Retinoblastoma-deficient mice show massive neuronal damage and deficits in both CNS and PNS tissue. Previous work in the field has shown that death is regulated through distinct processes where CNS tissue undergoes death regulated by the tumor suppressor p53 and the apoptosome component, APAF1. Death in the PNS, however, is independent of p53 and reliant on the death protease, caspase 3. In the present study, we more carefully delineated the common and distinct mechanisms of death regulation by examining the stress-activated kinases, JNK2 and 3, the conserved Bcl-2 member Bax, and the relationship among these elements including p53. By use of genetic modeling, we show that death in various regions of the CNS and DRGs of the PNS is reliant on Bax. In the CNS, Bax acts downstream of p53. The relevance of the JNKs is more complex, however. Surprisingly, JNK3 deficiency by itself does not inhibit c-Jun phosphorylation and instead, aggravates death in both CNS and PNS tissue. However, JNK2/3 double deficiency blocks death due to Rb loss in both the PNS and CNS. Importantly, the relationships between JNKs, p53, and Bax exhibit regional differences. In the medulla region of the hindbrain in the CNS, JNK2/3 deficiency blocks p53 activation. Moreover, Bax deficiency does not affect c-Jun phosphorylation. This indicates that a JNK–p53–Bax pathway is central in the hindbrain. However, in the diencephalon regions of the forebrain (thalamus), Bax deficiency blocks c-Jun activation, indicating that a Bax–JNK pathway of death is more relevant. In the DRGs of the PNS, a third pathway is present. In this case, a JNK–Bax pathway, independent of p53, regulates damage. Accordingly, our results show that a death regulator Bax is common to death in both PNS and CNS tissue. However, it is regulated by or itself regulates different effectors including the JNKs and p53 depending upon the specific region of the nervous system.

The tumor suppressor Retinoblastoma (Rb) influences a wide number of biological processes including cell growth, modulation of cell death, and differentiation (1). Rb is mutated in a third of human tumors and its role in oncogenesis is intensively studied (2). Its importance is further underscored by observations that Rb mutant mice display gross neuronal defects accompanied by ectopic S phase entry, and midgestation (E13–E14) lethality (3–5). Cell death is observed in Rb mutant embryos in PNS, CNS, muscle, lens, and fetal liver tissues.

Recent efforts have focused on understanding the mechanisms of neuronal loss induced in both PNS and CNS of developing Rb mutant mice. Neuronal death in these mice appears to occur through a complex tissue-specific signaling cascade, making this an intriguing model of death that occurs during development. Death is not cell autonomously regulated but likely due to other secondary events such as hypoxia (6, 7). In this paradigm, neuronal death in the PNS and CNS also appear to be regulated differentially. Death in the CNS is regulated by E2F1 and E2F3, two Rb-regulated transcription factors critical for cell cycle regulation (8–10). In addition, death in the CNS is entirely dependent upon the tumor suppressor p53 (11), and Apaf1 (12), a central component of the apoptosome critical for activation of the mitochondrial pathway of apoptosis. Finally, death is not affected by caspase 3 deficiency (13).

In contrast to the CNS, neuronal loss in PNS tissue of Rb-deficient mice is only partially dependent upon E2F1/3 (8–10) and Apaf1 (12), but completely dependent upon caspase 3 (13) and independent of p53 (11). These data suggest that both proximal (e.g. p53) and distal (e.g. caspase 3) effectors of death differ in the CNS and PNS. These findings highlight the important question of the identity(ies) of the central common mechanistic elements between CNS and PNS death in Rb-deficient embryos. As will be discussed in more detail below, Bax is thought to be critical in many forms of neuronal death. Accordingly, we initially hypothesized that the Bcl-2 member, Bax might be the central death regulator. We also postulated that there were different upstream regulators of Bax in the CNS and PNS tissue. In this regard, we examined both p53 and the JNKs, two known upstream regulators of Bcl-2 family members.

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2 The abbreviations used are: Rb, retinoblastoma; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; DRG, dorsal root ganglia; CNS, central nervous system; PNS, peripheral nervous system.
Bax and JNK Requirement in Rb-mediated Death

The Bcl-2 family member Bax has been shown to be central in neuronal death in a number of different paradigms (for example (14–16)). In many cases, Bax translocation to the mitochondria leads to release of cytochrome c, concomitant activation of the apoptosome, and in turn, activation of numerous effector caspases. However, Bax does not necessarily mediate death solely through activation of apoptosome. Bax may also regulate apoptosis-inducing factor (AIF) release from the mitochondria (17). As such, Bax represents a viable candidate to explain the Apaf1-independent death processes observed in the PNS. The factors which lead to activation of Bax are numerous. Of relevance, p53 (14) as well as the stress activated kinases JNKs, members of the larger MAPK superfamily, are a family of three (JNK1,2,3) kinases which are activated by multiple stimuli including death inducing insults such as growth factor deprivation, DNA damage, dopaminergic toxins, axotomy, and ischemic insult (18, 23–30). The JNK family members are key activators of the transcription factor c-Jun by phosphorylating c-Jun on Ser63 and Ser73 (31–33). In most cases in neurons, late activation of Bcl-2 family members such as Bim in models of death that are Bax-dependent, JNKs can also modulate p53 function (37, 38). The interplay between p53, JNK and Bax is therefore likely complex. We presently set out to delineate the potentially critical role of these signals in neuronal death due to Rb deficiency.

By use of genetic modeling using Bax- and JNK-deficient mice, we report that death in dorsal root ganglia (DRGs) of the PNS and various regions of the CNS is Bax-dependent. However, the relationship of JNKs to death is more complex. JNK3 deficiency alone exacerbates death in PNS and CNS tissue. However, JNK2/3 double deficiency is protective. Our data also provide a model by which JNKs act differentially in the CNS. JNKs can regulate both p53 and c-Jun upstream of Bax in the caudal region of the hindbrain (medulla oblongata). However, it can also act downstream of Bax in the diencephalic regions of the forebrain. In the DRGs of the PNS, death is dependent upon JNKs which acts upstream of Bax, independent of p53. Therefore we propose that a common central activator of death, Bax, which can also act downstream of Bax in the diencephalic regions, is regulated by and activates differential upstream and effector signaling pathways in the CNS and PNS tissues of Rb-deficient mice.

EXPERIMENTAL PROCEDURES

Transgenic Mice—All transgenic lines were maintained on a C57/Bl6 genetic background. Following interbreeding of various genotypes as described in the text, genotyping was performed by PCR analyses as follows: Bax knock-out embryos were genotyped using GCCGGCCGATAGTATCGAGTTACC (MJ2B3), GTTAGACAATCCCAGAGGTTGTGTG (MJ2F5), and CCAGCTATCCCTCCACTCATG (PGK1T1) in one PCR reaction. Both JNK2 and JNK3 were run under the following PCR conditions: 95 °C, 5 min (1 cycle); 95 °C, 1 min; 58 °C, 1 min (~1.0 °C/cycle); 72 °C, 1 min (10 cycles); 95 °C, 1 min; 48 °C, 1 min; 72 °C, 1 min (20 cycles); 72 °C, 5 min. For JNK2 and JNK3, this reaction amplifies a 270-bp and 250-bp product indicating the targeted allele and a 400-bp and a 430-bp product indicating the wild-type allele, respectively.

Bax embryos were genotyped using GTTGAGAGATGTAGCTGAG (BaxIN5R), GAGCTGATCAGAACCATCATG (BaxEX5F) and CCGCTATCTGCTACGCGG (NEOR) primers under the following PCR conditions: 94 °C, 5 min (1 cycle); 94 °C, 1 min; 62 °C, 1 min; 72 °C, 1.5 min (30 cycles); 72 °C, 7 min. BaxIN5R and BaxEX5F primers were used to amplify a 304-bp product indicating the wild-type allele whereas BaxIN5R and NEOR were used to amplify 504-bp fragment indicating the targeted allele.

Rb-deficient transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a C57BL/6 genetic background. Rb embryos were genotyped using AATTGGCGGCCATCTGCTACTTATATCGC (RX3), CCCATGTTCCCGCTCTAG (R13), and GAAGACGATCAGCG (PGK3)’s primers in two separate reaction tubes. RX3 and R13 primers were used to amplify the wild-type product and RX3 and PGK3’ primers were used to amplify the targeted allele product under the following conditions: 95 °C, 5 min (1 cycle); 94 °C, 1 min; 58 °C, 1 min; 72 °C, 1.5 min (29 cycles).

For TUNEL and immunofluorescence analyses, embryos were obtained at E13.5. Tissue samples were obtained for genotyping purposes. The embryos were fixed in 4% PFA/0.1 M phosphate buffer pH 7.4 overnight at 4 °C. The embryos were rinsed using 1× PBS, pH 7.4 (Invitrogen) and cryoprotected in 30% sucrose/0.1 M phosphate buffer pH 7.4 overnight at 4 °C. Embryos were equilibrated in a 50:50 mixture of 30% sucrose/OCT (TissueTek 4583) for ~1 h on a rocking platform. Finally, the embryos were embedded in the same 50:50 mixture and frozen on liquid nitrogen. The embryos were sectioned sagittally at 14 μm on a cryostat. In the Bax/Rb breedings, a subset of embryos was also collected at E18.5 to determine whether survival was enhanced with Bax deficiency.

TUNEL Labeling—TUNEL labeling of sections was performed as previously described (13). Briefly, sections were fixed with 1% glutaraldehyde for 15 min. Following washing with PBS, sections were permeabilized with 1:1 methanol/acetone mixture for 10 min. After washing with PBS, slides were incubated with Hoechst (1:4000 of 0.5 mg/ml soln), and TUNEL reaction was performed for 1 h 37 °C using the terminal transferase kit as per the manufacturer’s instructions (Roche Applied Science). TUNEL labeling was visualized by secondary labeling using Cy3-streptavidin (Jackson Laboratories). For quantification, regions of the thalamus in the forebrain, medulla in the hindbrain, trigeminal ganglion and caudal DRGs were quantitated as follows: areas were photographed using Northern Eclipse, Empix Imaging software. Magnification: 20 × 4 × 5 grid set up, positive neurons were counted and compared with...
TABLE 1
Genotype frequency of offspring from all BAXRb interbreedings
Two types of breedings were performed. These include double heterozygous crosses as well as breedings of double heterozygous mice with BAX−/− Rb+/− mice. Each of these crosses give different expected Mendelian ratios. The expected calculations listed here are ratios of these strategies combined taking into consideration the number of breedings for each strategy.

| Genotype of offspring | Expected frequency | Number of E13.5 embryosa | Frequency |
|-----------------------|-------------------|--------------------------|-----------|
| BAX++ Rb++           | 4.9               | 8                        | 5.3       |
| BAX++ Rb−            | 12.5              | 20                       | 13.3      |
| BAX−/− Rb++          | 10.4              | 7                        | 4.7       |
| BAX++ Rb−            | 9.8               | 20                       | 13.3      |
| BAX−/− Rb−           | 22.3              | 34                       | 22.7      |
| BAX−/− Rb−           | 12.5              | 24                       | 16.0      |
| BAX++ Rb−            | 4.9               | 13                       | 8.7       |
| BAX−/− Rb+           | 15.3              | 11                       | 7.3       |
| BAX−/− Rb+           | 7.6               | 13                       | 8.7       |

*a Number of embryos, n = 150.

their respective Hoechst-positive nuclei. Both TUNEL labeling as well as the total number of Hoechst-positive cells were evaluated. The data are expressed as TUNEL-positive cells/total Hoechst positive cells ± S.E. Statistical significance was analyzed by analysis of variance with Newman-Keuls multiple comparison.

Immunofluorescence Analysis—Sections were obtained as described above and immunofluorescence described previously (13). Briefly, sections were washed three times in PBS, incubated overnight in PBS containing 0.3% Triton X-100 and primary antibody. Primary antibodies utilized were c-Jun (Cell Signaling Technologies, 1:150), phospho-c-Jun Ser73 (Cell Signaling 1:150), and phospho-Ser15 p53 (Cell Signaling, 1:150), Nestin (RDI, 1:400), Active caspase 3 (BD Pharmingen. 1:500), B-III Tubulin (kind gift from D. Brown, 1:50). Following three washes in PBS, sections were incubated in PBS, 0.3% Triton X-100 solution containing Cy3-conjugated donkey anti rabbit secondary antibody (Jackson, 1:2000). Quantitation was performed similar to that described above for TUNEL analysis. In the case of active caspase 3, total counts were evaluated in relation to a defined region and not to the number of Hoechst cells to facilitate analyses.

RESULTS
Bax Deficiency Rescues PNS and CNS Death in Rb-deficient Mice—To determine whether Bax acts as a common mediator of PNS and CNS neuronal death in Rb-deficient neurons, we interbred Bax heterozygous deficient mice with RB heterozygous deficient mice to obtain double heterozygous progeny. These animals were then bred to produce double homozygous-deficient mice. Alternatively, Bax/Rb double heterozygous mice were also interbred with Bax−/− Rb−/− mice. The expected and actual frequencies of genotypes from E13.5 embryos obtained from these crosses are listed in Table 1. The frequencies of embryos were roughly as expected. The majority of the analyses were performed at this age since gross apoptosis in both PNS and CNS is observed at this point prior to embryonic lethality of Rb-deficient mice.

As shown in Supplemental Fig. S1, widespread death was observed in a variety of areas of the Rb-deficient CNS as previously reported (3–5). Death in the cortex (dorsal telencepha-
between B-III tubulin-positive and -negative areas are the most defined, we observed that active caspase 3 was reduced in both tubulin-positive and -negative regions (Fig. 2B) with Bax/Rb double-deficient embryos. This was not the case with JNK3 deficiency, which is not protective against death due to Rb loss as described below.

Because Bax deficiency dramatically reduced neuronal death in the brain regions examined, we asked whether Bax deletion would promote increased survival of Rb-deficient embryos. Accordingly, the number of Bax/Rb double-deficient embryos was analyzed at E18.5. However no viable Rb-deficient animals were found at this age (n = 39).

Previous evidence has indicated that p53 is induced in the CNS of Rb-deficient mice (11). In addition, cell death in this region is dependent upon p53 since p53/Rb double-deficient mice show less death when compared with Rb-deficient mice alone (11). Therefore, we next determined whether Bax may inhibit p53 activation or whether protection mediated by Bax would leave the p53 response intact. The latter would be expected if p53 acted upstream of Bax. As shown in Fig. 3, active phosphorylated p53 was detected utilizing a phosphospecific phosphoSer15 p53 antibody. Modification at this site has been associated with increased stabilization/activation of p53. Phosphorylated p53 is most clearly induced in the medulla of the CNS in Rb-deficient but not control mice. No significant induction of active p53 was observed in the DRGs and only minor induction in the thalamus (data not shown). In contrast to the protection observed with Bax deficiency, however, Bax/Rb-deficient mice showed no significant decrease in p53 levels in the hindbrain (medulla). Similar results were also obtained for total p53 staining in the medulla (Fig. 3D). Finally, it was also important to show that activated p53 also occurred in dying cells in Rb-deficient mice and that this was reversed with Bax deficiency. To do this, we quantified the percentage of phosphop53-positive cells, which also showed signs of nuclear condensation and fragmentation in the medulla. Our results show that ~30% was colocalized (Fig. 3F). Colabeling is not 100% because p53 induction likely precedes late death markers such as nuclear condensation. We then determined whether this colocalization was altered in Rb/Bax double-deficient animals. In fact, we observed that the colocalization with markers of damage decreased dramatically (less than 10%) as a result of Bax
FIGURE 3. Effects of Bax and JNK deficiency on p53 activation. E13.5 embryos were obtained from crosses of Rb-deficient mice with Bax or JNK2/3-deficient mice to obtain the indicated genotypes. A, representative immunofluorescence image of hindbrain region of the CNS stained with phospho-Ser15 p53 antibody. Inset in each panel is a higher magnification picture of immunofluorescent phospho-Ser15 p53 signal (top inset) in a different field of the same genotype along with its associated Hoechst staining (bottom inset). B, quantitative evaluation of the effects of Bax deficiency on phospho-Ser15 p53 staining induced by Rb deficiency. The number of Ser15-positive cells were quantified as described under “Experimental Procedures.” * indicates significance $p < 0.05$ of Bax+/− Rb+/− in comparison to the other genotypes except Bax+/− Rb+/−. C, quantitative evaluation of the effects of JNK deficiency on phospho-Ser15 p53 staining induced by Rb deficiency. Sections of the genotype indicated were analyzed for phospho-Ser15 p53 staining in the hindbrain in an identical fashion as in B. * indicates significance $p < 0.05$ of JNK3+/− Rb+/− in comparison to the other genotypes. D, quantitative evaluation of the effects of Bax deficiency on p53 staining induced by Rb deficiency. Sections of the genotype indicated were analyzed for p53 staining in the hindbrain in an identical fashion as in B. * indicates significance $p < 0.05$ of Bax+/− Rb+/− in comparison to the other genotypes except Bax+/− Rb+/−. E, quantitative evaluation of the effects of JNK deficiency on p53 staining induced by Rb deficiency. Sections of the genotype indicated were analyzed for p53 staining in the hindbrain in an identical fashion as in B. * indicates significance $p < 0.05$ of JNK3+/− Rb+/− in comparison to the other genotypes except JNK3+/− Rb+/− and JNK2+/− JNK3+/− RB+/− genotypes. F, quantitative evaluation of phospho-p53-positive cells which also displayed condensed/fragmented nuclei in the medulla regions of the hindbrain of the genotypes as indicated. * indicates significance $p < 0.05$ of Bax+/− Rb+/− in comparison to the other genotypes.
Bax and JNK Requirement in Rb-mediated Death

A. BAX-/ Rb-/ BAX+/ Rb+/ BAX-/ Rb+/ BAX+/ Rb-/

FOREBRAIN

DRG

B. 

| Condition         | DRG | TGN | FOREBRAIN | HINDBRAIN |
|-------------------|-----|-----|-----------|-----------|
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |

C. 

| Condition         | DRG | TGN | FOREBRAIN | HINDBRAIN |
|-------------------|-----|-----|-----------|-----------|
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |
| BAX +/- Rb +/-    |     |     |           |           |

D. 

| Condition         | DRG | TGN | FOREBRAIN | HINDBRAIN |
|-------------------|-----|-----|-----------|-----------|
| RB +/-            |     |     |           |           |
| Bax +/- Rb +/-    |     |     |           |           |
| JNK3 +/- Rb +/-   |     |     |           |           |

% dead phospho-jun positive cells

| Tissue | DRG | TGN | FOREBRAIN | HINDBRAIN |
|--------|-----|-----|-----------|-----------|
|        |     |     |           |           |
|        |     |     |           |           |
|        |     |     |           |           |
|        |     |     |           |           |
|        |     |     |           |           |
|        |     |     |           |           |
deficiency. This reduction did not occur with JNK3 deficiency which is not protective against cell death (see below for full results on JNKs). This is strong supportive evidence that cells which are p53 positive are the ones which are destined to die. Taken together, our data suggest that at least in the medulla, Bax acts at a point downstream of p53 and that inhibition of Bax still leaves p53 activation intact in this region.

The Role of JNKs in RB-mediated Death—The JNK/c-Jun pathway is reported to affect neuron survival under select conditions. In peripheral neurons such as sympathetic neurons deprived of NGF, it is thought that this pathway regulates Bax activation (18–21). Because Bax is a common mediator of death in both PNS and CNS tissues evaluated, we examined whether the JNK/c-Jun pathway might regulate neuronal death in both regions. We first examined whether c-Jun is phosphorylated on Ser73, a known JNK phosphorylation site in RB-deficient mice in the selected PNS and CNS regions as described above. As shown in Fig. 4, a substantial increase in phosphorylated c-Jun was observed in both forebrain and hindbrain of the CNS examined as well as in TG and rostral DRGs of the PNS. This induction of c-Jun phosphorylation observed in RB-deficient mice was not affected in the sensory ganglia by Bax deficiency. This suggests that in this tissue, JNKs act upstream of Bax. In the CNS, however, Bax deficiency blocks c-Jun phosphorylation observed in RB-deficient mice in the thalamus, but not in the medulla. This indicates that in various regions of the CNS, the linkage between Bax and JNKs differ. In the medulla, JNKs, like p53 likely act more proximally prior to Bax activation. However in the thalamus, Bax appears to regulate c-Jun phosphorylation. Similar results were obtained for total c-Jun staining (Fig. 4).

Importantly, similar to the results discussed above for p53, c-Jun phosphorylation colabeled to a significant degree with markers of nuclear condensation/fragmentation in RB-deficient cells (Fig. 4D). As shown in Fig. 4D, this colocalization was dramatically reduced with Bax deficiency, which is protective, but not with JNK3 deficiency, which fails to protect (see below). This supports the notion that c-Jun phosphorylation occurs in dying cells.

We next asked whether JNKs might be important for neuronal death observed in RB-deficient embryos. To do this, we first examined the effects of loss of JNK3, the JNK isoform present predominately in the brain and most associated with neuronal death (29, 30). We interbred JNK3 heterozygous-deficient mice with RB heterozygous-deficient mice to obtain double heterozygous progeny. These animals were then bred to produce double homozygous-deficient mice. Alternatively, JNK3/RB double heterozygous mice were also interbred with JNK3-deficient RB heterozygous mice. The expected and actual frequencies of genotypes from E13.5 embryos obtained from these crosses in combination are listed in Table 2. As shown for Bax/Rb embryos (Table 1), the frequencies of embryos for all JNK3 and RB genotype combinations were roughly as expected.

We next determined whether JNK3 deficiency protects from neuron damage induced by RB loss in the DRGs of the PNS and select CNS regions by TUNEL analysis. In this regard, JNK3/RB double-deficient embryos did not show reduced TUNEL reactivity when compared with RB-deficient embryos alone in any CNS or PNS regions (Figs. 1 and 5). In fact, JNK3 deficiency caused a dramatic increase in death caused by RB deficiency in both CNS (hindbrain—medulla 19% forebrain—thalamus 17 versus 6.5 and 9.5%, respectively) and PNS (DRG 41 versus 14%) tissues (Fig. 5). This suggests that JNK3 by itself may even have some protective function or that there is compensation by other JNK members in the absence of RB. No other gross phenotype other than increased TUNEL was detected in Bax/Rb double knock-out embryos when compared with just RB-deficient animals. JNK3 deficiency by itself had little effect in death in CNS and PNS tissues. Similarly, JNK2 deficiency also failed to provide significant protection in the PNS, but did provide a slight protective effect in the CNS (Fig. 5). Importantly, when compared with JNK3 deficiency, JNK2 deficiency did not result in increased TUNEL positivity.

Because JNK 2 and 3 family members may compensate for each other, we also determined whether a deficiency of both JNK2 and 3 would ameliorate death induced by loss of RB. As shown in Fig. 5, death in the sensory PNS was reduced in JNK2/3/Rb triple-deficient mice when compared with RB deficiency alone (14% versus 6%). Similarly, death in the CNS regions examined was also significantly reduced in JNK2/3/Rb-deficient embryos. This decrease was more dramatic when com-

**TABLE 2**

Genotype frequency of offspring from all JNK3 RB interbreedings

| Genotype of offspring | Expected frequency | Number of E13.5 embryos* | Frequency |
|------------------------|--------------------|--------------------------|----------|
| JNK3+/+ Rb–/– | 2.2 | 1 | 1.2 |
| JNK3+/- Rb–/– | 12.5 | 15 | 18.1 |
| JNK3–/- Rb–/– | 17.8 | 9 | 10.8 |
| JNK3+/- Rb–/– | 4.4 | 5 | 6.0 |
| JNK3–/- Rb–/– | 18.1 | 19 | 22.9 |
| JNK3–/- Rb–/– | 2.5 | 20 | 24.1 |
| JNK3+/- Rb–/– | 2.2 | 3 | 3.6 |
| JNK3–/- Rb–/– | 20.6 | 2 | 2.4 |
| JNK3+/+ Rb–/– | 10.3 | 8 | 9.6 |

* Number of embryos, n = 83.

**FIGURE 4. Effects of Bax deficiency on c-Jun activation.** E13.5 embryos were obtained from crosses of RB-deficient mice with Bax deficient mice to obtain the indicated genotypes. A, representative immunofluorescence image of forebrain (thalamus) CNS or DRG PNS regions stained with phospho-Ser73 c-Jun antibody. Inset in each panel is higher magnification picture of the phospho-Ser73 c-Jun immunofluorescent signal from a different field (bottom inset) of the same genotype along with its associated Hoechst staining (top inset). B, quantitative evaluation of the effects of Bax deficiency on phospho-Ser73 c-Jun staining induced by RB deficiency. * indicates significance p < 0.05 of Bax+/+/Rb–/– in comparison to Bax+/+/Rb–/–, Bax+/+/- Rb+/–, and Bax–/- Rb+/– but not Bax+/+/- Rb–/– genotypes for TGN, TG, and hindbrain sections. For the forebrain, * indicates significance p < 0.05 of Bax+/+/- Rb–/– in comparison to the other genotypes. C, quantitative evaluation of the effects of Bax deficiency on c-Jun staining induced by RB deficiency. * indicates significance p < 0.05 of Bax–/- Rb–/– in comparison to Bax+/+/- Rb–/– in the DRG. For TGN and hindbrain, * indicates significance p < 0.05 of Bax–/- Rb–/– in comparison to Bax+/+/- Rb–/–, Bax+/+/- Rb+/–, and Bax–/- Rb+/– but not Bax+/+/- Rb–/– genotypes. For the forebrain, * indicates significance p < 0.05 of Bax+/+/- Rb–/– in comparison to the other genotypes. D, quantitative evaluation of phospho-c-Jun-positive cells, which also displayed condensed/fragmented nuclei in the various regions as indicated. * indicates significance p < 0.05 of Bax+/+/- Rb–/– in comparison to the other genotypes.

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pared with JNK2/Rb double-deficient embryos in the CNS. This indicates that death in the PNS and CNS is dependent upon the combination of JNK2/3.

We next determined how JNK deficiency is associated with the level of c-Jun and c-Jun phosphorylation. As shown in Fig. 6, JNK3 deficiency, which did not prevent the loss of neurons in Rb-deficient mice, also did not prevent c-Jun phosphorylation or c-Jun increase in any tissues examined. Interestingly, in some tissues (sensory DRG, Forebrain-thalamus), JNK3 deficiency by itself appeared to increase c-Jun levels. The reason for this is unclear, but could be one explanation of why JNK3 deficiency might exacerbate damage as shown in Fig. 5. JNK2 deficiency alone or JNK2/3 double deficiency significantly blocked c-Jun phosphorylation or c-Jun increase in the CNS and PNS tissue of Rb-deficient mice. Interestingly, JNK2 deficiency by itself fails to block death in the PNS while at the same time blocking c-Jun phosphorylation. This indicates that at least in this tissue, factors other than c-Jun phosphorylation are critical for death.

The JNKs are also thought to mediate activation of p53 under select conditions (37, 38). Therefore we asked whether p53 may act downstream of JNKs in the hindbrain, the region where p53 activation is most pronounced. Similar to TUNEL labeling, JNK3 deficiency failed to down-regulate p53 (Fig. 3, B and E). However, JNK 2- or JNK2/3-deficient mice did show reduced phospho-p53 and total p53 labeling (Fig. 3, B and E). This suggests that JNK2/3 act upstream of p53 activation in the hindbrain.

**DISCUSSION**

Rb-deficient embryos die early around E13.5 and display massive neuronal loss in various regions of the CNS and PNS. As such, this model is an important system to study death processes, which occur in the developing nervous system (3–5). The mechanism(s) by which neuronal death occurs in this paradigm is not fully defined but there is increasing evidence that the pathways differ in the two tissues. Accordingly, we embarked to determine whether there might be common elements in the death pathways of the CNS and PNS and whether there may be additional death promoting factors, which might explain the different regulatory pathways. The present results indicate a number of conclusions as follows: 1) Bax is a common critical factor regulating death in both DRGs of the PNS and CNS tissues examined. 2) However, Bax does not regulate all cell loss due to Rb deficiency and does not extend the life of the embryo. 3) Bax deficiency does not affect p53 activation suggesting that it acts downstream of p53 in the medulla of the CNS. 4) Interestingly, Bax deficiency also promotes a reduction in c-Jun activation, but only in the thalamus (in the forebrain) of the CNS. 4) JNK3 deficiency exacerbates death in both the CNS or PNS tissues examined. However, double-deficient JNK2/3 mice display reduced death in these tissues. 5) This protection is correlated with reduced c-Jun phosphorylation in the CNS areas examined, but not the sensory ganglia of the PNS. 6) JNK2/3 deficiency also reduces p53 activation in the medulla (hindbrain) of the CNS. Taken together our results suggest a region specific linkage of the JNKs, Bax, p53, and c-Jun. We propose a model by which death in the DRGs of the PNS is regulated by a JNK-Bax pathway, which acts to promote death in an Apaf1/caspase 3-dependent fashion. This pathway is also partially dependent upon the E2Fs, although the linkage at this point is not clear. Death in the CNS is region specific. In the medulla-hindbrain, JNKs regulate both p53 and c-Jun activation which acts upstream of Bax. In this region, E2Fs also regulate p53. Death is promoted more distally by an Apaf1 dependent pathway independent of caspase3. In the thalamus-forebrain, in contrast, JNK/c-Jun involvement appears downstream of Bax (see Fig. 7).

**CNS Pathway**—Death in the CNS by Rb deficiency has been previously reported to be dependent upon both E2F1 and E2F3 (8–10). Mice deficient for both Rb and E2Fs show almost complete inhibition of death and ectopic proliferation observed in the CNS (8–10). This observation is consistent with the well established role of E2Fs as Rb targets (39). However, it is important to note that death in the nervous system due to Rb deficiency is not cell autonomous and likely due to secondary events such as hypoxia (6, 7). Therefore, it is unclear whether E2Fs mediate death in the CNS directly in damaged neurons or whether E2F deficiency rescues more primary defects associated with Rb loss. In the CNS, cell death of Rb-deficient embryos is also completely dependent upon p53 (11). This p53-
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Figure 6. Effects of JNK deficiency on c-Jun activation. E13.5 embryos were obtained from crosses of Rb-deficient mice with JNK2 and/or JNK3-deficient mice to obtain the indicated genotypes. A, quantitative evaluation of the effects of JNK deficiency on phospho-Ser73 c-Jun staining induced by Rb deficiency. For the DRG, TGN, and forebrain tissues, * indicates significance $p < 0.05$ of JNK3 $^+/\text{Rb}^+$ in comparison to the other genotypes except JNK3 $^-/\text{Rb}^-$. For the hindbrain, * indicates significance $p < 0.05$ of JNK3 $^+/\text{Rb}^+$ and JNK2 $^+/\text{JNK3}^-/\text{Rb}^+$ in comparison to the other genotypes except JNK3 $^+/\text{Rb}^+$ in comparison to the other genotypes except JNK3 $^-/\text{Rb}^-$. B, quantitative evaluation of the effects of JNK deficiency on c-Jun staining induced by Rb deficiency. * indicates significance $p < 0.05$ of JNK3 $^+/\text{Rb}^+$ in comparison to JNK3 $^-/\text{Rb}^-$ and JNK3 $^+/\text{Rb}^+$ in comparison to all genotypes except JNK3 $^+/\text{Rb}^+$. For hindbrain, * indicates significance $p < 0.05$ of JNK3 $^+/\text{Rb}^+$ in comparison to all genotypes except JNK3 $^+/\text{Rb}^+$.

dependent death is also associated with a dependence on the ced4 homologue Apaf1 (12).

Previously, we showed that Bax is also a critical mediator of CNS death in the Rb-deficient embryo. Rb/Bax-deficient embryos show dramatically reduced TUNEL labeling when compared with Rb-deficient controls in both hindbrain and forebrain regions examined. Bax deficiency however, does not affect p53 induction suggesting that Bax acts downstream of p53. These results are consistent with our previous reports indicating that death in models of stroke and ischemia (29, 30). A final level of complexity is illustrated by how JNKs may be related to Bax activation in the CNS. In the forebrain, Bax deficiency abolishes c-Jun activation indicating that the JNK/c-Jun pathway is downstream of Bax. However, in the hindbrain, this is not the case and the linkage is reversed.

Interestingly, while death appears to be regulated by the apoptosome in the CNS, caspase 3 deficiency by itself is not sufficient to inhibit CNS damage (13). This suggests that Bax-mediated death signal likely activates and utilizes multiple caspases in the CNS to cause damage. From our present data as well as of others, we propose a model by which E2Fs, either in a direct cell autonomous or indirect fashion leads, to activation of...
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FIGURE 7. Model of the pathways which regulate CNS and PNS damage mediated by Rb deficiency. The data presented here are combined with reported data about Apaf1 and caspases as discussed in the text.

Bax. However, how Bax is regulated and the consequence of Bax regulation depends upon the region of the CNS. A caveat to our studies as well as those of others is that we have not firmly established the presence/absence of differences, which might occur between cell types in the areas of the CNS. However, we and others have observed that death is widespread between progenitors and newly differentiated neurons and have not noticed any qualitative changes in these two populations.

**PNS Pathway**—In contrast to the CNS, death in the DRGs of the PNS is less dependent upon the E2Fs and is independent of p53 (8–11). Mice which are double-deficient for both Rb and p53 do not display a reduction in damage when compared with Rb-deficient embryos alone (11). Interestingly, death in sensory ganglia is only partially dependent upon Apaf1 suggesting that death can also occur in independent of the classic apoptosome (12). As in the CNS, we presently show that Bax deletion abolishes death, which results from Rb deficiency in the PNS. This suggests that Bax plays a more central role than Apaf1. We propose that Bax in fact may mediate Apaf1-dependent and -independent processes. In support of this, Bax has been shown to participate in alternative apoptosome independent modes of neuronal death in vitro. These pathways include regulation of alternative mitochondrial factors, which also promote death such as AIF (17).

Death in the CNS appears to proceed through a JNK-p53-Bax-Apaf1- or Bax-JNK/APAF1-dependent process. In contrast, the proximal signals which regulate the mitochondrial pathway of death in the sensory ganglia of the PNS is independent of p53 but instead rely only upon the JNKs. Depletion of a combination of JNK2 and 3 reduces TUNEL labeling in the PNS associated with RB deficiency. The observation that Bax deficiency does not affect c-Jun phosphorylation also suggests that JNK acts upstream of Bax activation. Interestingly, the functional relevance of c-Jun phosphorylation by the JNKs in mediating PNS death is questionable. For example, our data show that JNK2 deficiency appears to inhibit c-Jun phosphorylation in the PNS, but does not prevent death. This is not the case in the CNS where inhibition of c-Jun and protection by JNK deficiency is tightly correlated. Again, this suggests that c-Jun has multiple potential functions outside of promotion of cell death (34–36, 40).

In summary, our data, in conjunction with other reports, support a model by which death in the PNS is initiated by a JNK/Bax pathway. However, death in the CNS is more complex where the involvement of JNK and p53 in Bax-mediated death differs depending upon the region of the brain. Once activated Bax likely regulates multiple pathways including but not limited to that of Apaf1/caspase 3. These results not only suggest common elements in neuronal loss in CNS and PNS tissue, it also delineates how these death elements differentially control damage in distinct regions of the nervous system.

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