extent of the cortex (Fig. 2A–C), with the largest decreases at the rostral level. We also measured the size of the lateral ventricles by determining total relative area at the same 3 rostrocaudal levels used for the cortical thickness measurements. This analysis showed that the lateral ventricles were enlarged throughout the rostrocaudal extent of the forebrain in p63fl/fl;R26YFPfl/fl;nestin-CreERT2C/Ø embryos compared with control p63wt/wt;R26YFPfl/fl;nestin-CreERT2C/Ø embryos (Fig. 2D). Thus, acute ablation of p63 in embryonic neural precursors and their progeny from E12 to E15 was sufficient to cause cortical tissue loss and ventricular enlargement.

Acute conditional deletion of p63 induces widespread apoptosis in the embryonic forebrain

One explanation for these morphological phenotypes is that p63 is essential for the survival of embryonic neural precursors and their neuronal progeny as we have shown for adult neural precursor cells. To address this possibility, we assessed cell death. Initially, we isolated cortices of E15 p63fl/fl;R26YFPfl/fl;nestin-CreERT2C/Ø and p63wt/wt;R26YFPfl/fl;nestin-CreERT2C/Ø embryos that were exposed to tamoxifen at E12, and performed qRT-PCR for p63 mRNA to confirm recombination. We also analyzed PUMA mRNA as an indicator of enhanced p53-dependent apoptotic signaling. This analysis showed that p63 mRNA levels were significantly decreased, as predicted, and that PUMA mRNA levels were coincidently increased almost 2-fold (Fig. 3A). We therefore immunostained E15 cortical sections from these embryos for cleaved caspase 3 (CC3), a marker for...
Figure 3. Conditional ablation of p63 in cortical precursors during the neurogenic period induces apoptosis throughout the embryonic cortex. p63<sup>WT/WT</sup>-R26<sup>YFP<sup>fl/fl</sup></sup>nestin-CreERT2<sup>-/-</sup>(p63WT) or p63<sup>fl/fl</sup>-R26<sup>YFP<sup>fl/fl</sup></sup>nestin-CreERT2<sup>-/-</sup>(p63FLOX) embryos were exposed to tamoxifen (injected into their mothers) at E12, and their cortices were analyzed 3 d later at E15. (A) Quantitative RT-PCR for p63 mRNA (left panel) and PUMA mRNA (right panel) comparing the relative levels of expression in p63WT and p63FLOX cortices at E15. Values are expressed as fold difference relative to the control group (p63WT). *p < 0.05; **p < 0.01; n = 4 and 5 embryos each for p63 and PUMA mRNAs respectively. (B) Representative images of coronal cortical sections from p63WT and p63FLOX embryos, immunostained for cleaved caspase-3 (CC3, red) and counterstained with Hoechst 33258 (blue). The boxed areas are shown at higher magnification to the right, and arrows denote CC3-positive cells. The cortex (Ctx), ganglionic eminences (GE), and lateral ventricles (LV) are all denoted. Scale bar = 200 µm. (C) Quantification of sections as shown in (B) for the total number of CC3-positive cells in the cortex per section, determined by counting 3 similar sections per embryo at rostral, medial and caudal cortical levels. ***p < 0.001; n = 3 animals each. (D) Representative images of coronal cortical sections from p63WT and p63FLOX embryos, analyzed by TUNEL (red) and counterstained with Hoechst 33258 (blue). The boxed areas are shown at higher magnification to the right, and arrows denote TUNEL-positive cells. The cortex (Ctx), ganglionic eminences (GE), and lateral ventricles (LV) are all denoted. Scale bar = 200 µm. (E) Quantification of sections as shown in (D) for the total number of TUNEL-positive cells in the cortex per section, determined by counting 3 similar sections per embryo at rostral, medial and caudal cortical levels. ***p < 0.001; n = 3 animals each. In all panels, error bars denote SEM.
apoptotic cell death. In control p63^wt/wt; R26YFP^fl/fl; nestin-CreERT2^+/0 embryos, very few cortical cells were positive for CC3, as we have previously shown for wildtype cortices.37,40 In contrast, large numbers of cells were CC3-positive in cortices of p63^fl/fl; R26YFP^fl/fl; nestin-CreERT2^+/0 embryos treated with tamoxifen (Fig. 3B). These CC3-positive cells were scattered throughout the cortical layers, with many positive cells in the precursor zones adjacent to the lateral ventricles, and many in the upper layers of the cortex, which contain newborn neurons. There were also many CC3-positive cells in the ganglionic eminence (GE) (Fig. 3B), consistent with the decreased size of this structure (Fig. 1C–E). Quantification of cortices at rostral, medial and caudal levels showed a robust increase in CC3-positive cells, with as many as 200 cortical CC3-positive cells per section following p63 ablation (Fig. 3C).

To confirm that this large increase in CC3-positive cells following acute p63 ablation was due to enhanced apoptosis, we performed TUNEL assays for DNA fragmentation. As seen with CC3, very few TUNEL-positive cells were observed in control p63^wt/wt; R26YFP^fl/fl; nestin-CreERT2^+/0 cortices following tamoxifen treatment, consistent with the low level of apoptosis in the wild-type embryonic cortex.37,40 In contrast, we observed many TUNEL-positive cells in cortices of p63^fl/fl; R26YFP^fl/fl; nestin-CreERT2^+/0 embryos treated with tamoxifen (Fig. 3D). As seen with CC3, these positive cells were located throughout the cortical layers, consistent with apoptosis of both precursors and newborn neurons. Quantification at rostral, medial and caudal levels indicated a highly significant increase in TUNEL-positive cells, with the magnitude similar to that seen with CC3 immunostaining (Fig. 3E). There were also many TUNEL-positive cells in the GE following p63 ablation (Fig. 3D), consistent with the CC3 data. Thus, acute ablation of p63 in neural precursors and their newborn progeny causes death of many developing forebrain cells.

**Acute conditional deletion of p63 depletes embryonic cortical radial precursors, intermediate progenitors, and newborn neurons**

To ask about the consequences of this increased apoptosis for cortical morphogenesis, we analyzed cell type-specific markers in cortical sections from E15 p63^fl/fl; R26YFP^fl/fl; nestin-CreERT2^+/0 and p63^wt/wt; R26YFP^fl/fl; nestin-CreERT2^+/0 embryos that were treated with tamoxifen at E12. To analyze cortical precursors, we immunostained sections for Pax6, a marker for radial precursors of the VZ/SVZ, and for Tbr2, a marker for intermediate progenitors that are found in the SVZ. This analysis (Fig. 4A) showed that both precursor populations were present and appropriately localized in the p63^fl/fl; R26YFP^fl/fl; nestin-CreERT2^+/0 cortices,
but that the VZ/SVZ itself was thinner following acute p63 ablation. Similarly, immunostaining for the pan-precursor marker Sox2, which delineates the VZ/SVZ and the neuronal marker βIII-tubulin, which delineates the IZ and CP (Fig. 4B) confirmed that cortical layers were appropriately organized, but that they were all relatively thinner following acute p63 ablation.

These data are consistent with the idea that cortical morphogenesis is normal following acute p63 ablation, but that the increased cell death results in decreased numbers of precursors and newborn neurons, leading to thinning of all cortical layers. To ask if this was the case, we quantified total neural precursors following immunostaining of similar sections for Sox2. To do this, we counted total marker-positive cells in a 200 μm wide strip of the medial-lateral cortex extending from the meninges to the ventricle. This analysis demonstrated that there were less than half as many Sox2-positive precursors in p63fl/fl;R26YFPfl/fl;nestin-CreERT2/Ø cortices relative to controls (Fig. 5A and B). To ask whether this was due to a reduction in radial precursors and/or intermediate progenitors, we performed a similar analysis for Pax6 (Fig. 5C and D) and Tbr2 (Fig. 5E and F). Both populations were significantly decreased by acute p63 ablation, with a larger decrease in Pax6-positive radial precursors. We performed a similar analysis for cortical neurons, immunostaining for Satb2, a marker for many of the cortical neurons that are born over the time period from E12 to E15.41 This analysis demonstrated that tamoxifen treatment caused a significant decrease in Satb2-positive neurons in p63fl/fl;R26YFPfl/fl;nestin-CreERT2/Ø versus control p63wt/wt;R26YFPfl/fl;nestin-CreERT2/Ø cortices (Fig. 5G and H), confirming the reduction in cortical neurons suggested by the decrease in βIII-tubulin immunostaining (Fig. 4B).

Embryonic cortical precursors display a cell-autonomous deficit in cell survival following acute p63 ablation

To further confirm that acute ablation of p63 in neural precursors causes enhanced cell death, we turned to cell culture experiments. Initially, we treated p63fl/fl;R26YFPfl/fl;nestin-CreERT2/Ø vs. control p63wt/wt;R26YFPfl/fl;nestin-CreERT2/Ø embryos with tamoxifen at E12 and then isolated neurospheres from the embryonic cortices. Quantification of the number of primary
nevosphere-initiating cells, a surrogate measure of neural precursor numbers, demonstrated that they were decreased more than 2-fold following acute p63 ablation in vivo (Fig. 6A). qRT-PCR analysis of the RNA from these neurospheres confirmed that p63 mRNA was highly reduced, but that expression of the other family members was unaltered (Fig. 6B).

To further establish a role for p63 in cortical precursor survival, we performed inducible ablation experiments in primary adherent cultures of developing cortical radial precursors. Specifically, we cultured p63fl/fl;R26YFPfl/fl;nestin-CreERT2+/+ versus control p63wt/wt; R26YFPfl/fl;nestin-CreERT2+/+ cortical precursors from E12.5 mice, and after 2 d added increasing concentrations of tamoxifen to induce ablation of p63 and expression of YFP. We immunostained these cultures one day later for CC3 and YFP (Fig. 6C). Quantification demonstrated a robust increase in apoptosis of YFP-positive precursors that was dependent upon the dose of tamoxifen (Fig. 6D).

\[ \Delta Np73 \text{ mRNA is increased in cortical precursors when p63 is constitutively knocked-out} \]

These data provide strong support for the idea that p63 is required in a cell-autonomous fashion to support the survival of cortical precursors and newborn neurons both in culture and in vivo. However, a previous study examined the cortices of embryonic p63−/− mice and concluded that p63 was not necessary for the survival of either precursors or neurons.38 One explanation for this discrepancy is that in the constitutive knockout situation, other family members compensate for the loss of p63.42 To test this idea, we cultured E15 cortical precursors from p63+/+ vs. p63−/− mice as neurospheres. As previously reported,38 and in contrast to the robust decrease in cortical neurosphere-initiating cells in tamoxifen-treated p63fl/fl;R26YFPfl/fl;nestin-CreERT2+/+ embryos (Fig. 6A), there was no difference in the number of neurosphere-initiating cells cultured from p63−/− versus p63+/+ cortices (Fig. 6E). However, while qRT-PCR analysis showed that the levels of TAfp73 and p53 mRNA were unaltered in p63−/− neurospheres, the levels of \( \Delta Np73 \) mRNA, a pro-survival member of the p53 family, were upregulated by 2-fold (Fig. 6F).

To ask if this compensatory increase in \( \Delta Np73 \) mRNA also occurred in vivo, we crossed p63+/+ mice, and then analyzed cortical precursors in their p63+/+ vs. p63+/− embryonic progeny. To do so, we analyzed E15 coronal cortical sections by immunostaining for Sox2 to identify cortical precursor cells, combined with in situ hybridization with a probe specific for \( \Delta Np73 \) mRNA (Fig. 6G). Quantification of this data demonstrated that while similar numbers of Sox2-positive cortical precursors expressed \( \Delta Np73 \) mRNA in p63−/− versus p63+/+ embryos, approximately 64% and 59%, respectively, \( \Delta Np73 \) mRNA levels were increased in p63−/− cortical precursors, 3.7 vs. 2.0 \( \Delta Np73 \) mRNA grains per Sox2-positive cell in p63−/− versus p63+/+ cortices, respectively. Since \( \Delta Np73 \) is a key prosurvival protein in the embryonic cortex,19,23,37,43-45 then this compensatory increase likely provides an explanation for the lack of an apoptotic phenotype in the p63−/− embryonic cortex as shown by Holembowski et al.38

**Discussion**

The work presented here definitively establishes that p63 plays an important prosurvival role in the embryonic central nervous system (CNS). Inducible ablation of p63 in embryonic neural precursors and their newborn neuronal progeny led to a robust increase in apoptosis of forebrain precursors and neurons, and perturbed cortical morphogenesis. The overall organization of the cortical layers was unchanged, but there were many fewer radial precursors, neurogenic intermediate progenitors and neurons, decreases that caused thinning of the cortex and enlargement of the ventricles. This cell death phenotype was cell autonomous, since inducible ablation of p63 in cultured precursors also caused apoptosis. These findings are very similar to those obtained using in utero electroporation to knockdown p63 in the embryonic cortex,37 but are different from those obtained by

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**Figure 5 (See previous page).** Cortical precursors and newborn neurons are reduced in number following conditional ablation of p63. p63wt/wt; R26YFPfl/fl;nestin-CreERT2+/+(p63WT) or p63fl/fl;R26YFPfl/fl;nestin-CreERT2−/−(p63FLOX) embryos were exposed to tamoxifen (injected into their mothers) at E12, and their cortices were analyzed 3 d later at E15. (A) Representative images of the VZ/SVZ of coronal cortical sections from p63WT and p63FLOX embryos, immunostained for Sox2 (green; the endogenous YFP is not seen under these excitation/emission conditions). Insets show higher magnification images and the arrows denote Sox2-positive cells. The boundaries of the VZ/SVZ are indicated by dotted white lines. Scale bar = 100 μm. (B) Quantification of the total number of Sox2-positive cells in a strip of the lateral-medial cortex extending from the meninges to the ventricle, determined from sections as in (A). ***p < 0.001; n = 3 sections per embryo and 3 embryos per genotype (9 sections total). (C) Representative images of the VZ/SVZ of coronal cortical sections from p63WT and p63FLOX embryos, immunostained for Pax6 (green; the endogenous YFP is not seen under these excitation/emission conditions). Insets show higher magnification images and the arrows denote Pax6-positive cells. The boundaries of the VZ/SVZ are indicated by dotted white lines. Scale bar = 100 μm. (D) Quantification of the total number of Pax6-positive cells in a strip of the medial-lateral cortex extending from the meninges to the ventricle, determined from sections as in (C). ***p < 0.001; n = 3 sections per embryo and 3 embryos per genotype (9 sections total). (E) Representative images of the VZ/SVZ of coronal cortical sections from p63WT and p63FLOX embryos, immunostained for Tbr2 (red). Insets show higher magnification images and the arrows denote Tbr2-positive cells. The boundaries of the VZ/SVZ are indicated by dotted white lines. Scale bar = 100 μm. (F) Quantification of the total number of Pax6-positive cells in a strip of the medial-lateral cortex extending from the meninges to the ventricle, determined from sections as in (E). ***p < 0.001; n = 3 sections per embryo and 3 embryos per genotype (9 sections total). (G) Representative images of the cortical plate of coronal cortical sections from p63WT and p63FLOX embryos, immunostained for Satb2 (red). Insets show higher magnification images and the arrows denote Satb2-positive cells. Scale bar = 100 μm. (H) Quantification of the total number of Satb2-positive cells in a strip of the medial-lateral cortex extending from the meninges to the ventricle, determined from sections as in (G). ***p < 0.001; n = 3 sections per embryo and 3 embryos per genotype (9 sections total). The cortical plate (CP), ventricular/subventricular zones (VZ/SVZ), intermediate zone (IZ) and lateral ventricles (LV) are all denoted. In all panels, error bars denote SEM.
studying p63/−/− embryos, where no CNS phenotype was observed. We show here that the lack of a phenotype when p63 is constitutively ablated is likely due to upregulation of the related prosurvival protein, ΔNp73, in neural precursors.

We previously showed that when p63 was haploinsufficient or was inducibly ablated in adult neural precursor cells (NPCs), there was apoptosis of forebrain and hippocampal NPCs, reduced adult neurogenesis, and deficits in hippocampus-dependent memory formation. These findings, together with the current work, indicate that p63 promotes NPC survival throughout murine life. We propose that it is the ΔNp63 isoform that promotes NPC survival, since the death of embryonic cortical precursors caused by p63 knockdown was rescued by ectopic expression of ΔNp63 but not TAp63, and since inducible knockout of ΔNp63 but not of TAp63 caused death of adult forebrain NPCs cultured as neurospheres. How then does ΔNp63 promote survival of cortical precursor cells during embryogenesis? Our previous work indicates that it does so by antagonizing the pro-apoptotic actions of p53. In particular, when p63 was acutely knocked down in cortical precursors, this caused increased apoptosis (as seen here with acute genetic ablation), and this cell death was rescued both in culture and in vivo by coincidently knocking down p53. This prosurvival mechanism appears to persist throughout life in neural precursors, since coincident p53 knockout completely rescued the deficits in adult NPCs and adult neurogenesis that occur in p63+/− mice. Thus, the ΔNp63 isoform acts to promote NPC survival by antagonizing p53 and potentially other full-length family members. In this regard, ectopic expression of TAp63 induces the death of both NPCs and neurons, indicating that in the nervous system, TAp63 promotes and ΔNp63 inhibits cell death.

These findings demonstrate that ΔNp63 and ΔNp73 are both key prosurvival proteins in the mammalian CNS, with ΔNp73 important for neuronal survival, and ΔNp63...
important for both NPC and newborn neuron survival.\textsuperscript{31,36,37} This difference is exemplified by the finding that knockdown of \(\Delta Np73\) in the embryonic cortex caused the death of newborn neurons but not precursors,\textsuperscript{37} while a similar knockdown of \(p63\) caused the death of cortical precursors.\textsuperscript{37} Thus, under normal circumstances, \(\Delta Np63\) is the most important family member with regard to NPC survival. However, our data showing upregulation of \(\Delta Np73\) expression in cortical NPCs of \(p63^{-/-}\) mice argue that when \(p63\) is constitutively ablated, \(\Delta Np73\) can replace the pro-survival function of \(\Delta Np63\), thereby explaining the lack of an apparent embryonic NPC phenotype in the \(p63^{-/-}\) mice. In normal circumstances, \(p73\) promotes 2 other important roles in NPCs. Firstly, \(TAp73\) enhances NPC self-renewal,\textsuperscript{26,27} at least in part by suppressing neurogenic transcription by upregulating the inhibitor bHLH Hey2.\textsuperscript{26} Secondly, \(p73\) regulates the balance of \(p53\)-mediated senescence vs. apoptosis of NPCs. In particular, coincident haploinsufficiency for \(p73\) inhibited the enhanced apoptosis of \(p63^{+/+}\) adult NPCs, causing them instead to senesce.\textsuperscript{31} Thus, it is the interplay between \(p63\), \(p73\) and \(p53\) that ultimately determines NPC numbers by regulating their survival, self-renewal, and senescence.

Materials and Methods

Animals and tamoxifen treatment

This study was approved by the Hospital for Sick Children Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines. \(p63^{+/+}\) mice\textsuperscript{4} were maintained on a C57BL/6 background as described.\textsuperscript{36} \(p63^{fl/fl}\) mice\textsuperscript{46} were crossed with R26YFP\textsuperscript{fl/fl} reporter mice\textsuperscript{47} and nestin-CreERT2\textsuperscript{2+/+} mice\textsuperscript{39} and then maintained through homozygous breeding pairs on a C57BL/6 background, as we have previously described.\textsuperscript{36} For neuroanatomical analyses, adult pregnant female \(p63^{fl/fl}\), R26YFP\textsuperscript{fl/fl},nestin-CreERT2\textsuperscript{2+/+} and p63\textsuperscript{wt/wt},R26YFP\textsuperscript{fl/fl},nestin-CreERT2\textsuperscript{2+/+} mice were injected intraperitoneally once with tamoxifen (180 mg/kg in sunflower seed oil) at gestational day 12. Mice had free access to rodent chow and water in a 12 hour dark-light cycle room.

Immunocytochemistry and histological analysis

For morphometric analysis, immunostaining of tissue sections was performed as described.\textsuperscript{36,48} Briefly, brain sections were washed with TBS buffer, permeabilized with TBS, 0.3% Triton X-100 solution, and then incubated in TBS, 5% BSA, 0.3% Triton X-100 for 1 hour as a blocking solution. Brain slices were incubated with primary antibodies in blocking solution at 4°C overnight. After TBS washes, the sections were incubated with secondary antibodies in blocking solution for 1 hour at room temperature. Finally, after TBS washes, sections were mounted in Permount solution (Thermo). In most cases, sections were counterstained with Hoechst 33258 (Sigma). TUNEL staining (Millipore) was performed according to manufacturer’s instructions. Digital image acquisition was performed with Zen software (Carl Zeiss) on a Zeiss Axio Imager M2 microscope with a Hamamatsu Orca-Flash 4.0 CCD video camera. For quantification of embryonic cortical thickness, rostral, medial and caudal cortical sections were measured at 3 different points (dorsal, lateral and ventral), analyzing at least 3 similar sections/embryo from 3 different animals per genotype (for a total of at least 9 sections per genotype) using ImageJ software (NIH). For quantification of the embryonic ventricle area, a line was drawn around the perimeter of the ventricle at rostral, medial and caudal levels from at least 3 similar sections/embryo from 3 different animals per genotype. The area was calculated and values expressed as arbitrary units (AU) using ImageJ software. For quantification of the number of apoptotic cells, or of precursor cells and neurons, serial coronal 18 μm sections were collected spanning the rostro-caudal extent of the E15 embryonic cortex and these were sampled and immunostained or TUNEL-stained (as relevant). For quantification of precursor and neuron numbers, we analyzed sections at the medial-lateral level, counting all marker-positive...
cells in a 200 μm wide strip of the cortex extending from the meninges to the ventricle. For cell death analysis, every TUNEL or cleaved caspase-3 positive cell was counted on sections at rostral, medial and caudal levels. In all cases, we analyzed at least 3 similar cortical sections/embryo from 3 different embryos per genotype (for a total of at least 9 sections per genotype).

In situ hybridization
Fluorescent in situ hybridization (FISH) was performed with probes targeting ΔNp73 (National Center for Biotechnology Information [NCBI] Nucleotide Reference Sequence [RefSeq] database accession number NM_001126330.1) using the RNA-scope kit (Advanced Cell Diagnostics), according to the manufacturer’s instructions. Briefly, freshly dissected brains of E15 embryos in OCT were snap-frozen in liquid nitrogen and cryosectioned coronally at 18 μm. Sections were 4% post-fixed with paraformaldehyde and washed with ethanol, followed by tissue pretreatment, probe hybridization and signal amplification. Positive hybridization was identified as punctate dots. After FISH, immunostaining was performed for Sox2. Z-stack images were taken with an optical slice thickness of 0.1 μm, with a 40X objective on a Zeiss Axio Imager M2 microscope with an Apo-Tome device and with a Hamamatsu Orca-Flash 4.0 CCD video camera. The proportion of ΔNp73 mRNA-Sox2-double positive cells was quantified from z-stacked images from random regions of the VZ/SVZ. The number of ΔNp73 mRNA foci per Sox2-positive cell was quantified from 200–300 z-stacked images from random regions of the VZ/SVZ.

Cortical precursor cell cultures and quantification
E12.5 cortical precursors were cultured from p63 wt/wt; R26YFPfl/fl;nestin-CreERT2+/- embryos, or p63 fl/fl;nestin-CreERT2+/- embryos, previously injected with tamoxifen on E12, were mechanically triturated in serum-free medium containing 20 ng/mL EGF (Sigma), 10 ng/mL FGF2 (Sigma), and 2 μg/mL heparin (Sigma). For quantification, cells were plated at clonal density in 6-well dishes (2 ml/well). After 6 d in culture, primary neurospheres containing at least 50 cells were counted, and were then collected for mRNA expression analysis. A similar procedure was used to make embryonic cortical neurospheres from E15 p63+/- or p63-/- embryos.

Quantitative RT-PCR
RNA was isolated from primary neurospheres or dissected E15 cortices using the E.Z.N.A. Total RNA Kit I (Omega Bio-tek), and was treated with DNase I using the E.Z.N.A RNase-Free DNase I Set (Omega Bio-tek). cDNA was synthesized from 1 μg of total RNA using RevertAid H Minus M-MulV Reverse Transcriptase (Fermentas), and quantitative PCR was performed using Lightcycler 480 SYBR Green I Master mix (Roche), following the manufacturer’s instructions. The following primers were used for quantitative PCR: TAp73F – GCACCTACTTTGACCTCCCC, TAp73R – GCACTGCTGAGCAGAATTGAC, Δp73F – CTA CCCCTACCCCACCTAG, Δp73R – CTGAGCAAATTGACTGGGC, pan-p63F – GGGATCTCCGTTTCTTGATGG, pan-p63R – GGGATCTCCGTTTCTTGATGG, p53R – CTCT CCCCGGCAGAAAGAAAA, p53R – CCTGAGACTCTGCAAGGGTTA, PUMAF – GTGACCACGGCATTCTTG, PUMAR – CTCTCCCTCTCTGAGACTT, GAPDH – GG GTGTGAACCACGAGAAATA, GAPDHR – CTGTGGTCATGGCGTACATGAGCCTTC. GAPDH mRNA was used as an endogenous control for all reactions, and all reactions were performed in triplicate. Quantitative PCR was performed using CFX Manager Software (Bio-Rad), and analyzed using Bio-Rad CFX Manager Software (Bio-Rad).

Statistical analysis
Statistics were performed using two-tailed Student’s t-test unless otherwise indicated in the text. To analyze the multi-group neuroanatomical studies, we used one-way ANOVA unless otherwise indicated in the text. Significant interactions or main effects were further analyzed using Newman-Keuls post-hoc tests. All tests were performed using GraphPad Prism 5. In all cases, error bars indicate standard error of the mean.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
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