Published in final edited form as:

Brain Res. 2009 November 3; 1296: 164–175. doi:10.1016/j.brainres.2009.07.101.

The DNA-PK Catalytic Subunit Regulates Bax-mediated Excitotoxic Cell Death by Ku70 Phosphorylation

Jia Liu^{a,b}, Janice R. Naegele^{a,*}, and Stanley L. Lin^{a,*}

^a Program in Neuroscience and Behavior, Department of Biology, Wesleyan University, Middletown CT 06459-0170

Abstract

DNA repair deficiency results in neurodegenerative disease and increased susceptibility to excitotoxic cell death, suggesting a critical, but undefined role for DNA damage in neurodegeneration. We compared DNA damage, Ku70-Bax interaction, and Bax-dependent excitotoxic cell death in kainic acid-treated primary cortical neurons derived from both wildtype mice and mice deficient in the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) encoded by the *Prkdc* gene. In both wildtype and *Prkdc*^{-/-} neurons, kainic acid treatment resulted in rapid induction of DNA damage (53BP1 foci formation) followed by nuclear pyknosis. Bax deficiency, by either Bax shRNA-mediated knockdown or gene deletion, protected wildtype and heterozygous, but not *Prkdc*^{-/-} neurons from kainate-induced excitotoxicity. Co-transfection of DNA-PKcs with Bax shRNA restored Bax shRNA-mediated neuroprotection in *Prkdc*^{-/-} neurons, suggesting that DNA-PKcs is required for kainate-induced activation of the pro-apoptotic Bax pathway. Immunoprecipitation studies revealed that the DNA-PKcs-non-phosphorylatable Ku70 (S6A/S51A) bound 3- to 4-fold greater Bax than wildtype Ku70, suggesting that DNA-PKcs-mediated Ku70 phosphorylation causes release of Bax from Ku70. In support of this, kainic acid induced translocation of a Bax-EGFP fusion protein to the mitochondria in the presence of a co-transfected wildtype, but not mutant Ku70 (S6A/S51A) gene when examined at 4 and 8 h following kainate addition. We conclude that DNA-PKcs links DNA damage to Bax-dependent excitotoxic cell death, by phosphorylating Ku70 on serines 6 and/or 51, to initiate Bax translocation to the mitochondria and directly activate a pro-apoptotic Bax-dependent death cascade.

Keywords

NHEJ; DNA damage; cell death; apoptosis; cerebral cortex

1. Introduction

Non-homologous end joining (NHEJ) is the predominant form of DNA double-strand break repair in post-mitotic neurons [47]. NHEJ is initiated by the DNA-dependent protein kinase

© 2009 Elsevier B.V. All rights reserved.

* Corresponding Authors: Dr. Stanley L. Lin Department of Biology Wesleyan University Middletown CT, 06459-0170 Phone: (860) 685-5984 Fax: (860) 685-3279 slin@wesleyan.edu Dr. Janice R. Naegele Department of Biology Wesleyan University Middletown CT, 06459-0170 Phone: (860) 685-3232 Fax: (860) 685-3279 jnaegele@wesleyan.edu.

^bCurrent address: Department of Neuroscience, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(DNA-PK), a heterotrimeric molecule comprised of Ku70, Ku80, and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the latter encoded by the *Prkdc* gene. Following the generation of DNA double-strand breaks, the Ku70/Ku80 dimer binds to the free DNA ends and recruits DNA-PKcs to form the DNA-PK complex that initiates double-strand break repair [34]. Loss of NHEJ activity in the developing brain can lead to pronounced apoptosis and prenatal lethality, whereas DNA repair deficiency in adulthood predisposes individuals to neurodegenerative disease [6,40,69]. The high metabolic rate of neurons, required for maintaining ionic gradients across the plasma membrane, can generate excessive oxygen radicals that damage DNA and ultimately trigger neuron death. Signal transduction pathways by which DNA damage triggers excitotoxic neuronal death are not well defined, in particular the regulation of p53 and Bcl-2 family members, such as Bax.

The p53 tumor suppressor is a DNA damage sensor known to play a role in excitotoxic cell death [4,31,63]. DNA-PK can activate p53 by phosphorylating the p53 amino terminus [55, 60], and p53 in turn can induce or activate Bax, a pro-apoptotic protein that translocates to the mitochondria and initiates the intrinsic death pathway [70]. Prior investigators have suggested a requirement for DNA-PKcs in apoptotic cell death via mechanisms involving DNA-PKcs-mediated activation of p53 [7,11,41,65,67]. However, p53 is often activated by ATM kinase [2,28,29], suggesting that the DNA-PK and ATM kinases have partially overlapping roles in activating p53. Furthermore, we have shown that excitotoxic cell death in *Prkdc*^{-/-} neurons still proceeds via a p53-dependent pathway [43]. Thus, the role of DNA-PK in excitotoxic cell death remains unknown.

DNA-PKcs has been shown to phosphorylate Ku70 on serines 6 and 51 [10,30]. The functional consequences of such Ku70 phosphorylation are unknown, but both DNA-PKcs-non-phosphorylatable Ku70 (S6A/S51A) and phosphomimetic Ku70 (S6D/S51D) function identically to the wildtype Ku70 in NHEJ, suggesting that DNA-PKcs-mediated phosphorylation of Ku70 is unnecessary for DNA repair [18]. Recent studies have implicated Ku70 as an important regulator of Bax activity. Ku70 binds and sequesters Bax in the cytosol, but releases Bax following apoptotic stimuli [14,61]. In addition, previous investigators have shown that ubiquitin-mediated Ku70 degradation induces Bax-dependent cell death [21], and pentapeptides corresponding to the Ku70 Bax-binding domain suppress cytotoxin- and polyglutamine-induced cell death [35,71]. However, the potential for DNA-PKcs to regulate Ku70-Bax interactions has not been explored. To explain the requirement for DNA-PKcs in apoptosis and define a role for DNA-PKcs-mediated phosphorylation of Ku70, we hypothesized that DNA-PKcs phosphorylates Ku70 to mediate the release of Bax during apoptosis. To test this hypothesis, we examined the ability of kainic acid to induce DNA damage, alter Ku70-Bax interaction, and initiate Bax-dependent apoptosis and excitotoxic cell death in neuronal cultures derived from wildtype, DNA-PKcs heterozygous (*Prkdc*^{+/-}), and DNA-PKcs null (*Prkdc*^{-/-}) mice. We show that kainic acid treatment of murine cortical neurons results in rapid DNA damage and subsequent neuronal death. Bax gene deletion or Bax shRNA-mediated knockdown of Bax expression was neuroprotective for neurons homo- or heterozygous for wildtype *Prkdc*, but not neuroprotective for *Prkdc*^{-/-} neurons. Introduction of a functional *Prkdc* gene restored Bax shRNA-mediated neuroprotection in *Prkdc*^{-/-} neurons, indicating that DNA-PKcs is required for neurons to undergo Bax-dependent excitotoxic cell death. Transfection of wildtype neurons with a DNA-PKcsnon-phosphorylatable Ku70 (S6A/S51A) revealed Ku70 (S6A/S51A) to display an elevated basal level of Bax binding, as compared to wildtype Ku70, that was unaltered by excitotoxic stimulation. Also, kainic acid treatment induced translocation of a Bax-GFP fusion protein to the mitochondria in neurons transfected with wildtype Ku70, but not with Ku70 (S6A/S51A). Our findings suggest that DNA-PKcs links excitotoxic cell death to the pro-apoptotic Bax pathway by phosphorylating Ku70, resulting in Bax release from Ku70-Bax complexes, and permitting Bax to translocate to the mitochondrion and initiate cell death.

2. Results

2.1. Kainic acid treatment of cortical neurons results in rapid induction of DNA damage

In response to DNA damage, the p53-binding protein, 53BP1, rapidly forms nuclear foci comprised of 53BP1, γ -H2AX, and activated DNA-PKcs [8,46]. We utilized 53BP1 foci formation as a marker to determine whether kainic acid treatment results in DNA damage in cortical neurons. In wildtype and *Prkdc*^{-/-} neurons, 53BP1 displayed a diffuse nuclear staining pattern, with faint puncta occasionally observed at the nuclear periphery (Fig. 1 insets). Treatment with camptothecin, a strong inducer of DNA breaks, resulted in rapid formation of 53BP1 foci (Fig. 1). Similarly, within 30 min of excitotoxic injury induced by addition of kainic acid, 53BP1 formed nuclear foci with similar kinetics in both wildtype and *Prkdc*^{-/-} neurons (Fig. 1). The number of neurons displaying foci peaked by 1 h, and persisted through 4 h. However, by 8 h the number of neurons displaying foci was significantly decreased (not shown). These results suggest that excitotoxic insult rapidly induces DNA damage. Since significant increases in nuclear pyknosis are not observed at these times, these results further suggest both that foci formation is transient and that DNA-PKcs is not required for either the appearance or disappearance of foci.

2.2. DNA-PKcs is required for Bax-dependent excitotoxic cell death

We previously demonstrated that kainate-induced excitotoxic death of wildtype, *Prkdc*^{+/-}, and *Prkdc*^{-/-} cortical neurons involves p53 and increased mitochondrial membrane permeability [43], processes that are linked by Bax [43,45]. Using hippocampal cultures from Bax knockout mice, prior investigators have demonstrated that Bax deletion is neuroprotective [68]. To determine whether *Prkdc*^{-/-} neurons display a similar requirement for Bax, we initially utilized RNA interference to knock down Bax expression. To quantify the ability of the Bax shRNA to knock down Bax expression, we co-transfected Hela cells with plasmids encoding murine Bax- α and β -galactosidase along with either the Bax shRNA, the parental mU6pro vector, or a scrambled Bax shRNA M3 vector. Three days after transfection, cells were lysed, and murine Bax expression was quantified by Western blot, using an antibody specific for the murine form of Bax. Following normalization to β -galactosidase levels, Bax shRNA was found to reduce murine Bax expression by $78 \pm 1.6\%$ of the mU6pro vector control (Fig. 2A,B). In comparison, Bax levels in the Bax shRNA M3-transfected cultures were not significantly different from that of the mU6pro control ($121 \pm 27.8\%$) (Fig. 2A,B). Therefore, Bax shRNA decreased Bax expression by approximately 78%. Due to a lack of specificity of the different Bax antibodies we examined, we were unable to perform immunocytochemistry to examine Bax down-regulation on an individual cell basis. Instead, we co-transfected murine Bax-EGFP fusion protein and RFP plasmids along with either Bax shRNA or Bax shRNA M3, as a control, and examined knockdown of EGFP fluorescence in RFP-positive neurons. Under our conditions, $>80\%$ of Bax shRNA co-transfected neurons were EGFP-negative (Fig. 2A'-C'). In contrast, $<10\%$ of the neurons that were co-transfected with the Bax shRNA M3 control were EGFP-negative (Fig. 2D'-F'). Therefore Bax shRNA knocks down Bax expression, whereas the scrambled Bax shRNA M3 does not.

To determine whether DNA-PKcs is involved in regulating Bax, we next asked whether Bax shRNA-mediated neuroprotection differs in neurons with or without DNAPKcs. We quantified kainate-induced nuclear pyknosis in wildtype, *Prkdc*^{+/-}, and *Prkdc*^{-/-} neurons co-transfected with either Bax shRNA or the empty mU6 vector (Fig. 3A). In neurons transfected with the mU6 vector, levels of kainate-induced cell death were essentially equivalent in wildtype and *Prkdc*^{+/-} neurons (wildtype pyknosis $14.6 \pm 2.9\%$ vs. *Prkdc*^{+/-} pyknosis $15.5 \pm 1.8\%$). Similar levels of kainate-induced toxicity have been previously observed by other investigators [39]. Transfection of wildtype or *Prkdc*^{+/-} neurons with Bax shRNA significantly increased survival under excitotoxic conditions (wildtype pyknosis $9.2 \pm 2.6\%$; *Prkdc*^{+/-} pyknosis $8.0 \pm 1.4\%$)

(Fig. 3A). In contrast, Bax shRNA had little effect on kainate-mediated excitotoxic cell death of *Prkdc*^{-/-} neurons; control vector-transfected neurons exhibited $20.7 \pm 4.4\%$ pyknosis compared to $17.6 \pm 3.4\%$ pyknosis in neurons transfected with Bax shRNA (Fig. 3A). Thus, Bax knockdown reduced neuronal death on average by ~51% and 44% in wildtype and *Prkdc*^{+/-} neurons, respectively, consistent with prior reports using *Bax*^{-/-} neurons [68], but only by 15% in *Prkdc*^{-/-} neurons.

Kainate-induced cell death in neurons transfected with the mutated Bax shRNA M3 control was comparable to levels in mU6-transfected neurons (Fig. 3B), suggesting that the Bax shRNA-mediated neuroprotection we observed in the wildtype and *Prkdc*^{+/-} neurons was due specifically to Bax knockdown.

Western blot analysis of whole cell extracts from untransfected neurons revealed that Bax levels did not differ between genotypes and remained unchanged by kainate treatment (not shown). Therefore, failure to rescue *Prkdc*^{-/-} neurons by Bax knockdown was not due to a lack of Bax expression in the cells. Additionally, transfection of an mU6 promoter-driven doublecortin shRNA [3] in *Prkdc*^{+/-} vs. *Prkdc*^{-/-} neurons knocked down doublecortin expression in greater than 90% of the neurons, and the extent of knockdown was equivalent in both genotypes, demonstrating function of the shRNA vector in *Prkdc*^{-/-} neurons (Supplemental Figure 1). Therefore, the fact that robust RNAi-mediated knockdown of Bax does not reduce excitotoxic cell death in *Prkdc*^{-/-} neurons suggests that these neurons utilize a Bax-independent cell death pathway.

To independently confirm that DNA-PKcs is required for Bax-mediated excitotoxic cell death, we compared excitotoxicity in cortical cultures derived from double knockout mice deficient for both DNA-PKcs and Bax. *Prkdc*^{+/-}/*Bax*^{-/-} neurons exhibited lower cell death ($13 \pm 2.8\%$) after kainate treatment, compared to *Prkdc*^{+/-}/*Bax*^{+/-} ($24.6 \pm 2.3\%$) (Fig. 3C), comparable to our findings with Bax knockdown in the *Prkdc*^{+/-} neurons. However, as shown in Figure 3D, Bax deletion in *Prkdc*^{-/-} neurons did not enhance survival following excitotoxic insult (*Prkdc*^{-/-}/*Bax*^{+/-} = $18.9 \pm 1.3\%$ pyknosis; *Prkdc*^{-/-}/*Bax*^{-/-} = $18.8 \pm 2.6\%$ pyknosis). These experiments confirm that DNA-PKcs mediates Bax-dependent excitotoxic cell death.

2.3. Lack of Bax shRNA-mediated rescue from excitotoxicity in *Prkdc*^{-/-} neurons is due to loss of DNA-PKcs activity

To determine whether the lack of Bax-dependent cell death in *Prkdc*^{-/-} neurons is due directly to a loss of DNA-PKcs activity, we next asked whether restoration of DNA-PKcs expression would confer Bax-dependent cell death. Triple transfections were performed in which a DNA-PKcs expression vector (or CMV promoter-driven *lacZ* vector control) was co-transfected with the Bax shRNA plasmid (or mU6 vector control), along with an EGFP gene, into *Prkdc*^{-/-} neurons. Kainate induced similar levels of neuronal death when the control CMV vector was co-transfected with either the parental mU6 vector ($26.7 \pm 2.4\%$ pyknosis) or Bax shRNA ($26.0 \pm 2.6\%$ pyknosis), confirming that Bax shRNA did not rescue *Prkdc*^{-/-} neurons from excitotoxicity (Fig. 4). Importantly, co-transfection of Bax shRNA with the DNA-PKcs gene, into *Prkdc*^{-/-} neurons, restored Bax shRNA-mediated neuroprotection by ~31.2% ($25.5 \pm 2.2\%$ pyknosis in the mU6 vector transfection vs. $17.6 \pm 2.0\%$ pyknosis in the Bax shRNA transfection, $p < 0.001$) (Fig. 4). This shows that Bax shRNA-mediated neuroprotection from kainate-induced excitotoxicity requires DNA-PKcs activity, consistent with a model in which DNA-PKcs regulates Bax-dependent excitotoxic cell death.

2.4. DNA-PKcs-mediated phosphorylation of Ku70 regulates Ku70-Bax interaction

DNA-PKcs is a serine-threonine kinase that phosphorylates Ku70 on serines 6 and 51 [10, 30]. Our findings led us to hypothesize that DNA-PKcs regulates the ability of Bax to induce

cell death by phosphorylating Ku70. To test this, we initially examined Bax binding to Ku70 in wildtype vs. *Prkdc*^{-/-} neurons following kainate addition, by probing Ku70 immunoprecipitates for Bax. Surprisingly, in wildtype cultures transfected with Ku70, kainate treatment increased Bax binding to Ku70 nearly 4-fold (3.7 ± 1.3 fold), when examined at 24 h following kainate addition (Fig. 5A). In contrast, kainate treatment did not significantly change the level of Bax bound to Ku70 (S6A/S51A), a DNA-PKcs-non-phosphorylatable mutant (treated/untreated = 1.4 ± 0.2) (Fig. 5A), suggesting that DNA-PKcs-mediated phosphorylation is critical in regulating Ku70-Bax interaction. However, basal levels of Bax bound to Ku70 (S6A/S51A) were elevated, comparable to that observed with wildtype Ku70 following kainate addition (Fig. 5A). These results suggest that when phosphorylation of Ku70 by DNA-PKcs is prevented, Ku70 binding to Bax is enhanced, suggesting that DNA-PKcs-mediated phosphorylation of Ku70-Bax complexes causes Bax release and translocation to the mitochondria to initiate Bax-mediated apoptosis. This is supported by the converse experiment where kainate treatment did not alter Ku70-Bax interaction in *Prkdc*^{-/-} neurons transfected with wildtype Ku70 (Fig. 5B).

2.5. Inhibition of kainic acid-induced Bax-EGFP translocation by Ku70 (S6A/S51A)

To directly demonstrate DNA-PKcs-mediated Ku70 phosphorylation in regulating Bax translocation, we co-transfected neurons with Bax-EGFP and either wildtype Ku70 or Ku70 (S6A/S51A). Following transfection, we treated neurons with kainic acid and examined the translocation of Bax-EGFP at 0, 1, 4, and 8 h post-treatment. In untreated neurons, Bax-EGFP displayed a diffuse distribution throughout the cell (Fig. 6A). By 4 h and persisting through 8 h following kainate addition, Bax-EGFP was found to accumulate in the cytosol and co-localize with cytochrome oxidase, a mitochondrial marker (Fig. 6). Similar translocation of an EGFP-Bax fusion protein has been observed following induction of apoptosis in other cell types [66]. In contrast, in kainate-treated neurons transfected with Ku70 (S6A/S51A), Bax-EGFP maintained a diffuse distribution throughout the neuron at all times, consistent with an inability of Ku70 (S6A/S51A) to release Bax. These results suggest that during excitotoxic cell death in cortical neurons, DNA-PKcs-mediated phosphorylation of Ku70 at serines 6 and/or 51 is required to release Bax and allow Bax to translocate to the mitochondria.

3. Discussion

The present study provides a mechanistic link between the DNA-dependent protein kinase catalytic subunit and the pro-death Bax pathway during excitotoxicity. DNA damage results in rapid phosphorylation of histone H2AX, to form γ -H2AX, that recruits activated DNA-PKcs and 53BP1 to form nuclear foci, at sites of DNA double-strand breaks, and initiate DNA repair [8,46]. Prior investigators have demonstrated rapid γ -H2AX nuclear foci formation following glutamate addition to cortical neurons [16]. We demonstrate rapid incorporation of 53BP1 into nuclear foci following kainate exposure. The number of neurons displaying 53BP1 foci dramatically increased by 30 min, peaked at 1 h, and persisted through 4 h, consistent with a rapid induction of DNA damage and activation of DNA-PKcs. The number and percentage of neurons displaying DNA damage declined substantially by 8 h following kainate addition (not shown), which precedes or coincides with the time at which kainate-induced nuclear pyknosis was previously found to become detectable [43]. These results suggest that DNA damage is an early event in kainate-induced excitotoxicity, and is consistent with our previous results indicating that DNA damage is a trigger for excitotoxic cell death [43]. That 53BP1 foci can form in *Prkdc*^{-/-} neurons also indicates that DNA-PKcs activity is not required for 53BP1 foci formation.

Bax shRNA-mediated neuroprotection from excitotoxin-triggered neuronal death was greatly reduced in the absence of DNA-PKcs, but restored by expression of DNA-PKcs in *Prkdc*^{-/-}

neurons. Bax deletion reduced kainate-induced excitotoxic cell death in wildtype and *Prkdc*^{+/-} neurons, but not in *Prkdc*^{-/-} neurons, indicating that DNA-PKcs regulates Bax-dependent excitotoxic cell death. Based on our previous finding that p53 is involved in excitotoxic cell death of both wildtype and *Prkdc*^{-/-} neurons [43], we assert that excitotoxic cell death in *Prkdc*^{-/-} cortical neurons is p53-dependent, but Bax-independent. Possibilities include activation of a p53-dependent extrinsic pathway leading to activation of caspase-3 [62], or through pathways involving poly (ADP-ribose) polymerase and apoptosis-inducing factor, which have been linked to caspase-independent forms of neuronal death [5,13,15]. Favoring the former possibility, we have observed caspase-3 activation following kainate addition in both wildtype and *Prkdc*^{-/-} neurons (Supplemental Fig. 2).

A prior investigation has reported decreased Bax levels in the M059J glioblastoma cell line, lacking DNA-PKcs expression, as compared with its isogenic M059K control [12]. We have not observed differences in Bax levels, by Western blot analysis, in either murine cortices or in cultured cortical neurons, regardless of DNA-PKcs status, consistent with others [28]. Therefore, the requirement for DNA-PKcs in Bax-dependent excitotoxic cell death led us to hypothesize that DNA-PKcs regulates a Bax-dependent pro-apoptotic pathway by phosphorylating Ku70, a known target of DNA-PKcs, to cause release of Bax from Ku70-Bax complexes. We supported this model by four lines of evidence: 1) kainic acid treatment induced rapid nuclear 53BP1 foci formation, indicative of DNA damage and DNA-PK activation, 2) DNA-PKcs nonphosphorylatable Ku70 (S6A/S51A) bound more Bax than wildtype Ku70, 3) Ku70 (S6A/S51A) occluded kainic acid-induced alterations in Ku70-Bax interaction, and 4) transfection of Ku70 (S6A/S51A) blocked kainate-induced translocation of a Bax-GFP fusion protein to the mitochondria. To explain the increase in binding of Bax to wildtype Ku70, observed at 24 h after kainate addition (Fig. 5A), it should be noted that loss of DNA-PKcs activity can occur during excitotoxic cell death, including ischemic models, where DNA-PKcs is rapidly cleaved and inactivated following injury [11,23,38,53,59]. DNA-PKcs cleavage is mediated by caspase-3/CPP32 [23,38,58], and we have shown caspase-3 to be dramatically elevated at 24 h after kainate addition to either wildtype or *Prkdc*^{-/-} cortical neurons (Supplementary Fig. 2). In addition, DNA-PKcs has been shown to self-inactivate by autophosphorylation on threonine 3950 following DNA damage [9,17,37,38]. Indeed, this self-inactivation is thought to be required for the DNA-PK complex to release the tethered DNA ends and allow NHEJ to proceed [64]. Therefore, the increased interaction between Ku70 and Bax, observed at late times after kainate addition, is not a consequence of DNA-PKcs activity, but rather results from DNA-PKcs degradation or inactivation, enabling dephosphorylation of Ku70 and a concomitant increase in Bax binding. Thus down-regulation of DNA-PKcs levels could represent a feedback mechanism to prevent excessive Bax release under certain conditions.

Phosphorylation of Ku70 by DNA-PKcs to release Bax from Ku70-Bax complexes defines a previously unknown role for DNA-PKcs-mediated phosphorylation of Ku70. Interestingly, PCAF- and CBP-mediated acetylation of Ku70, on lysines 539 and 542, has been implicated in Bax dissociation from Ku70 [14,35]. Our results raise the intriguing possibility that phosphorylation of Ku70 could create a docking site for acetylases in a manner similar to that described for the CREB transcription factor, which binds CBP/p300 acetylases following phosphorylation by protein kinase A [48].

Excitotoxic cell death is a fundamental mechanism underlying a variety of neurodegenerative disorders, including epilepsy, stroke, Parkinson's and Alzheimer's diseases, and amyotrophic lateral sclerosis [1,19,24,27,49]. DNA double-strand breaks, which activate the NHEJ pathway, are the most lethal form of DNA damage and have been observed in several neurodegenerative disorders, including Alzheimer's and Huntington's diseases [26,52]. In contrast to single-strand repair processes, activation of NHEJ leads to cell cycle arrest and

apoptosis [20,42]. However, studies indicate that although excitotoxic in nature, the mode of cell death in neurodegenerative disease exists as a continuum between apoptosis and pathological cell death, the latter being a major contributor to persistent and severe pathological tissue damage [25,57]. Results presented here suggest that DNA-PKcs plays a critical role in the decision to favor apoptotic over more pathological forms of cell death following injury. Consequently, deficits in NHEJ, observed in aging and Alzheimer's disease [51,52,54], could play a prominent role in age-dependent and disease-related neuropathology. Future studies delineating the mechanisms that regulate DNA-PKcs activity to enhance pro-apoptotic forms of cell death can be expected to have a major impact on neurodegenerative disease.

4. Experimental procedure

4.1. Animals

Prkdc^{+/-} mice (*Prkdc*^{tm1Fwa}), on a 129-6J-background strain, have been previously described [22,43], and were the generous gift of Dr. Frederick Alt (Harvard Medical School). Colonies were maintained as a heterozygous stock. Wildtype C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) or Harlan Sprague Dawley Inc. (Indianapolis, IN). Bax heterozygous (*Bax*^{tm1Sjk}) mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Prkdc*/*Bax* mice on a C57Bl/6J background strain were generated by crossing *Prkdc*^{-/-} mice to *Bax*^{+/-} mice. Mice were bred in the Wesleyan University animal facility in a pathogen-free environment. All procedures and methods followed an established protocol that was approved by the Wesleyan University Institutional Animal Care and Use Committee, and conformed to guidelines established in the NIH Guide for the Care and Use of Laboratory Animals. Mice were genotyped as described previously [22] or as suggested by Jackson Laboratories.

4.2. Plasmids

A Bax shRNA vector was engineered by inserting complementary oligonucleotides encoding murine Bax shRNA into the mU6pro vector (David Turner, University of Michigan) [72]. The Bax shRNA sequence inserted into mU6pro was 5'-AATTGGAGATGAACTGGACAG-3'. As a control, a scrambled Bax shRNA, denoted Bax shRNA M3, of sequence 5'-AATTGGAGTTGTACAGGATAG-3', was inserted into the mU6pro vector.

To test shRNA efficiency, Bax shRNA (or control vector) was co-transfected with pORF5-mBax- α (Invitrogen, San Diego, CA) and a CMV promoter-driven *lacZ* gene (pSLICK-Z) [36] (Bax shRNA:pORF-mBax- α :pSLICK-Z at a 3:1:1 ratio) into HeLa cells. At 3 d following transfection, cultures were lysed and extracts were subjected to Western blot analysis for murine Bax and normalized to β -galactosidase. The murine doublecortin shRNA plasmid, mU6pro-DCX30TRhp (Joseph LoTurco, University of Connecticut), and plasmids encoding a CMV promoter-driven full-length DNA-PKcs gene (Kathryn Meek, Michigan State University) and murine Bax-green fluorescent protein (Bax-EGFP) (Apurva Sarin, National Center for Biological Sciences, India) fusion genes, as well as EF1 α promoter-driven, V5 epitope-tagged Ku70, Ku70 (S6A/S51A), and Ku70 (S6D/S51D) (Kathryn Meek) were previously described and were generous gifts of the indicated investigators [3,18,44,56]. To ensure that translational initiation of wildtype and mutant Ku70 occurred at the correct start codon [32], we inserted a purine at the -3 position from the start codon by PCR, using the high-fidelity ProofStart DNA polymerase (Qiagen, Valencia, CA), and forward (5'-TCGGATCCACCATGTCAGGGTGGGAG-3') and reverse (5'-TAGAATTCGTTTTGCACCTGG-3') Ku70 primers, and inserting the 352 bp BamHI/EcoRI-restricted PCR product into the corresponding BamHI/EcoRI region in the original vector. All plasmids were sequenced through the primer junctions. An EF1 α promoter-driven enhanced green fluorescent protein gene (pEF-EGFP) was engineered by inserting the EGFP-

encoding 734 bp Nco1/Xba1 fragment of pEGFP-N1 (Clontech, Palo Alto, CA) into the 4.6 kb Nco1/Xba1 fragment of pEFm-B-Raf.6 [36].

4.3. Cell Culture

Timed pregnancies were scored by day of mating. The day that a plug was found was designated Embryonic Day 0.5 (E0.5). Embryos were obtained from timed-pregnancies in which breeding pairs were housed together for a 24-hour period. Pregnant dams were euthanized by CO₂ exposure, followed by cervical dislocation. Cortical-hippocampal cultures were derived from E14.5 *Prkdc*^{-/-} mice and heterozygous littermates as previously described [43]. Dissociated cells were plated at a density of 5.5×10^5 cells/ml in Neurobasal medium containing B27 supplement, 0.5mM L-glutamine (GIBCO-BRL, Grand Island, NY), 0.025mM L-glutamic acid, 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), and 50 U/ml penicillin-streptomycin (MediaTech, Inc., Herndon, VA). Cultures were fed with glutamate and serum-free medium on the day following plating (DIV 1) and DIV 4. Cultures were maintained at 37°C under 5% CO₂ in a humidified incubator.

4.4. Transfections and Quantification of Excitotoxic Cell Death

To quantify cell death, pyknosis was scored. Transfections were performed in 4-well Permanox chamber slides (Nunc, Rochester, New York) with the addition of 1.6 µg total DNA/ml. The Bax shRNA, mU6pro-DCX30TRhp, or the mU6pro control vector was co-transfected with a human EF1 promoter-driven EGFP at a 2:1 ratio, using Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's instructions. For triple transfections, EGFP, DNA-PKcs, and Bax shRNA were transfected at a 1:4:2 ratio. Transfection reagents were removed after 5 h and cultures were fed with conditioned medium. All transfections were performed on DIV 4. Three days following transfection (DIV 7), kainic acid (Ocean Produce International, Shelburne, Nova Scotia, Canada) was added directly to the culture medium to a final concentration of 100 µM. For quantification of cell death, cells were fixed, at 24 h following kainate addition, in warmed 4% paraformaldehyde in 0.1M phosphate buffer, rinsed, and stained by immunocytochemistry for EGFP and MAP2, and counterstained with Hoechst 33342. Neuronal death was quantified by an investigator blind to genotype, who scored the percentage of pyknotic neurons that were EGFP and MAP2-positive. For Bax-EGFP translocation, cells were fixed at 0, 1, 4, and 8 h following kainate addition, then stained for cytochrome oxidase. Transfections of primary cortical cultures for immunoprecipitation experiments were performed in 60 mm cell culture dishes (Corning, Oneonta, NY) on DIV 7 with 8 µg DNA total of Ku70 and Bax at a Ku70:Bax ratio of 23:1. At no time were differences in transfection efficiency observed between wildtype and *Prkdc*^{-/-} neurons.

4.5. Immunocytochemistry

Non-specific staining was blocked by preincubation in 5% normal goat serum (Vector Laboratories Inc., Burlingame, CA) containing 0.1% Triton X-100 in PBS. Cells were incubated overnight in primary antibodies, including Alexa 488-conjugated rabbit anti-GFP (1:1000; Molecular Probes, Eugene, OR), mouse anti-MAP2 (1:1000, Sigma-Aldrich), cytochrome oxidase (1:50, Molecular Probes), or guinea pig anti-doublecortin (1:1000, Chemicon, Temecula, CA). A fluorescent secondary antibody of either Alexa 546-conjugated goat anti-guinea pig IgG or Alexa 568-conjugated goat anti-mouse IgG (1:1000; Molecular Probes) was applied for 1 h at room temperature. Sections were counterstained with Hoechst 33342 (1 g/ml; Molecular Probes), then mounted in Vectashield medium for quantification (Vector Laboratories Inc.). The 53BP1 antibodies were the generous gift of Dr. Junjie Chen (Yale University) and immunocytochemistry was performed as previously described with modifications [46]. Briefly, cultures were fixed and then permeabilized with ethanol/acetic acid (2:1) at -20°C for 5 min. Cultures were then incubated with rabbit anti-53BP1 (1:1500)

at 37°C for 20 min, followed by incubation with Alexa 488-conjugated goat anti-rabbit IgG secondary antibody (1:1000, Molecular Probes).

4.6. Quantification of Fluorescent Labeling, Statistics and Photography

Stained cells were examined under epifluorescent wavelengths with a Zeiss Axiovert fluorescence microscope or a Zeiss LSM 510 metaconfocal microscope. The percentage of pyknotic nuclei was quantified as previously described [43]. An observer, blind to the genotype of the cells, counted at least 200 neurons per treatment.

All statistics and graphs were generated in Microsoft Excel. P values were calculated using Student's *t*-tests. Figures were created with Adobe Photoshop software with minor changes to brightness and contrast.

4.7. Immunoprecipitation and Western Blot Analysis

Kainic acid was added directly to the culture medium to a final concentration of 100 M, and cultures were incubated an additional 24 h. For immunoprecipitation of Ku70 and Bax, transfected neurons were lysed using detergent-free hypotonic buffer (PBS containing 5 mM sodium chloride at pH 7.4) [50]. Briefly, after clearing 1 ml of samples with 50 µl Protein G-Sepharose (Sigma-Aldrich), immunoprecipitates were incubated overnight at 4°C with 2 µg murine anti-V5 antibody (Invitrogen). Immunocomplexes were precipitated with 50 µl of Protein G-Sepharose followed by extensive washing in the buffer. Beads were boiled in 50 µl Laemmli buffer [33] and proteins were separated on 4-20% SDS-PAGE gels prior to immunoblotting as previously described [36]. Mouse anti-murine Bax (5B7) (1:1000, Sigma-Aldrich) antibodies were used to detect Bax bound by Ku70. Goat anti-Ku70 (1:1000, Santa Cruz, Santa Cruz, CA) was used to detect Ku70 by Western blot. Protein levels on Western blots were imaged using a Syngene G:Box and quantified using GeneTools analysis software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

NIH RO1 NS42826 and a McKnight Brain Disorders Award to JRN, and funds provided by Wesleyan University (SLL) supported this research. We thank members of the Naegele lab, Paul Lombroso, and Marina Picciotto for helpful comments on the manuscript. Drs. Kathryn Meek (Michigan State University), David Turner (University of Michigan), and Joseph J. LoTurco (University of Connecticut) generously provided plasmids. Dr. Junjie Chen (Yale) provided the 53BP1 antibodies. We also thank Ron Gordon, Sera Brown, and Angela Lentini of the Wesleyan Animal Facility.

Abbreviations

DIV, days in vitro; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; shRNA, short-hairpin RNA; EGFP, enhanced green fluorescent protein; NHEJ, non-homologous end joining; RFP, red fluorescent protein; MAP2, microtubule-associated protein 2.

REFERENCES

1. Alexi T, Borlongan CV, Faull RL, Williams CE, Clark RG, Gluckman PD, Hughes PE. Neuroprotective strategies for basal ganglia degeneration: Parkinson's and Huntington's diseases. *Prog Neurobiol* 2000;60:409–470. [PubMed: 10697073]
2. Araki R, Fukumura R, Fujimori A, Taya Y, Shiloh Y, Kurimasa A, Burma S, Li GC, Chen DJ, Sato K, Hoki Y, Tatsumi K, Abe M. Enhanced phosphorylation of p53 serine 18 following DNA damage

- in DNA-dependent protein kinase catalytic subunit-deficient cells. *Cancer Res* 1999;59:3543–3546. [PubMed: 10446957]
3. Bai J, Ramos RL, Ackman JB, Thomas AM, Lee RV, LoTurco JJ. RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat Neurosci* 2003;6:1277–1283. [PubMed: 14625554]
 4. Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 1998;281:1674–1677. [PubMed: 9733514]
 5. Bredesen DE. Key note lecture: toward a mechanistic taxonomy for cell death programs. *Stroke* 2007;38:652–660. [PubMed: 17261709]
 6. Brooks PJ. DNA repair in neural cells: basic science and clinical implications. *Mutat Res* 2002;509:93–108. [PubMed: 12427533]
 7. Chakravarthy BR, Walker T, Rasquinha I, Hill IE, MacManus JP. Activation of DNA-dependent protein kinase may play a role in apoptosis of human neuroblastoma cells. *J Neurochem* 1999;72:933–942. [PubMed: 10037464]
 8. Chan DW, Chen BP, Prithivirajasingh S, Kurimasa A, Story MD, Qin J, Chen DJ. Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. *Genes Dev* 2002;16:2333–2338. [PubMed: 12231622]
 9. Chan DW, Lees-Miller SP. The DNA-dependent protein kinase is inactivated by autophosphorylation of the catalytic subunit. *J Biol Chem* 1996;271:8936–8941. [PubMed: 8621537]
 10. Chan DW, Ye R, Veillette CJ, Lees-Miller SP. DNA-dependent protein kinase phosphorylation sites in Ku 70/80 heterodimer. *Biochemistry* 1999;38:1819–1828. [PubMed: 10026262]
 11. Chechlacz M, Vemuri MC, Naegele JR. Role of DNA-dependent protein kinase in neuronal survival. *J Neurochem* 2001;78:141–154. [PubMed: 11432981]
 12. Chen GG, Sin FL, Leung BC, Ng HK, Poon WS. Glioblastoma cells deficient in DNA-dependent protein kinase are resistant to cell death. *J Cell Physiol* 2005;203:127–132. [PubMed: 15493013]
 13. Cheung EC, Melanson-Drapeau L, Cregan SP, Vanderluit JL, Ferguson KL, McIntosh WC, Park DS, Bennett SA, Slack RS. Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. *J Neurosci* 2005;25:1324–1334. [PubMed: 15703386]
 14. Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C, Frye R, Ploegh H, Kessler BM, Sinclair DA. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* 2004;13:627–638. [PubMed: 15023334]
 15. Cregan SP, Fortin A, MacLaurin JG, Callaghan SM, Cecconi F, Yu SW, Dawson TM, Dawson VL, Park DS, Kroemer G, Slack RS. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J Cell Biol* 2002;158:507–517. [PubMed: 12147675]
 16. Crowe SL, Movsesyan VA, Jorgensen TJ, Kondratyev A. Rapid phosphorylation of histone H2A.X following ionotropic glutamate receptor activation. *Eur J Neurosci* 2006;23:2351–2361. [PubMed: 16706843]
 17. Douglas P, Cui X, Block WD, Yu Y, Gupta S, Ding Q, Ye R, Morrice N, Lees-Miller SP, Meek K. The DNA-dependent protein kinase catalytic subunit is phosphorylated in vivo on threonine 3950, a highly conserved amino acid in the protein kinase domain. *Mol Cell Biol* 2007;27:1581–1591. [PubMed: 17158925]
 18. Douglas P, Gupta S, Morrice N, Meek K, Lees-Miller SP. DNA-PK-dependent phosphorylation of Ku70/80 is not required for non-homologous end joining. *DNA Repair (Amst)* 2005;4:1006–1018. [PubMed: 15941674]
 19. Emerit J, Edeas M, Bricaire F. Neurodegenerative diseases and oxidative stress. *Biomed Pharmacother* 2004;58:39–46. [PubMed: 14739060]
 20. Frappart PO, McKinnon PJ. Mouse models of DNA double-strand break repair and neurological disease. *DNA Repair (Amst)* 2008;7:1051–1060. [PubMed: 18458002]
 21. Gama V, Gomez JA, Mayo LD, Jackson MW, Danielpour D, Song K, Haas AL, Laughlin MJ, Matsuyama S. Hdm2 is a ubiquitin ligase of Ku70-Akt promotes cell survival by inhibiting Hdm2-dependent Ku70 destabilization. *Cell Death Differ* 2009;16:758–769. [PubMed: 19247369]

22. Gao Y, Chaudhuri J, Zhu C, Davidson L, Weaver DT, Alt FW. A targeted DNA-PKcs-null mutation reveals DNA-PK-independent functions for KU in V(D)J recombination. *Immunity* 1998;9:367–376. [PubMed: 9768756]
23. Han Z, Malik N, Carter T, Reeves WH, Wyche JH, Hendrickson EA. DNA-dependent protein kinase is a target for a CPP32-like apoptotic protease. *J Biol Chem* 1996;271:25035–25040. [PubMed: 8798786]
24. Heath PR, Shaw PJ. Update on the glutamatergic neurotransmitter system and the role of excitotoxicity in amyotrophic lateral sclerosis. *Muscle Nerve* 2002;26:438–458. [PubMed: 12362409]
25. Hou ST, MacManus JP. Molecular mechanisms of cerebral ischemia-induced neuronal death. *Int Rev Cytol* 2002;221:93–148. [PubMed: 12455747]
26. Illuzzi J, Yerkes S, Parekh-Olmedo H, Kmiec EB. DNA breakage and induction of DNA damage response proteins precede the appearance of visible mutant huntingtin aggregates. *J Neurosci Res* 2009;87:733–747. [PubMed: 18831068]
27. Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol* 2003;53(Suppl 3):S26–36. [PubMed: 12666096]discussion S36–28
28. Jhappan C, Yusufzai TM, Anderson S, Anver MR, Merlino G. The p53 response to DNA damage in vivo is independent of DNA-dependent protein kinase. *Mol Cell Biol* 2000;20:4075–4083. [PubMed: 10805749]
29. Jimenez GS, Bryntesson F, Torres-Arzayus MI, Priestley A, Beeche M, Saito S, Sakaguchi K, Appella E, Jeggo PA, Taccioli GE, Wahl GM, Hubank M. DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage. *Nature* 1999;400:81–83. [PubMed: 10403253]
30. Jin S, Weaver DT. Double-strand break repair by Ku70 requires heterodimerization with Ku80 and DNA binding functions. *Embo J* 1997;16:6874–6885. [PubMed: 9362500]
31. Jordan J, Galindo MF, Prehn JH, Weichselbaum RR, Beckett M, Ghadge GD, Roos RP, Leiden JM, Miller RJ. p53 expression induces apoptosis in hippocampal pyramidal neuron cultures. *J Neurosci* 1997;17:1397–1405. [PubMed: 9006981]
32. Kozak M. Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* 2005;361:13–37. [PubMed: 16213112]
33. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
34. Lees-Miller SP, Meek K. Repair of DNA double strand breaks by nonhomologous end joining. *Biochimie* 2003;85:1161–1173. [PubMed: 14726021]
35. Li Y, Yokota T, Gama V, Yoshida T, Gomez JA, Ishikawa K, Sasaguri H, Cohen HY, Sinclair DA, Mizusawa H, Matsuyama S. Bax-inhibiting peptide protects cells from polyglutamine toxicity caused by Ku70 acetylation. *Cell Death Differ*. 2007
36. Lin SL, Le TX, Cowen DS. SptP, a *Salmonella typhimurium* type III-secreted protein, inhibits the mitogen-activated protein kinase pathway by inhibiting Raf activation. *Cell Microbiol* 2003;5:267–275. [PubMed: 12675684]
37. Mandal M, Adam L, Kumar R. Redistribution of activated caspase-3 to the nucleus during butyric acid-induced apoptosis. *Biochem Biophys Res Commun* 1999;260:775–780. [PubMed: 10403841]
38. McConnell KR, Dynan WS, Hardin JA. The DNA-dependent protein kinase catalytic subunit (p460) is cleaved during Fas-mediated apoptosis in Jurkat cells. *J Immunol* 1997;158:2083–2089. [PubMed: 9036952]
39. McDonald JW, Bhattacharyya T, Sensi SL, Lobner D, Ying HS, Canzoniero LM, Choi DW. Extracellular acidity potentiates AMPA receptor-mediated cortical neuronal death. *J Neurosci* 1998;18:6290–6299. [PubMed: 9698321]
40. McKinnon PJ, Caldecott KW. DNA strand break repair and human genetic disease. *Annu Rev Genomics Hum Genet* 2007;8:37–55. [PubMed: 17887919]
41. Mukherjee B, Kessinger C, Kobayashi J, Chen BP, Chen DJ, Chatterjee A, Burma S. DNA-PK phosphorylates histone H2AX during apoptotic DNA fragmentation in mammalian cells. *DNA Repair (Amst)* 2006;5:575–590. [PubMed: 16567133]
42. Myers K, Gagou ME, Zuazua-Villar P, Rodriguez R, Meuth M. ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. *PLoS Genet* 2009;5:e1000324. [PubMed: 19119425]

43. Neema M, Navarro-Quiroga I, Chechlac M, Gilliams-Francis K, Liu J, Lamonica K, Lin SL, Naegle JR. DNA damage and nonhomologous end joining in excitotoxicity: neuroprotective role of DNA-PKcs in kainic acid-induced seizures. *Hippocampus* 2005;15:1057–1071. [PubMed: 16216017]
44. Parikh N, Sade H, Kurian L, Sarin A. The Bax N terminus is required for negative regulation by the mitogen-activated protein kinase kinase and Akt signaling pathways in T cells. *J Immunol* 2004;173:6220–6227. [PubMed: 15528359]
45. Polster BM, Fiskum G. Mitochondrial mechanisms of neural cell apoptosis. *J Neurochem* 2004;90:1281–1289. [PubMed: 15341512]
46. Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol* 2001;153:613–620. [PubMed: 11331310]
47. Rass U, Ahel I, West SC. Defective DNA repair and neurodegenerative disease. *Cell* 2007;130:991–1004. [PubMed: 17889645]
48. Ravnskjaer K, Kester H, Liu Y, Zhang X, Lee D, Yates JR 3rd, Montminy M. Cooperative interactions between CBP and TORC2 confer selectivity to CREB target gene expression. *Embo J* 2007;26:2880–2889. [PubMed: 17476304]
49. Rothman SM, Olney JW. Glutamate and the pathophysiology of hypoxic--ischemic brain damage. *Ann Neurol* 1986;19:105–111. [PubMed: 2421636]
50. Sawada M, Sun W, Hayes P, Leskov K, Boothman DA, Matsuyama S. Ku70 suppresses the apoptotic translocation of Bax to mitochondria. *Nat Cell Biol* 2003;5:320–329. [PubMed: 12652308]
51. Seluanov A, Danek J, Hause N, Gorbunova V. Changes in the level and distribution of Ku proteins during cellular senescence. *DNA Repair (Amst)* 2007;6:1740–1748. [PubMed: 17686666]
52. Shackelford DA. DNA end joining activity is reduced in Alzheimer's disease. *Neurobiol Aging* 2006;27:596–605. [PubMed: 15908050]
53. Shackelford DA, Tobaru T, Zhang S, Zivin JA. Changes in expression of the DNA repair protein complex DNA-dependent protein kinase after ischemia and reperfusion. *J Neurosci* 1999;19:4727–4738. [PubMed: 10366606]
54. Sharma S. Age-related nonhomologous end joining activity in rat neurons. *Brain Res Bull* 2007;73:48–54. [PubMed: 17499636]
55. Shieh SY, Ikeda M, Taya Y, Prives C. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* 1997;91:325–334. [PubMed: 9363941]
56. Shin EK, Rijkers T, Pastink A, Meek K. Analyses of TCRB rearrangements substantiate a profound deficit in recombination signal sequence joining in SCID foals: implications for the role of DNA-dependent protein kinase in V(D)J recombination. *J Immunol* 2000;164:1416–1424. [PubMed: 10640757]
57. Silva MT, do Vale A, dos Santos NM. Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. *Apoptosis* 2008;13:463–482. [PubMed: 18322800]
58. Song Q, Burrows SR, Smith G, Lees-Miller SP, Kumar S, Chan DW, Trapani JA, Alnemri E, Litwack G, Lu H, Moss DJ, Jackson S, Lavin MF. Interleukin-1 beta-converting enzyme-like protease cleaves DNA-dependent protein kinase in cytotoxic T cell killing. *J Exp Med* 1996;184:619–626. [PubMed: 8760815]
59. Song Q, Lees-Miller SP, Kumar S, Zhang Z, Chan DW, Smith GC, Jackson SP, Alnemri ES, Litwack G, Khanna KK, Lavin MF. DNA-dependent protein kinase catalytic subunit: a target for an ICE-like protease in apoptosis. *Embo J* 1996;15:3238–3246. [PubMed: 8670824]
60. Soubeyrand S, Schild-Poulter C, Hache RJ. Structured DNA promotes phosphorylation of p53 by DNA-dependent protein kinase at serine 9 and threonine 18. *Eur J Biochem* 2004;271:3776–3784. [PubMed: 15355354]
61. Subramanian C, Pipari AW Jr, Bian X, Castle VP, Kwok RP. Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. *Proc Natl Acad Sci U S A* 2005;102:4842–4847. [PubMed: 15778293]
62. Tun C, Guo W, Nguyen H, Yun B, Libby RT, Morrison RS, Garden GA. Activation of the extrinsic caspase pathway in cultured cortical neurons requires p53-mediated down-regulation of the X-linked inhibitor of apoptosis protein to induce apoptosis. *J Neurochem* 2007;102:1206–1219. [PubMed: 17488272]

63. Uberti D, Belloni M, Grilli M, Spano P, Memo M. Induction of tumour-suppressor phosphoprotein p53 in the apoptosis of cultured rat cerebellar neurones triggered by excitatory amino acids. *Eur J Neurosci* 1998;10:246–254. [PubMed: 9753133]
64. Uematsu N, Weterings E, Yano K, Morotomi-Yano K, Jakob B, Taucher-Scholz G, Mari PO, van Gent DC, Chen BP, Chen DJ. Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks. *J Cell Biol* 2007;177:219–229. [PubMed: 17438073]
65. Wang S, Guo M, Ouyang H, Li X, Cordon-Cardo C, Kurimasa A, Chen DJ, Fuks Z, Ling CC, Li GC. The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. *Proc Natl Acad Sci U S A* 2000;97:1584–1588. [PubMed: 10677503]
66. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997;139:1281–1292. [PubMed: 9382873]
67. Woo RA, Jack MT, Xu Y, Burma S, Chen DJ, Lee PW. DNA damage-induced apoptosis requires the DNA-dependent protein kinase, and is mediated by the latent population of p53. *Embo J* 2002;21:3000–3008. [PubMed: 12065413]
68. Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS. Bax involvement in p53-mediated neuronal cell death. *J Neurosci* 1998;18:1363–1373. [PubMed: 9454845]
69. Yang and K. Herrup Y. Loss of neuronal cell cycle control in ataxia-telangiectasia: a unified disease mechanism. *J Neurosci* 2005;25:2522–2529. [PubMed: 15758161]
70. Yee KS, Vousden KH. Complicating the complexity of p53. *Carcinogenesis* 2005;26:1317–1322. [PubMed: 15888490]
71. Yoshida T, Tomioka I, Nagahara T, Holyst T, Sawada M, Hayes P, Gama V, Okuno M, Chen Y, Abe Y, Kanouchi T, Sasada H, Wang D, Yokota T, Sato E, Matsuyama S. Bax-inhibiting peptide derived from mouse and rat Ku70. *Biochem Biophys Res Commun* 2004;321:961–966. [PubMed: 15358121]
72. Yu JY, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* 2002;99:6047–6052. [PubMed: 11972060]

Fig. 1. Kainic acid treatment causes rapid induction of 53BP1 foci formation in both wildtype and *Prkdc*^{-/-} neurons

Kainate (200 μ M final concentration) or camptothecin (10 μ M final concentration) was added directly to the culture medium. Cultures were fixed and stained for 53BP1 at the indicated times. Insets, staining of 53BP1 in untreated cultures. Upper panels, wildtype neurons; lower panels, *Prkdc*^{-/-} neurons. Staining was performed on two separate cultures per genotype. Scale bar=10 μ m.

Fig. 2. Bax shRNA-mediated knockdown of Bax expression

(A). Representative Western blot of Bax knockdown in HeLa cells co-transfected with plasmids encoding murine Bax, μ -galactosidase, and either mU6pro, Bax shRNA, or the scrambled Bax shRNA M3. (B) Bax levels in the Bax shRNA- and the Bax shRNA M3-transfected HeLa cell cultures were normalized to Bax levels in cultures transfected with mU6pro. Results show Bax shRNA M3 did not reduce Bax expression, whereas Bax shRNA reduced Bax expression by 80%. Data represent the means $\pm$ SE from three separate experiments. (C). Cortical neurons were co-transfected with plasmids encoding a Bax-EGFP fusion protein and either the Bax shRNA (A'-C') or Bax shRNA M3 (D'-F'). Bax shRNA co-transfected neurons were EGFP-negative, but Bax shRNA M3-cotransfected neurons were not.

Fig. 3. Bax shRNA mediated knockdown of Bax reduces excitotoxicity in wildtype and *Prkdc*^{+/-}, but not *Prkdc*^{-/-} neurons

(A) Wildtype, *Prkdc*^{+/-}, *Prkdc*^{-/-} cultures were transfected with either control mU6pro vector or Bax shRNA and treated with kainic acid. Bax shRNA reduced the percentage of pyknotic cells in wildtype and *Prkdc*^{+/-} cultures, but not *Prkdc*^{-/-} cultures. Black bars, *Prkdc*^{-/-} neurons (n=7); grey bars, *Prkdc*^{+/-} neurons (n=10); white bars, wildtype neurons (n=8). Data represent the means $\pm$ SE from three separate experiments. * $P < 0.05$, ** $p < 0.005$, *** $P < 0.0005$, determined by *t*-test. (B) Kainic acid treatment induced equivalent levels of pyknosis in primary cortical neurons transfected with mU6pro vs. Bax shRNA M3. White bars, mU6pro-transfected (n=4). Black bars, Bax shRNA M3-transfected (n=4). (C) Kainate-induced excitotoxic cell death was compared by quantifying pyknosis in heterozygous or double knockout neurons. White bar, *Prkdc*^{+/-}*Bax*^{+/-} neurons (n=4); light grey bars, *Prkdc*^{+/-}*Bax*^{-/-} neurons (n=3). These results show that Bax knockout is neuroprotective in heterozygous neurons expressing DNA-PKcs. * $P < 0.05$, determined by *t*-test. (D). Kainate-induced pyknosis was not affected in double knockout neurons deficient for both DNA-PKcs and Bax. Dark grey bar, *Prkdc*^{-/-}*Bax*^{+/-} neurons (n=7); black bars, *Prkdc*^{-/-}*Bax*^{-/-} neurons (n=5). These results show that Bax knockout does not reduce excitotoxicity in *Prkdc*^{-/-} neurons.

Fig. 4. Transfection of a wildtype DNA-PKcs gene into *Prkdc*^{-/-} neurons restores Bax shRNA-mediated rescue

Prkdc^{-/-} cultures were transfected with pEF-EGFP, Bax shRNA (or mU6 vector control, and a CMV promoter-driven full-length DNA-PKcs gene (or pSLICK-Z control). Transfection of the wildtype DNA-PKcs gene into *Prkdc*^{-/-} neurons results in 31.2% rescue by Bax shRNA following kainate treatment. Data represent the $\pm$ means SE from 14 embryos in two separate cultures. ** $p < 0.006$. *** $p < 0.001$, determined by *t*-test.

Fig. 5. Phosphorylation of serine 6 and/or serine 51 by DNA-PKcs controls the binding of Bax to Ku70 in kainate-induced excitotoxicity

Neurons were transfected with plasmids encoding V5 epitope-tagged Ku70 genes. At 24 h following kainate addition, neurons were lysed, transfected Ku70 was immunoprecipitated, and immunocomplexes were probed for Bax. (A) Representative Western blot for wildtype neurons transfected with Ku70 and Ku70 (S6A/S51A). Wildtype Ku70 showed an increase in binding to Bax, 24 h following kainate treatment, whereas Ku70 (S6A/S51A) displayed higher basal levels of bound Bax that were unchanged by kainate treatment. (B) Representative Western blot for *Prkdc*^{-/-} neurons transfected with wildtype Ku70. *Prkdc*^{-/-} cultures transfected with Ku70 did not show changes in Bax/Ku70 binding following kainate treatment. (C) Quantitation of Bax bound to Ku70 in kainate-treated neurons relative to vehicle-treated control; calculated by dividing Bax/Ku70 binding in kainate-treated cultures over vehicle-treated cultures. For each condition, n=3.

Fig. 6. Kainic acid induces early translocation of Bax-EGFP to the mitochondria

Primary neuronal cultures were co-transfected with Bax-EGFP and wildtype Ku70. On DIV7, kainic acid (100 μ M) was added directly to the media and cells were fixed at 0h (A, E, I), 1h (B, F, J), 4h (C, G, K), and 8h (D, H, L) following kainate addition, then stained for cytochrome oxidase (red). Arrows indicate neurons displaying Bax-EGFP translocation. Translocation was not observed in neurons co-transfected with Ku70 (S6A/S51A) (not shown). Scale bar=20 μ m.