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CYCLIN-DEPENDENT KINASE 4/6 ENABLES PUMA INDUCTION DURING NEURONAL APOPTOSIS

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ABSTRACT

DNA damage, oxidative stress, and ER stress are involved in the pathogenesis of acute and chronic neurodegenerative conditions. However, the signalling pathways that activate neuronal apoptosis in response to these stressors are not well understood. Here, we demonstrate that the Bcl-2 Homology Domain 3-only (BH3-only) family member Puma is transcriptionally upregulated in response to DNA damage, oxidative stress, and ER stress. Moreover, PUMA is essential for neuronal apoptosis in these contexts. Cyclin-dependent kinase 4/6 (cdk 4/6) activation has been implicated in triggering neuronal cell death processes. In this study, we have shown that the cdk 4/6 inhibitors flavopiridol, p16INK4a, and dominant negative-cdk 4 (DN-cdk 4) block stress-induced Puma transcription and apoptotic processes in neurons. Taken together, these results indicate that transcriptional induction of the Puma gene through cyclin-dependent kinases is critical for initiating apoptosis in response to neurodegenerative-associated stresses.

KEYWORDS

Neurons; Puma; BH3-only genes; qRT-PCR; transcription; DNA damage; oxidative stress; ER stress; neurodegeneration; cdk 4/6; Rb; flavopiridol; apoptosis; cell death; caspase-3

CO-AUTHORSHIP

The work for this thesis was done under the supervision of Dr. Sean Cregan. Meera Karajgikar and Diana Steckley are technicians in the lab who helped with management of the Puma knockout mouse colony and primary neuron cultures. Meggan Brine, a summer research student, acted as a blinded observer who performed apoptotic counts in the Puma knockout survival studies.

DEDICATION

This body of work is dedicated to the memory of my dearly departed mother, Anita Marie Fuerth, who lost her battle with cancer on October 21st, 2003. Her work as a psychologist touched the lives of many people, as she was the embodiment of patience, compassion, and virtue. I am forever grateful for her nurturing support over the years and for instilling in me a passion for learning. I would not be the person I am today without her love and guidance.

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°C:	degrees Celsius
µg:	Micrograms
µL:	Microlitres
µm:	Micrometres
xg:	times the force of gravity
A1:	Bcl-2 related protein A1
ACINUS:	Apoptosis chromatin condensation inducer in the nucleus
AD:	Alzheimer's disease
AIF:	Apoptosis inducing factor
AMPA:	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA:	Analysis of variance
ANT:	Adenine nucleotide translocator
Apaf-1:	Apoptotic protease-activating factor 1
APP:	Amyloid precursor protein
ATF6:	Activating transcription factor 6
ATM:	Ataxia telengiectasia-mutated
ATP:	Adenosine 5'-triphosphate
Bad:	Bcl-2 antagonist of cell death
Bak:	Bcl-2 antagonist/killer
Bax:	Bcl-2-associated protein X
Bcl-2:	B cell lymphoma/leukemia-2
Bcl-w:	Bcl-2 related protein w

Bcl-xl:	Bcl-2 related X-like protein
BH:	Bcl-2 homology
Bid:	Bcl-2 interacting domain death agonist
Bik:	Bcl-2 interacting killer-like
Bim:	Bcl-2 interacting mediator of cell death
BiP:	Immunoglobulin heavy chain-binding protein
BLM:	Bleomycin
Bmf:	Bcl-2 modifying factor
BRF-1:	Brain factor-1
Ca^{2+}:	Calcium ion
CAD:	Caspase-activated Dnase
CARD:	Caspase activation and recruitment domain
Cdk:	Cyclin-dependent kinase
CGN:	Cerebellar granule neuron
cki:	Cyclin-dependent kinase inhibitor
CPT:	Camptothecin
Cre:	Cre recombinase
CSF:	Cerebrospinal fluid
Cu^{+}:	Copper (I)
DBD:	DNA binding domain
DED:	Death effector domain
DNA:	Deoxyribonucleic acid
DN-cdk4:	Dominant negative-cyclin-dependent kinase 4

DSB:	Double-strand break
DTT:	Dithiothreitol
ETP:	Etoposide
EDTA:	Ethylenediamine tetraacetic acid
eIF2-α:	Eukaryotic initiation factor 2-alpha
endoG:	Endonuclease G
ER:	Endoplasmic reticulum
ETC:	Electron transport chain
FADD:	Fas-activating death domain
$FADH_2$:	Flavin adenine dinucleotide
Fe^{2+}:	Iron (II)
Flavo, Fl:	Flavopiridol
GSH:	Glutathione
H_2O_2:	Hydrogen peroxide
HBSS:	Hank's buffered saline solution
HD:	Huntington's disease
HNE:	4-hydroxy-2-nonenal
Hrk/DP5:	Harakiri/Death protein 5
IAP:	Inhibitor of apoptosis protein
ICAD:	Inhibitor of caspase-activated DNase
IMM:	Inner mitochondrial membrane
IMS:	Mitochondrial intermembrane space
INK4:	Inhibitor of cyclin-dependent kinase 4

IRE1:	Inositol requiring ER transmembrane RNase 1
JNK:	c-jun N-terminal kinase
Mcl-1:	Myeloid cell leukemia-1
MDA:	Malondialdehyde
mM:	Millimolar
M.O.I.:	Multiplicity of infection
mRNA:	messenger ribonucleic acid
Na^+:	Sodium ion
NADH:	Nicotinamide adenine dinucleotide
NES:	Nuclear export sequence
NLS:	Nuclear localization sequence
nM:	Nanomolar
NMDA:	N-methyl-D-aspartic acid
nNOS:	Neuronal nitric oxide synthase
$^{\cdot}NO$:	Nitric oxide
NOC-12:	3-ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene
Noxa:	NADPH oxidase activator 1
$O_2^{\cdot-}$:	Superoxide anion
$^{\cdot}OH$:	Hydroxyl radical
OMM:	Outer mitochondrial membrane
$ONOO^-$:	Peroxynitrite
PARP:	Poly ADP-ribose polymerase
PBS:	Phosphate-buffered saline

PCNA:	Proliferating cell nuclear antigen
PCR:	Polymerase chain reaction
PD:	Parkinson's disease
PERK:	PKR-like ER kinase
PMSF:	Phenyl- Methyl- Sulfonyl- Fluoride
PS1:	Presenilin-1
PTP:	Permeability transition pore
Puma:	p53-upregulated mediator of apoptosis
qRT-PCR:	Quantitative reverse transcriptase-polymerase chain reaction
Rb:	Retinoblastoma
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
RS:	Reactive species
RyR:	Ryanodine receptor
SCI:	Spinal cord injury
SDS:	Sodium dodecyl sulphate
SERCA:	Sarco-endoplasmic reticulum calcium ATPase
Smac/DIABLO:	Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
SOD:	Superoxide dismutase
TBH:	tert-butyl hydroperoxide
TBI:	Traumatic brain injury
TCA:	Tricarboxylic acid cycle

TG:	Thapsigargin
TGF-β:	Transforming growth factor-β
TNF:	Tumor necrosis factor
TU:	Tunicamycin
UPR:	Unfolded protein response
UV:	Ultraviolet
VDAC:	Voltage-dependent anion channel

CHAPTER 1- INTRODUCTION

Apoptosis is a programmed form of cell death that is important for the proper development of the nervous system. Two distinct periods of cell death have been observed during neural development, one during neurogenesis and the other during synaptogenesis, in which numerous neural precursor cells and young postmitotic neurons die by apoptosis (Lossi and Merighi, 2003). The production of excessive numbers of neurons only to have them eliminated shortly thereafter seems energetically expensive and counter-productive for a developing organism. Nevertheless, it was proposed that competition for growth factors between developing neurons could be a way to form coordinated networks of neuronal connections.

Apoptosis also plays a beneficial role in protecting organisms as a whole from toxic insults that inhibit normal cellular functions (Adams and Cory, 2007). For example, it can prevent the propagation of mutated DNA, which could potentially lead to a cancerous phenotype.

Despite all of its beneficial functions, apoptosis can be harmful when activated in a finite population of terminally differentiated cells, such as neurons. This seems to be the unfortunate case in acute neuronal insults, such as stroke and traumatic brain injury (TBI), and in chronic neurodegenerative diseases, like Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Ekshyyan and Aw, 2004;Jellinger, 2001;Mattson, 2000). The various forms of neurodegeneration all induce the same two types of stress in neurons, oxidative stress and ER stress, leading to the activation of apoptosis (Halliwell, 2006;Xu et al., 2005).

1.1- OXIDATIVE STRESS

1.11-Definition of Oxidative Stress

As aerobic organisms, humans require oxygen for metabolism and the production of energy in the form of adenosine 5'-triphosphate (ATP). ATP synthesis occurs in the mitochondria through a process known as oxidative phosphorylation. The electron transport chain (ETC) is integral to this process through generation of the proton motive force, an electrochemical gradient of protons across the inner mitochondrial membrane (IMM) which provides the necessary energy for ATP synthase function in the mitochondrial matrix (Buck and Pamenter, 2006). The carrier complexes 1-4 of the ETC, which extract electrons from the donor molecules NADH and $FADH_2$ of the tricarboxylic acid (TCA) cycle, generate the proton motive force by pumping protons into the mitochondrial intermembrane space (IMS) when the extracted electrons are passed between complexes (Krieger and Duchen, 2002). Oxygen acts as the final electron acceptor in the chain and is converted to water.

Consequently, the ETC in the mitochondria is also the most important source of oxidative stress, which is an imbalance in the redox state of the cell (Chong et al., 2005). When the production of reactive species (RS) outweighs their neutralization by the anti-oxidant defence, these molecules readily react with and damage important cellular components such as DNA, proteins, and lipids.

Oxidative stress is one of the most common and well studied types of stress encountered in neurodegenerative diseases. During a stroke, for example, the brain is deprived of oxygen and nutrients through interruption of its blood supply. As metabolism continues in these brain cells, depletion of the proton motive force

uncouples the ETC from ATP synthesis and a lack of oxygen allows electrons that are normally passed along the chain to react with neighbouring molecules producing RS (Moro et al., 2005). Furthermore, sudden reoxygenation of the brain, by the restoration of circulation in the cerebral vasculature of a stroke victim, causes a rise in the partial pressure of oxygen within the mitochondria of the cell. The ETC and anti-oxidant defence are quickly saturated and RS are generated by activated oxygen (Won et al., 2002).

1.12-Reactive Species: Reactive Oxygen Species & Reactive Nitrogen Species

Reactive species, which include reactive oxygen species (ROS) and reactive nitrogen species (RNS), can be subdivided into two categories: free radical and non-radical species. Free radical species can be defined as any chemical that can exist independently containing one or more atoms with an unpaired electron (Halliwell, 1992). The important point to note about free radicals is that they are highly reactive molecules with short half-lives. However, their presence can be maintained over an extended period of time when they react with a non-radical to produce a new radical species, which may result in a chain reaction of free radical formation (Antunes et al., 2005). Some examples of common free radical ROS encountered by the cell include superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$).

Superoxide anion appears to be a common free radical ROS by-product of the ETC, since it has been estimated to account for 2% of the oxygen consumed by the cell (Skulachev, 1997). It can be produced at an even higher level when the cell is placed under stress. However, superoxide anion is not membrane permeable and can be converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD) (Halliwell, 1978). Since superoxide anion is selectively reactive with a few

biomolecules, most of the damage it causes is indirect due to its conversion to other more indiscriminate RS. For this reason, it is often placed at the start of the oxidative stress cascade. For example, superoxide anion can produce the hydroxyl radical, a very potent ROS DNA damaging agent, through the Haber-Weiss reaction which involves its reaction with water (Mello Filho and Meneghini, 1984).

Non-radical ROS are also generated by the ETC at the mitochondria, of which hydrogen peroxide is the most common. On its own, hydrogen peroxide is not particularly reactive but, through the Fenton reaction which requires the presence of transition metals such as Fe^{2+} or Cu^{+}, it is reduced to form the hydroxyl radical (Dizdaroglu et al., 1991). Interestingly, these metals are found in association with DNA in trace amounts in the cell and provide an opportunity for damage to the nuclear material (Blakely et al., 1990). Furthermore, H_2O_2-mediated degradation of heme proteins can release iron (Chiu et al., 1996). Hydrogen peroxide, known to play a role in models of neurodegeneration, is usually detoxified into water in the cell through the actions of catalases and peroxidases (Hanrott et al., 2006).

Stressed neurons are prone to releasing excess amounts of glutamate, due to the depolarization of the plasma membrane and activation of the voltage-gated calcium channels. This neurotransmitter can activate the NMDA receptors at the postsynaptic membrane, allowing for an influx of calcium (White and Reynolds, 1997). Calcium can activate neuronal nitric oxide synthase (nNOS), an enzyme that synthesizes the RNS nitric oxide ($^{\cdot}NO$) from the amino acid L-arginine (Dawson et al., 1992).

Nitric oxide readily reacts with the superoxide anion to form the non-radical RNS known as peroxynitrite ($ONOO^-$) (Alvarez and Radi, 2003). The formation of peroxynitrite *in vitro* is three times faster than the rate of dismutation of the superoxide anion by SOD, thus enabling nitric oxide to easily compete with SOD for this molecule (Halliwell et al., 1999). Peroxynitrite is membrane permeable which enables it to move throughout the cell.

1.13-Reactive Species Cause Damage to Proteins, Lipids, and DNA

Oxidative stress caused by the RS superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), nitric oxide ($^{\cdot}NO$), and peroxynitrite ($ONOO^-$) is self-perpetuating as it stimulates a vicious cycle of free radical formation. Free radicals and non-radicals alike can cause extensive damage to all cellular components through many processes.

For example, lipid peroxidation generates a variety of unstable decomposition products known as α,β-unsaturated reactive aldehydes (e.g.- malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and 2-propenal (acrolein)) that react with amino acid groups of proteins and modify their properties (Dalle-Donne et al., 2006). Protein modification also occurs through oxidation and nitration, processes which respectively generate carbonylated and tyrosine-nitrated proteins (Alvarez and Radi, 2003;Lyras et al., 1997). Many of these protein modifications can cause their aggregation and toxic build-up in the cell.

Different types of DNA lesions in the nucleus and mitochondria, such as base modification (e.g.- 8-hydroxydeoxyguanosine), abasic sites, and strand breaks can result from exposure to oxidative stress (Barzilai and Yamamoto, 2004). However,

the double strand break (DSB) is one of the most lethal, since it can directly inactivate essential genes and is subject to error-prone repair processes.

The cell initiates signalling pathways to activate apoptosis in response to damage caused by oxidative stress, but these molecular events are poorly characterized. Activation of apoptosis is dependent on whether or not the initial insult was severe enough to generate RS in excess of the cell's anti-oxidant defence system, composed of free radical scavengers, such as glutathione (GSH), vitamins E & C, and detoxifying enzymes, like SOD, and cause damage to the cell beyond its reparative capacity (Contestabile, 2001).

1.14-DNA Damage and Cell Death

DNA damage caused by oxidative stress can activate a complex web of signalling pathways, collectively known as the DNA damage response (Barzilai and Yamamoto, 2004). This response activates cell cycle arrest at checkpoints in the cell cycle to prevent synthesis of mutated DNA and allow time to repair the damage (Shackelford et al., 2000).

The DNA damage response shifts from repair to activation of apoptosis when the DNA suffers irreparable damage, although this mechanism is still uncertain. Poly ADP-ribose polymerase (PARP) and ataxia telengiectasia-mutated (ATM) are proteins that play a dual role in both DNA repair and apoptosis since they have been shown to activate and stabilize the tumor suppressor protein, p53, which is a transcription factor involved in cell cycle arrest and the induction of many pro-apoptotic genes (Hong et al., 2004;Kurz and Lees-Miller, 2004).

1.2- ER STRESS

1.21-Calcium Deregulation

Another major stress associated with neurodegenerative diseases involves the impairment of intracellular calcium homeostasis, resulting in RS generation and dysfunctional protein folding and degradation (Culmsee and Landshamer, 2006).

The concentration of free calcium within the cytoplasm of a resting neuron is approximately 100 nM, whereas extracellularly it is estimated to be 1-2 mM, a 10,000-20,000 fold difference (Won et al., 2002). This huge concentration gradient is tightly regulated since Ca^{2+} is an important messenger molecule for the neuron. Calcium is involved in such processes as the activation of various types of proteins (e.g.- kinases, proteases, transcription factors) and the exocytosis of synaptic vesicles.

The intracellular levels of calcium in the neuron are maintained through several mechanisms: 1. Calcium entry from the extracellular space through neurotransmitter-activated receptors (e.g.- NMDA, AMPA, kainate) or voltage-gated channels. 2. Calcium release from the endoplasmic reticulum (ER) by the stimulation of inositol triphosphate (IP3) or ryanodine receptors (RyR), or from the mitochondria through the Na^{+}-Ca^{2+} exchanger. 3. The exclusion of calcium from the cytoplasm through the Ca^{2+}-ATPase or Na^{+}-Ca^{2+} exchanger at the plasma membrane. 4. The binding of Ca^{2+} to target proteins. 5. The sequestration of Ca^{2+} into the ER through the Ca^{2+}-ATPase, or into the mitochondria through a uniport mechanism (Won et al., 2002).

The disruption of any of the previously mentioned mechanisms for the regulation of intracellular free calcium levels will affect homeostasis. An increase in

calcium concentration in the cytoplasm can result in three major consequences. Firstly, the mitochondria of the cell become depolarized as they attempt to restore homeostasis in the cytoplasm by sequestering calcium in the mitochondrial matrix. The result is impaired ATP synthesis and the generation of RS through the previously described mechanisms of oxidative stress (Chinopoulos and Adam-Vizi, 2006).

Secondly, free calcium in the cytoplasm can increase the rate of catabolism of vital proteins through Ca^{2+}-dependent activation of proteases such as calpains (Sedarous et al., 2003). The activation of proteases can have multiple effects in neurons, such as dendritic remodelling, interruption of membrane and cytoplasmic transport, and modification of gene expression, all contributing to neuronal degeneration.

Finally, increases in intracellular calcium concentration will alter calcium-dependent signalling pathways within the cell (Scorrano et al., 2003). For example, elevated intracellular calcium concentration can activate cytosolic phospholipase A2 translocation to the plasma membrane, where it cleaves free fatty acids (e.g.-arachidonic acid) from the phospholipids and can result in ROS production (Sapirstein and Bonventre, 2000). Also, if high calcium levels are maintained, downstream effectors of calcium-dependent signalling pathways will remain constitutively activated resulting in an unregulated response.

1.22-Unfolded Protein Response

Toxicity to the cell through deregulation of calcium homeostasis in the ER, the main site of protein synthesis and maturation in the cell, occurs through a more complex mechanism. In an unstressed cell, resident protein chaperones such as

protein disulfide isomerase and *cis-trans* prolyl isomerase catalyze calcium-dependent protein folding reactions, while other chaperones like BiP maintain proteins in a folding-competent state and prevent protein aggregation (Shen et al., 2004). In model systems of ER stress, decreasing the calcium concentration in the lumen of the ER impairs calcium-dependent protein folding performed by the protein chaperones. Therefore, new proteins are unable to reach their mature conformations and accumulate in the ER, activating a cellular defense mechanism known as the unfolded protein response (UPR) (Kudo, 2003).

PKR-like ER kinase (PERK), a trans-membrane protein of the ER, acts as a sensor of ER stress via a luminal domain that senses the accumulation of unfolded proteins (Degracia and Montie, 2004). The activated luminal domain of PERK induces its oligomerization and autophosphorylation, activating the cytoplasmic kinase domain. Phosphorylation of the translation initiator, eukaryotic initiation factor 2-alpha (eIF2-α), by the PERK kinase domain transiently suppresses global protein synthesis (Wek et al., 2006). This molecular event prevents the accumulation of new proteins in the ER while the stress is being managed.

Two other resident ER stress sensors, inositol requiring ER transmembrane RNase-1 (IRE1) and activating transcription factor 6 (ATF6), mediate the transcriptional activation of genes encoding proteins that increase ER translocation, protein folding, export, and degradation (Shen et al., 2004). In addition, the expression of various calcium buffering proteins, such as the sarcoplasmic reticulum calcium ATPase (SERCA), calreticulin, and calnexin, is upregulated in an attempt to restore calcium levels in the ER lumen (Verkhratsky and Toescu, 2003).

If calcium homeostasis and subsequently, protein folding mechanisms, cannot be restored, then the irreparable damage caused by ER stress will initiate signals to activate the apoptotic cascade (Chen and Gao, 2002). However, these initial signalling pathways are poorly understood.

1.3- OXIDATIVE STRESS AND ER STRESS IN NEURODEGENERATION

The rationale for examining oxidative stress and ER stress in the context of neuronal apoptosis comes from a multitude of observations in the literature implicating their presence and active involvement in the pathogenesis of neurodegeneration (Kidd, 2005).

For example, the amyloid-β peptide is an aberrant cleavage product of 40 to 42 amino acids of the amyloid precursor protein (APP) and is found in abundance in AD brain tissue in extracellular deposits, known as senile plaques. This peptide has since been demonstrated to induce oxidative stress and neurotoxicity through its methionine-35 residue in *in vitro* and *in vivo* models (Misiti et al., 2004). Furthermore, the free radical scavenger, vitamin E, can prevent the amyloid-β peptide-mediated induction of RS, lipid peroxidation, protein oxidation, and neurotoxicity in these models (Butterfield and Boyd-Kimball, 2005).

Levels of nitrite/nitrate, metabolites representing an indirect measure of nitric oxide radical production, were found to be higher in cerebrospinal fluid (CSF) and serum samples taken from human stroke and subarachnoid hemorrhage victims than in control patients (Moro et al., 2005). In addition, it was shown that levels of the biosynthetic precursor of nitric oxide, L-arginine, were decreased in CSF and plasma samples following an ischemic event.

A common theme among most neurodegenerative diseases is the accumulation of aggregates of various kinds of proteins due to damage caused by oxidative stress, mutations, and altered processing. Protein aggregates are suspected to play a role in the loss of specific neuronal populations. For example, an abundance of α-synuclein and ubiquitin proteins are found in intracytoplasmic inclusions, known as Lewy bodies, in dopaminergic neurons from PD patients (Lyras et al., 1998). The huntingtin gene contains an expansion of trinucleotides (CAG) that produces a protein with an abnormally large number of polyglutamine repeats which forms aggregates containing TATA-binding protein and ubiquitin in cortical neurons of HD patients (Butterworth et al., 1998;Huang et al., 1998).

The familial Alzheimer's disease-linked mutation I213T of the presenilin-1 (PS1) protein, an ER membrane protein responsible for the gamma site cleavage of APP, delays the onset of the UPR (Katayama et al., 2004). This was determined by the increased amount of time needed for PERK and IRE1 phosphorylation *in vitro* following ER stress induced by DTT treatment in mutant PS1 knock-in versus PS1 wildtype cells. PS1 mutant protein also perturbs calcium homeostasis in the ER by promoting calcium release into the cytoplasm. Therefore, the mutant form of PS1 makes neurons more susceptible to misfolded protein accumulation and for a longer period of time.

1.4- THE ROLE OF APOPTOSIS IN NEURODEGENERATION

1.41-Characteristics of Apoptosis

Neurons can die by apoptosis in response to numerous intracellular stresses

such as unregulated calcium levels, oxidative stress, and DNA damage (Lossi and Merighi, 2003). However, a complex neurotoxic stress, like cerebral ischemia, induces a dichotomous response in the total population of neurons, wherein, some cells die by necrosis, while others undergo apoptosis (Won et al., 2002).

These two modes of cell death, apoptosis and necrosis, are distinguishable based on several different criteria. First of all, apoptosis is an active, organized process that requires energy and new protein synthesis for its execution whereas necrosis takes place in a seemingly passive and haphazard manner (Roy and Sapolsky, 1999).

The hallmark of classical apoptosis is the activation of specific caspases. Caspases are a family of fourteen known mammalian proteases that share a number of common features (Lossi and Merighi, 2003). For example, they are expressed as zymogens which become active enzymes during apoptosis. As active enzymes, caspases use their catalytic triad, composed of a cysteine residue, a histidine residue, and a carbonyl group, to specifically cleave substrates after any aspartate residue.

The caspase family of proteins is divided into three groups based on function (Jin and El-Deiry, 2005). The first group is known as the inflammatory caspases which includes caspase-1, -4, -5, -11, -12, -13, and -14 and are not involved in apoptosis. The second group is labelled the apoptotic initiator caspases which contain either a death effector domain (DED) (caspase-8 & -10) or a caspase activation and recruitment domain (CARD) (caspase-2 & -9) to mediate their interaction with adaptor molecules containing similar domains, such as FADD and Apaf-1, respectively. Apoptotic initiator caspases are named as such because they activate the

third group of caspases, the apoptotic effector caspases (caspase-3, -6, & -7). This is the executioner class of caspases that cleaves multiple cellular substrates. These proteases carry out the degradation of structural and effector proteins of the cell and activate DNases that cleave DNA into internucleosomal fragments (Jin and El-Deiry, 2005). Conditions during necrosis may or may not enable the activation of caspase-independent proteases leading to limited DNA degradation.

Apoptosis and necrosis are also differentiated based on morphological assessment. In necrosis, the plasma membrane and cytoplasmic organelles swell and eventually burst, releasing their intracellular contents (Yuan et al., 2003). Activation of a harmful inflammatory response ensues, further compounding damage to the area by affecting surrounding healthy cells. Apoptotic cells undergo an opposite process of shrinkage while maintaining the integrity of their organelles and plasma membrane, which can fold up on itself, a phenomenon known as membrane blebbing (Kerr et al., 1972). Several membrane-bound apoptotic bodies pinch off the cell, carrying various degraded intracellular components. The exposure of phosphatidyl serine at the extracellular surface of the plasma membrane is a signal for phagocytes to remove apoptotic bodies.

Nuclear morphology can also be used to differentiate which cells are undergoing either cell death pathway. The DNA pattern in necrotic nuclei appears non-uniform and diffuse, as opposed to a consistent and tightly condensed pattern of degraded DNA in apoptosis (Kerr et al., 1994).

1.42-Extrinsic and Intrinsic Pathways of Apoptosis

There are two distinct, but not mutually exclusive, ways of activating

apoptosis known as the extrinsic and intrinsic pathways. The extrinsic or 'death receptor' pathway can be activated through the binding of extracellular death ligands, which are released in an autocrine or paracrine fashion, to their respective receptors at the plasma membrane of the cell, thus directly initiating a caspase activation cascade and ultimately, the destruction of the cell (Jin and El-Deiry, 2005). A couple examples of these pro-apoptotic ligand-receptor combinations are the Fas ligand/Fas receptor and the TNF-alpha/TNF receptor.

The intrinsic or 'mitochondrial' pathway of apoptosis is activated when cell death signals are initiated in response to damage inside the cell. In this pathway, the mitochondria become depolarized and are targeted by the pro-apoptotic members of the Bcl-2 protein family to induce membrane permeability through the formation of the mitochondrial permeability transition pore (PTP) (van Gurp et al., 2003). Cytochrome c is released from its association with the IMM at the IMS into the cytoplasm via the PTP. This translocation allows cytochrome c to bind to Apaf-1 in the presence of ATP to form the apoptosome, which activates caspase-9 and proceeds to cleave procaspase-3 into caspase-3, an active effector caspase (Scorrano and Korsmeyer, 2003). Caspase-3 causes nuclear DNA degradation by cleaving the inhibitor of caspase-activated DNase (ICAD), allowing caspase-activated DNase (CAD) and apoptosis chromatin condensation inducer in the nucleus (ACINUS) to translocate from the cytoplasm into the nucleus and carry out their respective functions as DNA-degrading and DNA-condensing enzymes (Cregan et al., 2004b;Keramaris et al., 2000).

Simultaneously, the murine Smac/human ortholog DIABLO and the serine protease Omi/HtrA2 are released from the mitochondria and aid in promoting caspase activation by the apoptosome (van Gurp et al., 2003). They perform this function by binding and sequestering the inhibitors of apoptosis proteins (IAPs); proteins which prevent the activation of procaspases and inhibit the activity of mature caspases. Smac/DIABLO and Omi/HtrA2 are functionally redundant proteins but show differential tissue expression, as Omi/HtrA2 is the only one expressed in the brain.

Other factors released from the mitochondria, such as apoptosis inducing factor (AIF) and endonuclease G (endoG), mediate a caspase-independent apoptotic process (Stefanis, 2005). The mitochondrial pathway of apoptosis is illustrated below in figure 1.

1.43-Evidence for the Involvement of Apoptosis in Neurodegeneration

Apoptosis as the cell death mechanism driving the pathogenesis of neurodegenerative conditions remains a highly contentious issue in the literature. However, it is undeniably a significant contributor since a fair number of apoptotic neurons and glial cells have been positively identified based on nuclear and morphological assessment of post-mortem brain tissue sections from patients who suffered from various neurodegenerative conditions, such as AD (Overmyer et al., 2000), PD (Hartmann et al., 2000), HD (Butterworth et al., 1998), and TBI (Smith et al., 2000). Similar observations have been made from *in vivo* models of neurodegenerative conditions, such as TBI, spinal cord injury (SCI), and PD (Ekshyyan and Aw, 2004).

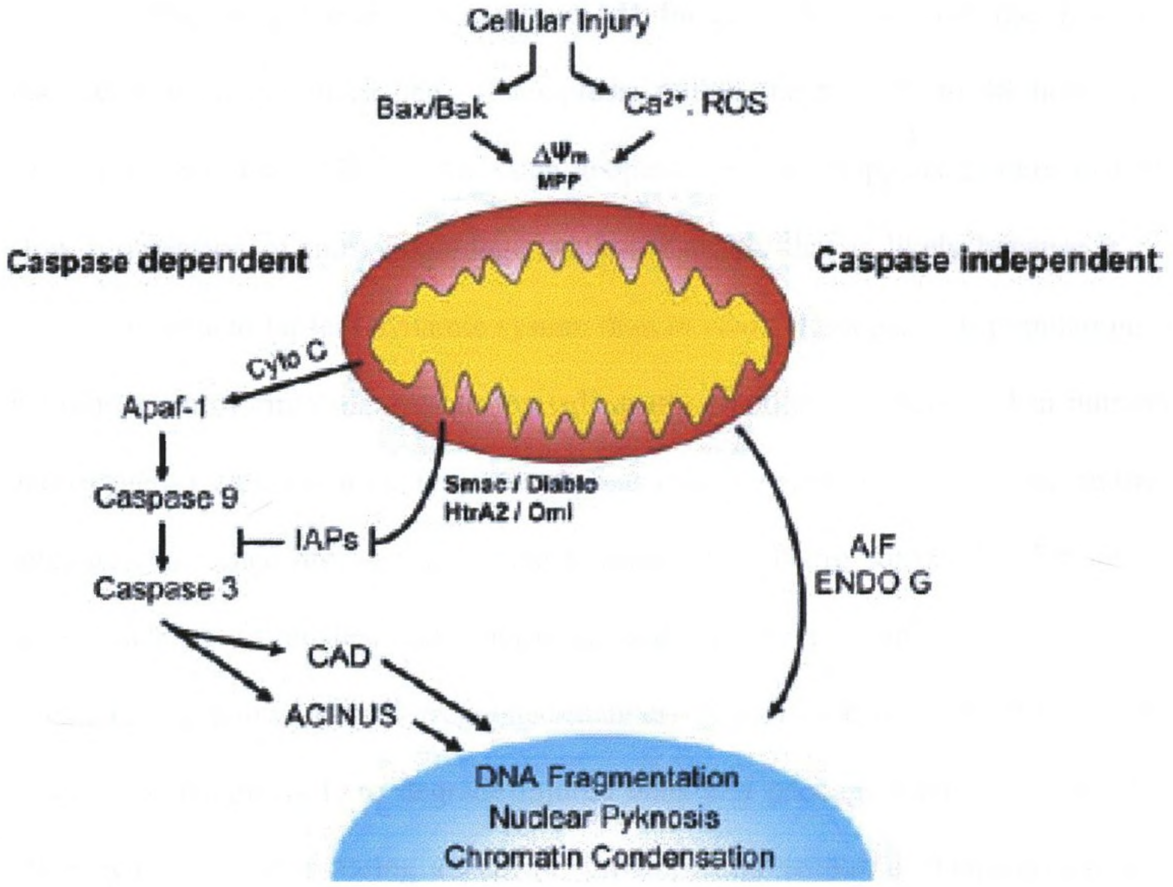


Figure 1- The Mitochondrial Pathway of Apoptosis. When the cell is challenged with intracellular toxic stimuli such as DNA damage, ER stress, or oxidative stress, apoptosis is initiated through formation of the mitochondrial permeability transition pore (MPP). The mitochondria are permeabilized through activation of the pro-apoptotic multi-BH domain proteins Bax/Bak, which translocate from the cytoplasm to the michtochondria where they can form homo-oligomeric pores in the OMM and interact with pre-existing mitochondrial pore proteins to deplete the mitochondrial transmembrane potential ($\Delta\Psi m$). This allows pro-apoptotic factors, such as Smac/Diablo and HtrA2/Omi, which inactivate the inhibitor of apoptosis proteins (IAPs), and cytochrome c (cyto c) to be released from the mitochondria and initiate the caspase activation cascade. Cytochrome c interacts with Apaf-1 and ATP to form the apoptosome, which cleaves procaspase-9 into active caspase-9, ultimately resulting in the activaton of the effector caspase, caspase-3. Caspase-3 activates caspase-activated DNase (CAD) and apoptosis chromatin condensation inducer in the nucleus (ACINUS) which respectively fragment and condense DNA. Other mitochondrial pro-apoptotic factors, like apoptosis inducing factor (AIF) and endonuclease G (endo G), can carry out similar functions in a caspase-independent manner.

Furthermore, the *in vitro* model of AD, for example, has shown the amyloid-β peptide to be a potent inducer of apoptosis within the first 24 to 48 hours after exposure (Loo et al., 1993). Although apoptosis *in vitro* happens quicker than the slow progression of most neurodegenerative diseases, that is likely because it is a much simpler and far less dynamic system than *in vivo*. Homogeneous populations of neurons are uniformly exposed to the cell death stimulus and incubated in nutrient- and oxygen- rich conditions, *in vitro*, whereas neurons *in vivo* are experiencing these internally mediated stresses at different times while being surrounded by support cells, such as astrocytes and microglia, and fed oxygen and nutrients by the circulatory system in a tightly regulated manner. Also, if one considers that the main function of apoptosis is to degrade injured cells and package them for disposal by phagocytes without inducing a harmful inflammatory response, then perhaps most apoptotic neurons are being phagocytosed by the brain's resident macrophages, microglia, throughout the lengthy course of neurodegeneration.

Other cell death mechanisms, such as necrotic excitotoxicity during stroke, can contribute to acute neurodegeneration but apoptosis is an organized process with a much larger window for potential therapeutic intervention.

1.5- THE Bcl-2 FAMILY OF PROTEINS

1.51-Anti-Apoptotic vs. Pro-Apoptotic Family Members

The Bcl-2 family of cell death proteins plays a key role in regulating the mitochondrial pathway of apoptosis (Tsujimoto, 2003). The members of this protein family all have certain conserved amino acid sequences in common known as Bcl-2 homology (BH) domains, of which there are four. Depending on the BH domain

content and function, the members of this family can be divided into three types: anti-apoptotic proteins containing BH domains 1-4 (BCL-2, BCL-W, BCL-XL, MCL-1, A1), pro-apoptotic multi-BH domain proteins containing BH domains 1-3 (principally BAX & BAK), and pro-apoptotic BH3-only domain proteins (BAD, BID, BIK, BIM, BMF, HRK/DP5, NOXA, PUMA) (Tsujimoto, 2003). The anti-apoptotic and pro-apoptotic members of the Bcl-2 protein family regulate the activation of the mitochondrial pathway of apoptosis through a balancing act of protein interactions in which the ratio between the two types acts like an on/off switch.

1.52-Function of Pro-Apoptotic Multi-BH Domain Proteins

Normally a monomeric, soluble factor in the cytosol of healthy cells, BAX/BAK can be activated in response to various apoptotic stimuli whereupon they translocate to the mitochondria and form homo-multimers for induction of mitochondrial membrane permeability (Kirkland and Franklin, 2003). The permeabilized membranes allow several pro-apoptotic factors, such as cytochrome c, to be released from the mitochondria and initiate the caspase activation cascade.

Two theories have been proposed for the capacity of multi-BH domain proteins to promote permeability of the mitochondrial membrane (Scorrano and Korsmeyer, 2003). The first theory claims that activated BAX/BAK can interact with the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM) and the adenine nucleotide translocator (ANT) and cyclophilin D in the IMM to promote formation of the PTP, through which pro-apoptotic factors can escape into the cytoplasm (Marzo et al., 1998;Shimizu et al., 1999). The second theory proposes that activated Bax/Bak can insert directly in the OMM as an oligomeric pore. This

pore has an estimated size of 22 angstroms, slightly bigger than a soluble cytochrome c molecule, and has been electrophysiologically detected in isolated patches of outer mitochondrial membranes from apoptotic cells (Pavlov et al., 2001).

Perhaps both of these and other still unknown mechanisms play a role in BAX/BAK-mediated mitochondrial membrane permeability. In any case, this class of multi-BH domain Bcl-2 family proteins is absolutely required for the activation of the mitochondrial pathway of apoptosis.

1.53-Function of Anti-Apoptotic Bcl-2 Family Proteins

The anti-apoptotic proteins of the Bcl-2 family protect the cell from activation of the mitochondrial pathway of apoptosis. Their anti-apoptotic effects are attributed to their ability to bind and inactivate the BH3 domain of multi-BH domain and BH3-only domain proteins via an interaction with their hydrophobic pocket (Cheng et al., 2001;Ke et al., 2001). The BH4 domain, found only in the anti-apoptotic Bcl-2 family proteins, has also been shown to have anti-apoptotic activity through an unknown BH3 domain heterodimerization-independent mechanism (Shimizu et al., 2000).

1.54-Function of BH3-only domain proteins

All BH3-only domain proteins act as sensitizers of apoptosis and do so by binding to anti-apoptotic Bcl-2 family proteins, thus inactivating these inhibitors of pro-apoptotic multi-BH domain proteins (Bouillet and Strasser, 2002). The BH3 domain, which is composed of a conserved 9-16 amino acid sequence between family members, is necessary for its cell killing function.

The presence of several different BH3-only domain proteins in mammalian cells seems functionally redundant. Interestingly, activation of specific BH3-only domain proteins has been shown to be cell type and stimulus specific, leading to speculation that perhaps each protein is responsible for sensing different stresses (Bouillet and Strasser, 2002). For example, Bid-deficient mice are developmentally normal, but their hepatocytes and not their lymphocytes are resistant to Fas antibody-induced cell death (Yin et al., 1999). Bim-deficient lymphocytes, a BH3-only domain protein required for hematopoietic cell homeostasis, are resistant to cytokine withdrawal and treatment with ionomycin or taxol but respond normally to induce cell death with phorbol ester or dexamethasone treatment (Bouillet et al., 1999;Bouillet et al., 2002). More studies of all the different BH3-only gene knockouts in various cell types will need to be performed in order to confirm if this is indeed the case.

A few BH3-only domain proteins, PUMA, NOXA, BIM, and HRK/DP5, are regulated at the transcriptional level. For example, Puma and Noxa were first identified as p53-inducible genes (Nakano and Vousden, 2001;Oda et al., 2000;Yu et al., 2001). The mechanism for p53 transactivation of these genes in neuronal cell death was subsequently characterized (Cregan et al., 2004a). Furthermore, the expression of Bim and Hrk/DP5 in a potassium withdrawal model of neuronal cell death is regulated by a c-jun N-terminal kinase (JNK)-dependent mechanism (Harris and Johnson, Jr., 2001).

The importance and regulation of BH3-only domain protein expression in pathologically relevant models of neuronal cell death, such as oxidative stress and ER stress, remain to be defined.

1.55-Evidence for the Involvement of Bcl-2 Family Proteins in Neurodegeneration

In one study of post-mortem brain sample extracts from AD patients, the levels and subcellular locations (i.e.-membranous vs. cytosolic) of the examined Bcl-2 family proteins, BCL-XL, BCL-2, BAX, BAK, and BAD were found, by western blotting, to be significantly altered, as compared to control samples (Kitamura et al., 1998). Other studies demonstrated an increase in the number of BAX-positive cells per unit area, by immunohistochemical staining of slice sections of the brain taken post-mortem, in the frontal cortex from AD and multiple infarct patients, and in the substantia nigra of PD patients compared to age-matched controls (Horowitz et al., 2003;Lu et al., 2005). Also, expression of the BH3-only gene Bim was recently shown to be increased at the mRNA and protein levels in post-mortem brain tissue from AD patients as compared to controls (Biswas et al., 2007).

1.6- CELL CYCLE MOLECULES AND NEURONAL CELL DEATH

1.61-Cell Cycle Reactivation Implicated in Neuronal Cell Death

Neurons are terminally differentiated cells that do not divide and remain quiescent outside of the cell cycle, in the G_0 phase. However, the upregulation of cell cycle molecules in neurons has been observed in several neurodegenerative conditions. For example, cyclin D, cdk 4, cyclin B1, and proliferating cell nuclear antigen (PCNA), an indirect marker of DNA synthesis, were found by

immunohistochemical staining to be abnormally expressed in several different regions of AD brains and almost undetectable in age-matched control samples (Busser et al., 1998). Cyclin B1 immunostaining was also detected in Lewy body-containing nigral neurons in brain tissue sections from PD patients (Lee et al., 2003).

Furthermore, in response to several different types of cell death stimuli in *in vitro* and *in vivo* models of neuronal apoptosis, the level and activity of particular cell cycle molecules are increased in dying neurons. For example, cyclin B1 levels and de novo DNA synthesis were increased by induced expression of the PD-associated protein α-synuclein in a stably transfected neuronal-like cell line (Lee et al., 2003).

The expression of cyclin D1 started to increase 3 to 6 hours after serum withdrawal, an apoptosis-inducing stimulus, in differentiated N1E-115 neuronal precursor cells (Kranenburg et al., 1996). Concomitantly, interactions between cyclin D1, its kinase binding partner, cdk4, and its substrate, Rb, started to increase during the same period of time, indicating that this complex was becoming activated.

In another study, the apoptosis-inducing paradigm of potassium withdrawal in primary murine cerebellar granule neurons (CGN) induced an increase in the level of cyclin D1 protein (Padmanabhan et al., 1999). Furthermore, the kinase activities of both the cyclin E and cyclin D1 immunoprecipitated complexes, as determined by Rb phosphorylation, were increased starting as early as a half hour post-treatment.

In a knock-in mouse model of HD, expressing mutant huntingtin protein with 111 glutamine residue repeats, cyclin D1 protein levels were two fold higher in striatal cell extracts from 8 month-old mutant mice compared to wildtype mice, which

expressed a normal huntingtin protein with 7 glutamine residue repeats (Gines et al., 2003).

An *in vivo* model of global cerebral ischemia in rats revealed, through microarray analysis, that the expression of the transcription factor E2F1 showed the greatest increase of all the genes studied from hippocampus after 72 hours of reperfusion (Jin et al., 2001). Significant increases in other related cell cycle genes included the E2F transcription co-factor DP-1 and the associated E2F inhibitor, Rb.

It is highly unlikely that these repeated observations of increases in cell cycle molecules across many different types of neurodegenerative conditions represent random occurrences. This has lead to the theory that during stress, reactivation of the cell cycle causes neurons to undergo apoptosis. Therefore, an underlying mechanism which ties together reactivation of the cell cycle and apoptotic machinery in stressed neurons probably exists, but has yet to be accurately defined.

1.62-Cyclins and cdks

Cyclins and their cyclin-dependent kinase (cdk) binding partners interact to form an active kinase complex that affects the function of key enzymes and transcription factors in order to coordinate the progression of the cell cycle in a highly ordered manner from interphase (G_1, S, G_2) to mitosis (M) (Vermeulen et al., 2003).

Cdk activity is regulated through its interaction with a specific cyclin subunit. Accordingly, cyclin expression is tightly regulated, as a specific cyclin protein is only expressed during the cell cycle phase that it controls and is then degraded by ubiquitin-mediated proteolysis (Pines, 1995). For instance, Cyclin D-cdk 4/6 are active during the G_1 phase, Cyclin E-cdk2 controls the G_1/S phase transition, Cyclin

A-cdk2 becomes active during the S phase, Cyclin A-cdk1 controls the G_2/M phase transition, and Cyclin B-cdk1 enables mitosis.

Cell cycle inhibitory proteins called cdk inhibitors (cki) can counteract cdk activity and are divided into two families, the INK4 family and the Cip/Kip family (Vermeulen et al., 2003). The INK4 family includes p15(INK4b), p16(INK4a), p18(INK4c), and p19(INK4d). These cki specifically inactivate G_1 cdk (cdk 4/6) by forming stable complexes with the cdk enzymes before cyclin binding, preventing association with Cyclin D. The Cip/Kip family of cki is comprised of p21(Waf1/Cip1), p27(Cip2), and p57(Kip2). These inhibitors of cdk function inactivate the G_1 cyclin-cdk complexes as a whole and, to a lesser extent, the cyclin B-cdk1 complex.

Both families of cki can be activated to mediate cell cycle arrest in response to external and internal signals. For example, p21 is under the transcriptional control of the p53 tumor suppressor and p15 and p27 are activated in response to transforming growth factor-β (TGF-β) (El-Deiry et al., 1993;Hannon and Beach, 1994;Reynisdottir et al., 1995).

1.63-E2F and Rb Protein Families

The E2F family of transcription factors regulates the expression of many genes throughout the cell cycle, including genes involved in DNA replication, cell cycle regulation, and chromosome mobilization (Helin, 1998).

There are currently eight known members of the E2F family, which have traditionally been divided into two categories: activators (E2F1-3) and repressors (E2F4-8) of gene expression (Degregori and Johnson, 2006). All E2F family proteins

have at least one DNA binding domain (DBD). E2Fs 1 to 6 have a dimerization domain that enables them to heterodimerize with one of three known DP co-factors (mainly DP1 & DP2, and DP4), which are required for high affinity DNA binding (Degregori and Johnson, 2006). E2Fs 7 and 8 have 2 DBDs and appear to associate with DNA in a DP-independent manner. Only the activators of transcription, E2Fs 1 to 3, have both a nuclear localization sequence (NLS) and a cyclin-cdk binding domain in the N-terminus (Dimova and Dyson, 2005). The repressors E2F4 and E2F5 possess instead a nuclear export sequence (NES) and lack a cdk binding domain. These differences may account for the disparity in transactivational activity between the two categories of E2F transcription factors.

The Rb protein family, comprised of Rb, p107, and p130, is otherwise known as the 'pocket protein' family due to the conserved domain structure that forms a molecular binding pocket covering the activation domain of E2F proteins (Du and Pogoriler, 2006). There is a non-homologous spacer region in the middle of the pocket domain, dividing it into pocket domains A and B. p107 and p130, but not Rb, have a cyclin-cdk binding domain in that spacer region (Du and Pogoriler, 2006). Fittingly, the E2F activators of transcription, E2Fs 1 to 3, bind the pocket protein Rb with the highest affinity, while the repressors, E2Fs 4 and 5, preferentially bind p107 or p130 (Attwooll et al., 2004). The transactivation and retinoblastoma (Rb) protein-binding domains are only found in E2Fs 1 to 5, at the C-terminus (Degregori and Johnson, 2006). During the cell cycle, hypophosphorylated pocket proteins bind their respective E2F factors as complexes and actively repress transcription of genes that contain E2F consensus binding sites in their promoters (Cobrinik, 2005).

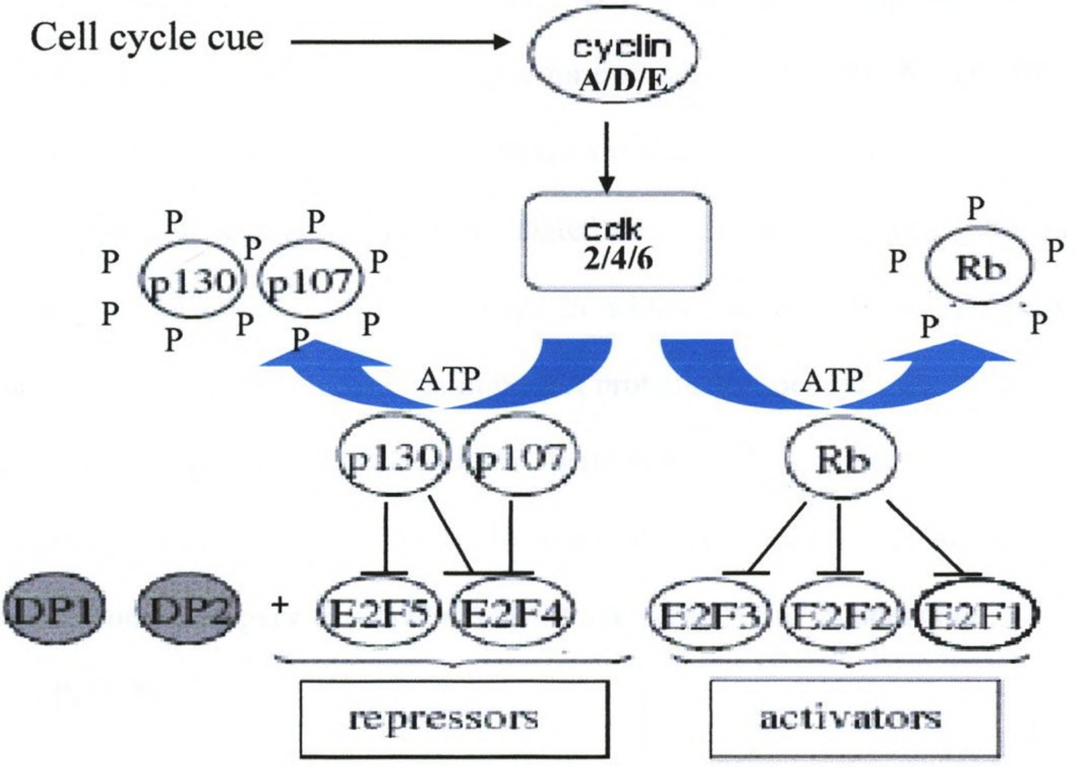


Figure 2- The Cyclin-cdk-Rb-E2F Cell Cycle Pathway of Transcriptional Regulation. Different cyclin proteins are upregulated at each stage of the cell cycle, which bind and activate their respective cyclin-dependent kinase (cdk) partners. The active G_1 (i.e.-cyclin D-cdk4/6) or G_1-S transition (i.e.-cyclin A/E-cdk2) cyclin-cdk complexes can phosphorylate and inactivate members of the Rb protein family, comprised of p130, p107, and Rb, which act as inhibitors of the E2F transcription factor family. The members of the E2F protein family require a DP protein co-factor to bind DNA and they are traditionally subdivided into two groups: repressors (E2F4 & 5) or activators (E2F1-3) of transcription. E2F transcription factors differentially control expression of a wide array of genes in various circumstances in order to affect such processes as proliferation, differentiation, and apoptosis.

However, when G_1 phase (i.e.-cyclin D-cdk4/6) or G_1-S transition (i.e.-cyclin A/E-cdk2) cyclin-cdk complexes are activated, they phosphorylate pocket proteins. In the hyperphosphorylated state, pocket proteins lose their binding affinity and are released from the E2F complexes (Cobrinik, 2005). Consequently, repression of gene transcription is relieved and made available for transactivation, either by the activator E2Fs or other transcription factors. This process is illustrated in figure 2.

Differential regulation of E2F-mediated transcription of various genes is an active area of research. A couple ways in which this may occur are through phosphorylation of different residues in pocket proteins by specific cdks and through DNA sequence-specific recruitment of particular E2F proteins (Tao et al., 1997;Zarkowska and Mittnacht, 1997). However, the exact mechanisms of regulation of the E2F-controlled gene expression network are yet to be fully understood.

1.7- HYPOTHESIS

Cyclin-dependent kinases mediate neuronal apoptosis by regulating the transcriptional induction of pro-apoptotic BH3-only genes.

CHAPTER 2- MATERIALS AND METHODS

2.1- CELL CULTURE

2.11-CD1 Cortical Neurons

Cortical neurons were dissected from the brains of day 14.5-15.5 mouse embryos of timed pregnant CD1 females purchased from Charles River Laboratories. The embryos were extracted from the female immediately following her sacrifice by an intra-peritoneal injection of euthanyl and placed in an ice cold solution of HBSS (Sigma- #H9394). Each embryo was dissected under a dissecting microscope and cerebral cortices were removed and subsequently stripped of their meninges.

Pooled cerebral cortices were treated with a solution of HBSS containing 1.2 mM $MgSO_4$ and 75 mg/ml trypsin (Sigma- #T4549) which was placed on a rotor in a 37°C incubator for 25 minutes to dissociate the cortical neurons. Trypsinization of the cortical neurons was subsequently stopped by addition of a solution containing 1.2 mM $MgSO_4$, 0.2 mg/ml trypsin inhibitor (Roche- #109878), and 0.25 mg/ml DNase I (Roche- #1284932) to each tube of cells suspended in HBSS. The cells were centrifuged for 5 minutes at 300xg and the supernatant was removed. The cell pellets were resuspended in a fresh solution of HBSS containing 3 mM $MgSO_4$, 1 mg/ml trypsin inhibitor, and 0.65 mg/ml DNase I.

Next, the solutions of trypsinized cells in suspension were subjected to 8-10 rounds of trituration through a flame polished glass pipette. Once triturated, the solutions of cells were left to stand for 5 minutes and the supernatant was removed carefully and transferred to a fresh tube. The solutions of triturated cells were spun down at 300xg for 5 minutes. The supernatant was then removed and the cells were

gently resuspended in complete neurobasal media, which contains 1x B27 (Invitrogen- #17504-044) and N2 (Invitrogen- #17502-048) supplements, 1 mM glutamax (Invitrogen- #35050-061), 50 U/ml penicillin:50 μg/ml streptomycin (Invitrogen- #15140-122).

The concentration of each solution of cells was determined by manual count using a hemocytometer under a light microscope. The solution of cells was then diluted to a concentration of 1 million cells per ml for plating of 5 ml per 60 mm (VWR- #CA25382-330) poly-D-lysine (VWR- #CACB354210) coated dish, or 0.5 million cells per ml for plating of 600 μl per well of a poly-D-lysine coated 4-well dish (VWR- #CA16777-539). After plating, the dishes were stored in a humidified 37°C incubator at 5% CO_2 and subjected to treatments following 4-5 days in culture.

2.12-CD1 Cerebellar Granule Neurons

These cells were extracted from 7-8 day old CD1 mouse pups born of females purchased from Charles River Laboratories. The pups were euthanized one at a time by decapitation and their cerebellums were dissected. Each cerebellum was cleaned of its meninges, diced into small fragments, and pooled together in one dish of ice cold HBSS. The cells were processed in the same manner as the CD1 cortical neurons. The media used to culture the cerebellar granule neurons (CGNs) was the same as for the cortical neurons except complete neurobasal media also contained 25 mM KCl.

The solutions of processed cells were diluted at the time of plating with media containing a pre-determined multiplicity of infection (M.O.I.) of the following adenoviral vectors, 20 M.O.I. of Ad-DN-cdk4, 25 M.O.I. of Ad-p16, or an equivalent

M.O.I. of Ad-EGFP, to a concentration of 1.5 million cells per ml for plating of 5 ml per 60 mm poly-D-lysine coated dish, or 0.75 million cells per ml for plating of 700 µl per well of a poly-D-lysine coated 4-well dish. After plating, the dishes were stored in a humidified 37°C incubator at 5% CO_2 and subjected to treatments following 48 hours in culture.

2.13- Puma KO & Conditional Rb KO Cortical Neurons

Cortical neurons were extracted from the dissected cortices of day 14.5-15.5 mouse embryos. Puma knockouts were produced from the pairing of male and female C57BL/6 mice heterozygous (+/-) for the Puma gene. The Rb gene was conditionally knocked out by the cre recombinase system. This system works on the principle of lox P sites (Flox), flanking exon 18 of the Rb gene, which are recognized and excised by cre recombinase. Cre was inserted in the genome under the control of the brain factor-1 promoter, an essential protein expressed only in the forebrain. The breeding scheme for crossing Rb-Flox and Cre mice is described in Appendix G.

A tail sample and the remainder of the brain were taken from each embryo and placed in separate 1.5 ml microcentrifuge tubes. The tail samples were used for genotyping (see 2.2- genotyping) and the remainder of the brains were stored at -80°C in case more tissue was needed.

Dissection of the cerebral cortices was performed in the same manner as for the CD1 cortical neurons, except the cerebral cortex from each embryo was processed individually in 1.5 ml microcentrifuge tubes. The processed cells from each individual embryo were resuspended in 3 ml of complete neurobasal media and the concentration of cells was determined as before. Rb cortical neurons were plated at

$2x10^6$ cells per poly-D-lysine coated 35 mm dish (VWR- #CA25382-334) and $3x10^5$ cells per well in a poly-D-lysine coated 4-well dish. After plating, the dishes were stored in a humidified 37°C incubator at 5% CO_2 and subjected to treatments following 4-5 days in culture.

2.2- GENOTYPING

The tail samples from each embryo were dissolved in DNA lysis buffer, which is composed of 100 mM Tris buffer, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 0.2 mg/ml Proteinase K (Invitrogen- #25530-015). Each tail sample was then vortexed and placed in a 55°C dry bath overnight until the tail was completely digested.

The DNA in solution was extracted by adding 500 µl of phenol-chloroform to each tail sample from a solution of phenol:chloroform:iso-amyl alcohol (25:24:1). Each tube was shaken forcefully for approximately 30 seconds, allowed to sit for a minute, and then centrifuged at 12,000xg for 10 minutes at room temperature. The resulting aqueous phase was carefully transferred to new tubes, avoiding contact with the interphase, and 500 µl of cold 95% isopropanol was added to each tube. The tubes were shaken until a white, stringy DNA precipitate appeared in solution. This DNA precipitate was spun down at 12,000xg at 4°C for 10 minutes.

After aspirating the supernatant, 1 ml of 70% ethanol was added to each tube and mixed by inversion. The DNA precipitate was spun down again at 12,000xg for 5 minutes at 4°C. This time, the supernatant was aspirated off carefully so as not to disturb the pellet and the tubes were left open to dry in a fume hood until no ethanol remained. The dried DNA pellets were resuspended in 1x TE buffer pH 8.0. All of the tubes were incubated at 37°C in a dry bath for 1 hour to help dissolve the DNA

pellet. The concentration and quality of each sample were determined by UV spectrophotometry (260nm/280nm) and the samples were used as template in PCR for genotyping.

The embryonic DNA samples, as well as positive and negative control samples, were set up for one PCR, in the case of Puma genotyping, and two separate PCRs: one for Rb-Flox and the other for Cre, in the case of Rb. The reagents, cycling conditions, primer sequences, and expected products for these PCRs can be found in appendices A & B, respectively.

Once the PCR was complete, 5 ul of 6x loading dye was added to each sample. The samples, along with a 1 kb+ DNA ladder (Fermentas), were subsequently loaded onto a 1.5% agarose gel submersed in 1x TAE buffer. The gel was run at 120V for approximately 25-30 minutes, or until the positive control bands were adequately separated. The ethidium bromide stained gel was visualized under UV light inside a gel dock system and a hard copy photograph was taken.

In the case of survival studies with Puma embryos, only neurons with the homozygous wildtype and null Puma genotypes were used for experimentation. As for studies involving Rb knockouts, only the cells from the embryos with the genotypes Flox/Flox Cre negative and Flox/Flox Cre positive were used for experimentation. Excision of exon 19 of the Rb gene by Cre recombinase under the control of the BRF1 promoter in the cortex of Flox/Flox Cre positive embryos was verified by PCR with DNA samples extracted from the cortical neurons (shown in Appendix B) harvested in Trizol® (Invitrogen- #15596-026), as per the manufacturer's instructions.

2.3- DRUG TREATMENTS

Drug treatments were applied to the cortical neurons four to five days after plating and to the CGNs two days after plating. Each drug was diluted with the appropriate volume of media to achieve its desired final concentration. The NMDA receptor antagonist MK-801 and the glutamine derivative glutamax were applied to the cells during all treatments in order to prevent any secondary excitotoxic cell death. All of the pertinent information for each drug used in this study is summarized in Table 1.

Table 1- Summary of Drug Treatments

Name	Effects	Company	Catologue Number	Stock conc. (mM)	Solvent	Final conc. (μM)
Camptothecin	DNA damage-Topoisomerase I inhibitor	Sigma	C9911	10	DMSO	10
Etoposide	DNA damage-Topoisomerase II inhibitor	Sigma	E1383	10	DMSO	10
TBH	Oxidative stress-Peroxide radical generator	Sigma	B2633	100	Water	200
NOC-12	Oxidative stress-Nitric oxide donor	Calbiochem/ VWR	80053-450	100	0.1M NaOH	200
Bleomycin	Oxidative stress-Superoxide generator	Sigma	B8416	1.5 U/ml	Water	0.05 U/ml
Tunicamycin	ER stress-N-glycosylation inhibitor	Sigma	T7765	10	DMSO	2
Thapsigargin	ER stress-SERCA inhibitor	Sigma	T9033	1	DMSO	2
Flavopiridol	cki (cdk4/6)	NIH-NCI	Gift	25	DMSO	1
MK-801	NMDA receptor antagonist	Sigma	M107	10	Water	10

DMSO was tested as a solvent control. The volumes of diluted drug added to the different types of dishes were 1/26th of the final volume of media, as follows: 80 µl/35 mm dish, 200 µl/60 mm dish, 24 µl/well in a 4-well dish for cortical neurons, and 28 µl/well in a 4-well dish for CGNs.

2.4- SURVIVAL STUDIES

2.41-CD1, Puma KO, & Conditional Rb KO Cortical Neurons

After 24 hours of treatment with DNA damaging agents or 30 hours of treatment with ER & oxidative stressors, the 4-well dishes were removed from the incubator. The media in each well was aspirated off and replaced with 300 µl of Lana's fixative, which is composed of 4% paraformaldehyde (Sigma- #P6148), 160 mM dibasic and monobasic sodium phosphate, and 0.2% picric acid (Sigma- #80456) dissolved in double distilled water. The fixative was left on the cells for 30 minutes, at which point it was aspirated off and the cells were washed twice in a solution of 1x PBS pH 7.4. Hoechst 33258 stain (Sigma- #861405) was diluted to 1µg/mL in 1x PBS pH 7.4 and 300 µl of this solution was added to each well. The 4-well dishes were left to sit in the stain for 30 minutes and covered to protect the stain from light exposure. The staining solution was aspirated off from each well and replaced with 300 µl of 1x PBS pH 7.4 for long term storage. Each 4-well dish was wrapped in parafilm, covered in aluminum foil, and stored at 4°C until they were imaged.

Imaging was done with a fluorescence microscope and digital camera connected to a computer that runs the Northern Eclipse 7.0 software. The labels on each 4-well dish were covered and coded blindly by another lab member and subsequently decoded following scoring. Pictures at five representative locations per

well were taken at 40x magnification and at least 300 nuclei per treatment were manually counted in each experiment. A neuron was considered apoptotic if the Hoechst-stained nucleus was condensed and intensely fluorescent. The number of apoptotic nuclei were divided by the total number of nuclei counted per well to get the percentage of apoptosis for the treatment.

2.42-CD1 Cerebellar Granule Neurons

The cells infected with the EGFP and DN-cdk4 adenoviruses were Hoechst 33258 stained in the same manner as the cortical neurons, since the visualization of DN-cdk4 expression was made possible by its GFP tag. However, the adenovirus-delivered p16 protein was not tagged with GFP and therefore, had to be immunostained in order to be seen (see immunocytochemistry).

Following immunostaining, imaging was done with a fluorescence microscope and digital camera connected to a computer that runs the Northern Eclipse 7.0 software. Pictures at five representative locations per well were taken at 40x magnification and only the GFP positive cells in each picture were manually counted. At least 300 nuclei per treatment were scored in each experiment. The number of apoptotic nuclei was divided by the total number of nuclei in the GFP positive population to get the percentage of apoptotic cells in each treatment.

2.43-Immunocytochemistry- cytochrome c and p16INK4a

Neurons were fixed in Lana's fixative and washed in three changes of 1x PBS, after which they were incubated for 2 hours with monoclonal antibodies directed against cytochrome c or p16INK4a (both BD-Pharmingen) diluted in 1x PBS containing 0.1% Triton-X100 and 2% bovine serum albumin. Neurons were then

washed in three changes of 1x PBS and incubated for 45 minutes with Alexa-488 conjugated secondary antibodies (Molecular Probes) diluted in 1x PBS containing 0.1% Triton-X100 and counterstained with Hoechst 33258 (1μg/mL). Immunostaining for p16INK4a was used to identify positively infected cells during scoring for survival studies. In order to evaluate mitochondrial membrane permeabilization, cytochrome c was visualized in the cells by fluorescence microscopy. Those cells displaying a punctate, cytoplasmic staining pattern were considered to have maintained mitochondrial membrane integrity. A minimum of 300 cells was scored per treatment in each separate experiment.

2.5- qRT-PCR

The RNA samples were harvested and extracted from cells using Trizol® (Invitrogen), according to the manufacturer's instructions. The concentration of each sample was determined in duplicate by UV spectrophotometry. RNA samples were diluted in DNase, RNase-free H_2O at a concentration of 5 ng/μl.

20 ng of RNA template was used in quantitative, one step RT-PCR using the reagents from the QuantiTect SYBR green RT-PCR kit from QIAGEN. A unit composed of the PTC-200 thermocycler and Chromo4 fluorescence detector, from MJ Research, performed the thermocycling and fluorescence detection. The reagents, cycling conditions, and primer sequences for qRT-PCR are summarized in Appendix C.

Analysis of the qRT-PCR results was performed using the software provided for the Chromo4 fluorescence detector, Opticon Monitor 3.0. The delta cycle threshold (ΔCt) method was employed to obtain the fold change in mRNA levels

relative to untreated control samples for each transcript normalized to ribosomal S12. The cycle threshold was set arbitrarily within the exponential phase of the reaction for each independent experiment. The equation used to calculate fold change in the gene of interest (G.O.I.) mRNA levels is as follows:

$$\text{Fold change} = \frac{2^{(\text{G.O.I. Ct CTRL} - \text{G.O.I. Ct Treated})}}{2^{(\text{S12 Ct CTRL} - \text{S12 Ct Treated})}}$$

All PCR reactions exhibited similar efficiencies. RT-PCR products of each transcript from preliminary experiments were sequenced in order to confirm primer specificity. Subsequently, a melting curve was run to conclude every qRT-PCR in order to verify that only the correct product was produced during the reaction.

2.6- CASPASE-3-LIKE ACTIVITY ASSAY

Cortical neurons were harvested in fresh PBS with a cell scraper and transferred to a 1.5 ml microcentrifuge tube. The cells were spun down at 400xg for 5 minutes at 4°C and resuspended in approximately 100 µl of caspase lysis buffer, which is composed of 10 mM Hepes buffer, 1 mM KCl, 1.5 mM MgCl2, 10% glycerol, 0.1% NP40, 1 µM DTT, and fresh protease inhibitor cocktail made up of 0.2 mg/ml PMSF (BIOSHOP- #PMS123), 5 ug/ml aprotinin (Sigma- #A1153), and 2 ug/ml leupeptin (Sigma- #L9783).

The cells were left to lyse on ice for half an hour, with periodic agitation. Cell lysates were then cleared by centrifugation at 13,000xg for 5 minutes at 4°C. The supernatant containing the protein was transferred to a fresh tube and protein concentration was determined by a Bio-Rad® Bradford protein assay, as per the manufacturer's instructions. 5 µg of protein was added to 200 µl of caspase assay buffer, which contains 25 mM Hepes buffer, 10 mM DTT, 10% sucrose, 0.1%

CHAPS (Sigma- #C9426), and 15 μM of the caspase-3 substrate Ac-DEVD-AFC (BIOMOL- #P409).

Fluorescence for each sample was recorded on a WALLAC 1420 plate reader, in which the samples were continuously incubated at 37°C. Fluorescence was measured (Excitation 400 nm, Emission 505 nm) every 15 minutes over a one hour interval.

The raw data for these experiments was recorded as arbitrary units of fluorescence. In order to obtain meaningful data to compare the differences between samples, the data was transformed by subtracting the value of the complete assay buffer control from the value of each sample at the same time point to get the background corrected value. Then, the background corrected value of each sample at the initial time point was subtracted from the background corrected value of the same sample at the half hour time point. Finally, each of these values was divided by the value of the control sample to get a fold change in caspase-3 activity over a half hour period.

2.7-STATISTICS

Data from the Puma knockout survival, cytochrome c, and caspase-3 activity assays were analyzed by a one-tailed t-test. The results from the survival, BH3-only gene qRT-PCR, and caspase-3 activity studies on CD1 cortical and cerebellar granule neurons were analyzed by one-way ANOVA and the reported p-values are results from Tukey's post-hoc test. The results from the survival and PUMA transcription studies of the conditional Rb knockout mice were analyzed by two-way ANOVA and the reported p-values are results from the Bonferroni's post-hoc test.

CHAPTER 3- RESULTS

3.1-DNA damage, Oxidative Stress, and ER Stress Trigger the Transcriptional Induction of Puma

Our laboratory has determined that the multi-BH domain pro-apoptotic Bcl-2 family protein BAX is a critical regulator of DNA damage-, oxidative stress-, and ER stress- induced neuronal apoptosis. Therefore, in the present study we investigated the mechanisms that regulate BAX activation in these neuronal death paradigms.

Previous studies in our lab demonstrated that the induction of cell death required *de novo* gene expression as the transcription and translation inhibitors actinomycin D and cycloheximide efficiently blocked cell death (see Appendix A). Since BH3-only proteins are known to be key regulators of BAX activation, we examined the expression profiles of various BH3-only genes in neurons following exposure to DNA damage, oxidative stress, or ER stress.

As shown in figures 3 A, B, and C, the oxidative stressors NOC-12, t-butyl hydroperoxide, and bleomycin all triggered a sustained induction of the BH3-only member Puma (approximately 5-fold at 10 hrs) that was evident within 5 hours of drug exposure. It was also noted that the BH3-only members Bim and Noxa were differentially induced in response to the different types of oxidative stress. Specifically, the nitric oxide generator NOC-12 triggered the induction of Bim whereas the peroxyl- and superoxide- free radical generators t-butyl hydroperoxide and bleomycin triggered the induction of Noxa. In contrast, the expression of other BH3-only family members including Bid and Bad or the multi-BH domain member Bax was not altered in response to oxidative stress.

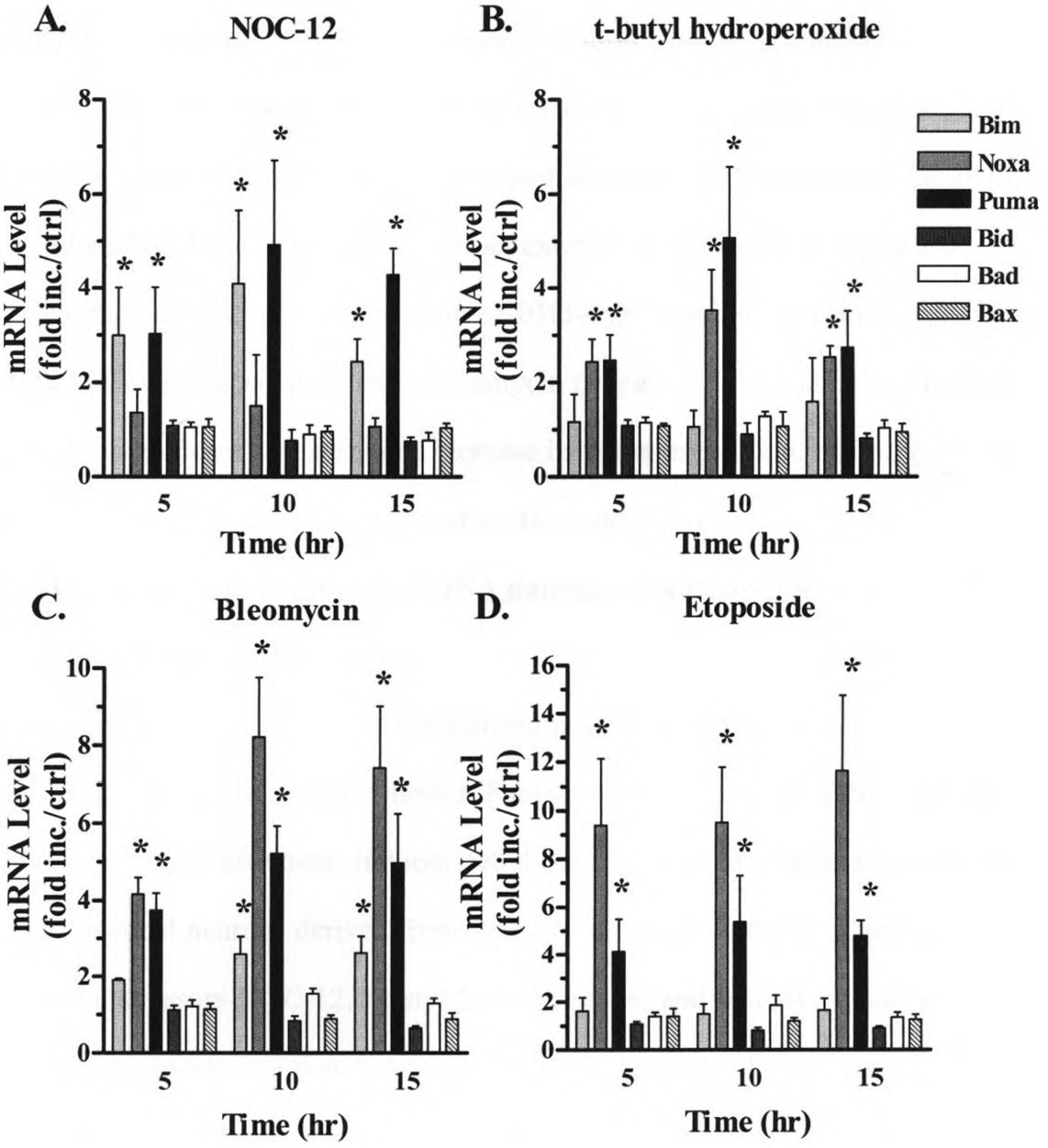


Figure 3. Puma expression is induced in neurons following exposure to oxidative stress and DNA damage. RNA was isolated from cortical neurons at the indicated times following treatment with oxidative stressors: (A) NOC-12 (200 μM), (B) t-butyl hydroperoxide (200 μM), (C) Bleomycin (0.05 U/ml), or the DNA damaging agent: (D) Etoposide (10 μM) and mRNA levels of Bcl-2 family members were determined by qRT-PCR. Expression was normalized to S12 mRNA levels and is reported as mean ± sem of fold increase over corresponding untreated control cells ($n \geq 3$). Significant increases over controls as compared to Bax mRNA levels were determined by one-way ANOVA followed by Tukey's post hoc test at each time point ($p<0.05$).

Since DNA damage is thought to be a prominent effect of oxidative stress we also examined the expression of BH3-only genes in neurons following treatment with the topoisomerase II inhibitor etoposide, which is known to generate double-stranded DNA lesions. Etoposide also triggered a robust and selective induction of the BH3-only members Noxa and Puma and to a lesser extent Bim, as shown in figure 3 D.

We then examined the expression of BH3-only members in neurons exposed to the ER stressors thapsigargin and tunicamycin (Figure 4). Interestingly, ER stress also led to a significant and sustained increase in Puma expression, and an early but transient induction of the BH3-only members Bim and Noxa.

3.2-PUMA is a Critical Regulator of DNA damage-, Oxidative Stress-, and ER Stress- induced Neuronal Apoptosis

Since Puma expression was upregulated in response to all of the death stimuli tested, we sought to determine whether Puma was necessary for DNA damage-, oxidative stress-, and ER stress- induced cell death. In order to address this question, we treated cortical neurons derived from Puma-/- mice and wildtype littermates with the oxidative stressors NOC-12, t-butyl hydroperoxide, and bleomycin and assessed the extent of apoptosis as a function of time (Figure 5). All of these treatments led to a robust induction of apoptosis within 24 hours in wildtype neuronal cultures, but not in the Puma-deficient cultures. Even after 48 hours, very few Puma-/- neurons were found to exhibit apoptotic morphology (Figure 5 B). Similarly, Puma-deficient neurons were found to be remarkably resistant to ER stress- and DNA damage-induced neuronal apoptosis (Figure 6).

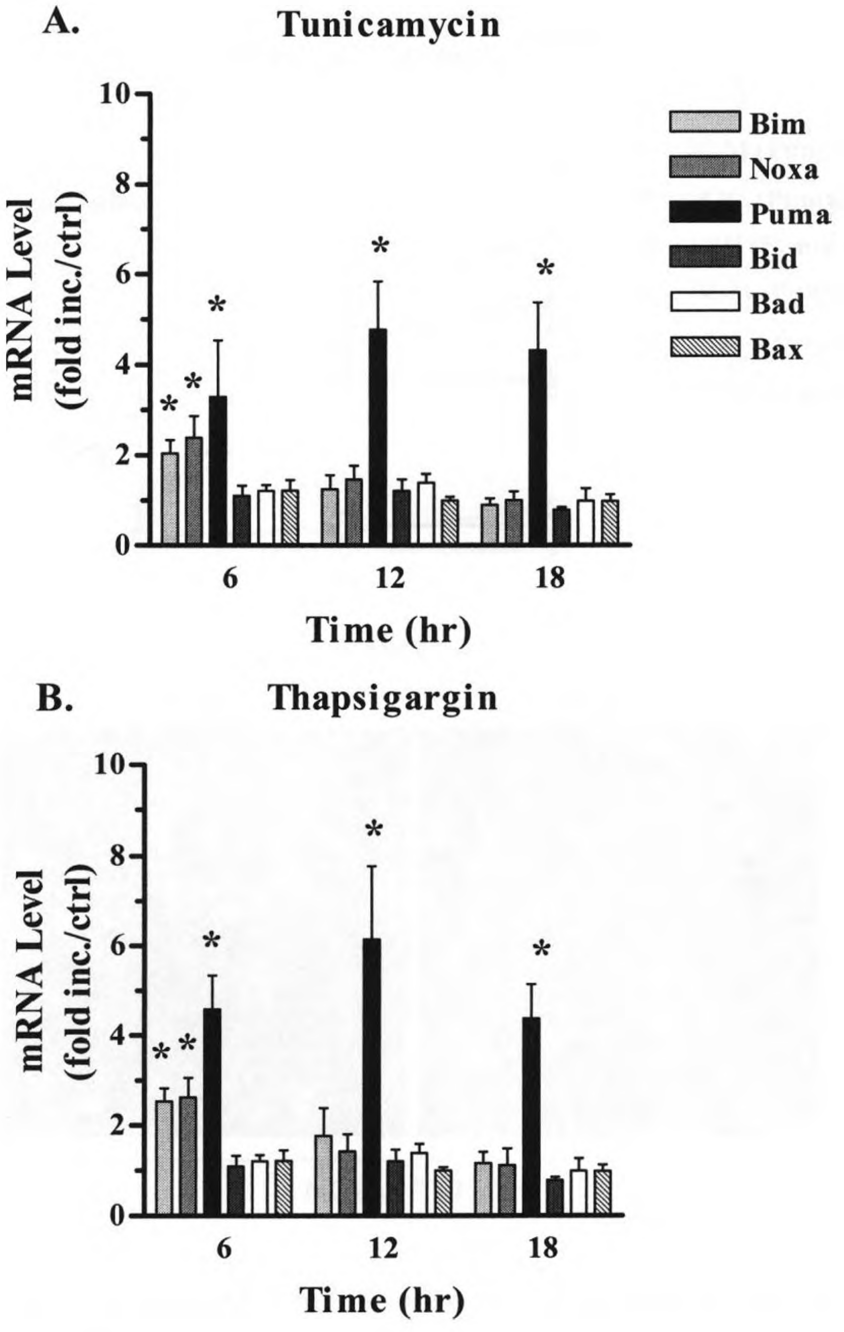


Figure 4. Puma expression is induced in neurons following exposure to ER-stress. RNA was isolated from cortical neurons at the indicated times following treatment with the ER-stressors: (A) Tunicamycin (2 μM) and (B) Thapsigargin (2 μM) and mRNA levels of Bcl-2 family members were determined by qRT-PCR. Expression was normalized to S12 mRNA levels and is reported as mean ± sem of fold increase over corresponding untreated control cells ($n \geq 3$). Significant increases over controls as compared to Bax mRNA levels were determined by one-way ANOVA followed by Tukey's post hoc test at each time point (* $p<0.05$).

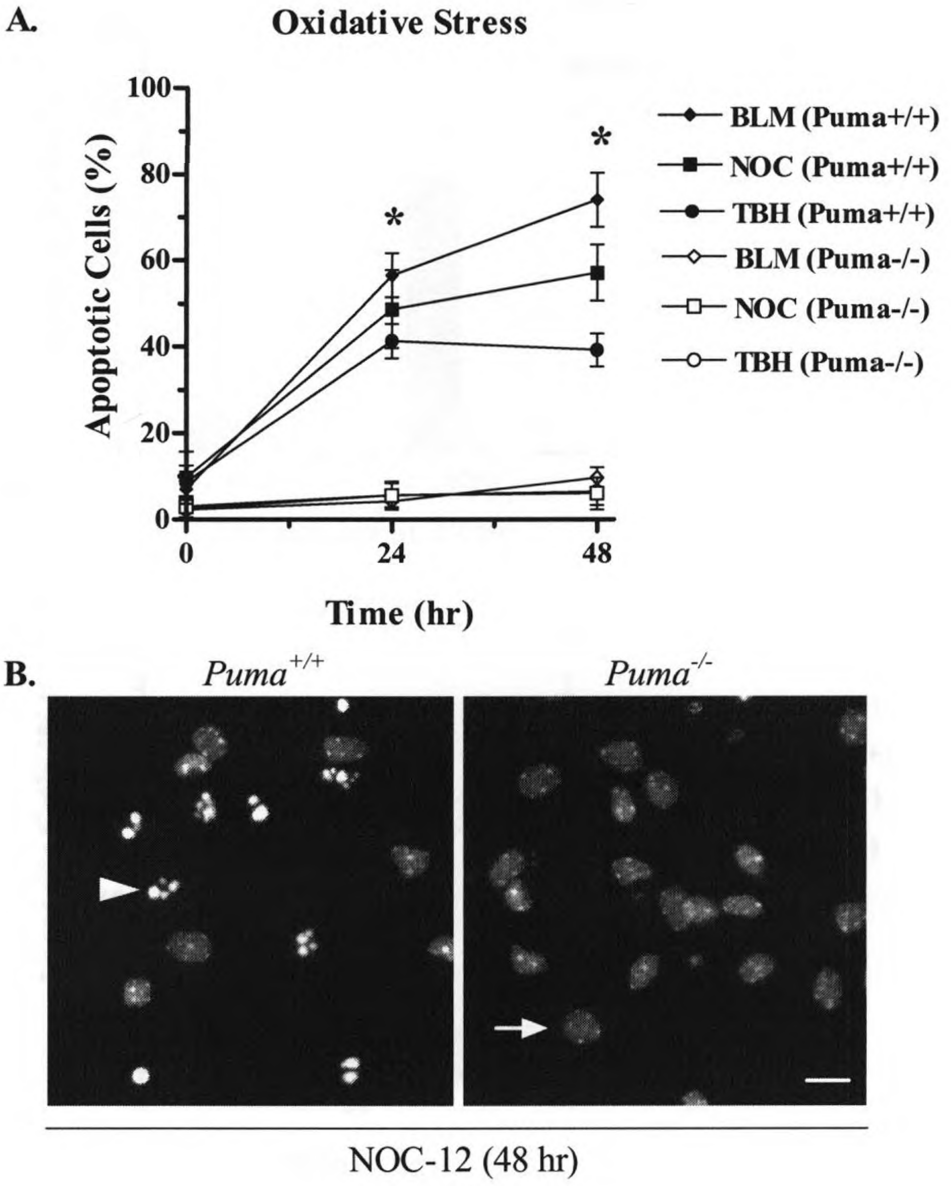


Figure 5. PUMA is essential for oxidative stress-induced neuronal apoptosis. (A) Cortical neurons cultured from Puma+/+ (closed symbols) and Puma-/- (open symbols) littermates were treated with TBH (200 μM), NOC-12 (200 μM), or BLM (0.05 U/ml). Data represents the mean ± sem of the fraction of apoptotic cells counted by Hoechst 33258 staining at 24 and 48 hr, ($n^3$3 per genotype). A one-tailed t-test determined NOC-12, TBH, and BLM all induced significantly less apoptosis in Puma-/- neuronal cultures than in Puma+/+ cultures at both 24 and 48 hr (all p values <0.01). (B) Representative images of Hoechst 33258 stained Puma+/+ and Puma-/- neurons captured 48hr after NOC-12 treatment, scale bar = 10 μm. Note that many Puma+/+ neurons (arrowhead), but not the Puma-/- neurons (arrow), exhibit an apoptotic nuclear morphology following NOC-12 treatment typified by chromatin condensation and fragmented bodies.

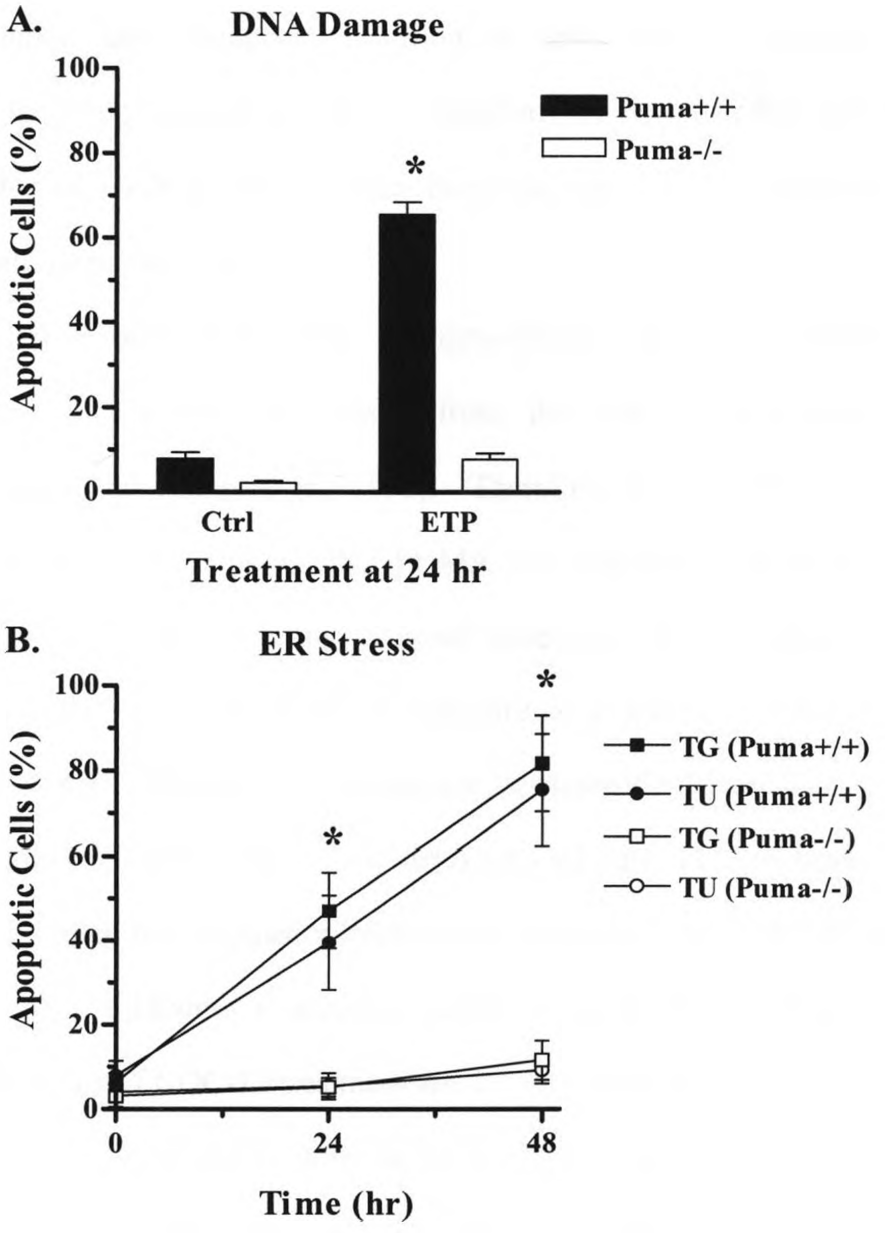


Figure 6. PUMA is essential for DNA damage- and ER stress- induced neuronal apoptosis. (A) Cortical neurons cultured from Puma+/+ and Puma-/- littermates were treated with the DNA damaging agent ETP (10 μM) and data represents the mean ± sem of the fraction of apoptotic cells counted by Hoechst 33258 staining at 24 hr, (n= 8 Puma+/+ and 9 Puma-/- mice). Etoposide induced significantly less apoptosis in Puma-/- neurons than in Puma+/+ neurons (*$p<0.01$). (B) Cortical neurons cultured from Puma+/+ (closed symbols) and Puma-/- (open symbols) littermates were treated with the ER stressors TG (2 μM) or TU (2 μM). Data represents the mean ± sem of the fraction of apoptotic cells counted by Hoechst 33258 staining at 24 and 48 hr, (n= 6 individual Puma+/+ and Puma-/- mice). A one-tailed t-test determined TG and TU induced significantly less apoptosis in Puma-/- neuronal cultures than in Puma+/+ cultures at both 24 and 48 hr (all *p values <0.01).

It has previously been determined that BAX is crucial for mitochondrial permeabilization and caspase-3 activation in stress-induced neuronal apoptosis (Cregan et al., 1999;Marzo et al., 1998). Therefore, we reasoned that if PUMA was a key regulator of BAX activation, then Puma-deletion should inhibit these BAX-mediated cell death processes.

It has previously been shown that cytochrome c immunoreactivity is rapidly lost in neurons following its release from the mitochondria (Cregan et al., 2002;Deshmukh and Johnson, Jr., 1998). Therefore, we examined cytochrome c immunostaining to determine whether PUMA was required for mitochondrial outer membrane permeabilization during neuronal apoptosis. As expected, a substantial fraction of wildtype neurons had lost cytochrome c staining within 30 hours of exposure to NOC-12, bleomycin, thapsigargin, or etoposide (Figure 7 A). In contrast, the vast majority of Puma-/- neurons (>90%) retained punctate cytochrome c staining indicating that they had retained mitochondrial membrane integrity. Representative images of the cytochrome c staining pattern in wildtype and Puma-/- neurons following 30 hours of NOC-12 treatment are shown in Figure 7 C.

Furthermore, consistent with the role of cytosolic cytochrome c in Apaf1-mediated caspase activation we found that, unlike wildtype neurons, Puma-/- neurons did not exhibit caspase-3-like activity following exposure to DNA damage, oxidative-, or ER- stressors (Figure 7 B). Taken together, PUMA appears to be a critical regulator of BAX activation during neuronal apoptosis induced by these stresses.

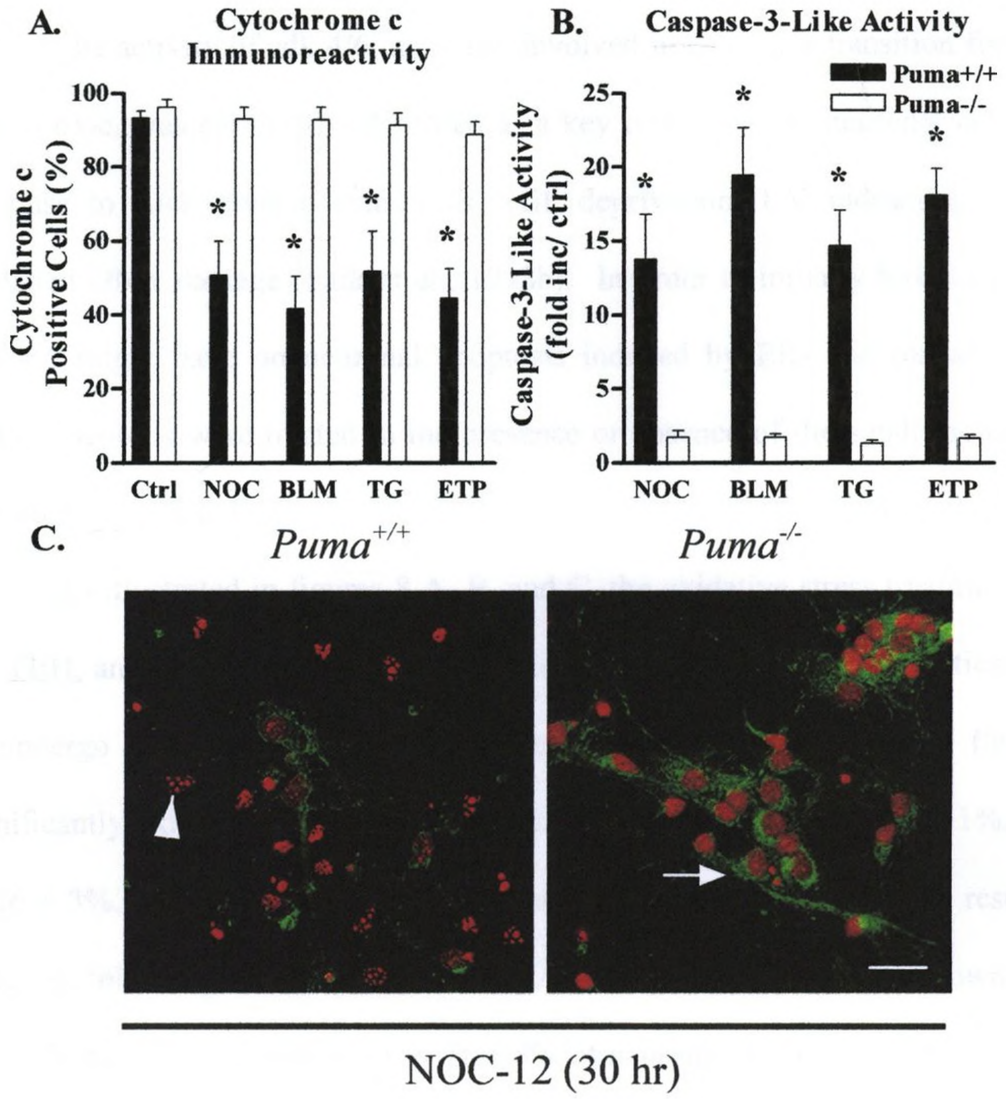


Figure 7. PUMA regulates cytochrome c release and caspase-3-like activation during neuronal apoptosis. (A) Cortical neurons derived from Puma+/+ and Puma-/- littermates were treated with NOC-12 (200 μM), BLM (0.05 U/ml), TG (2 μM), or ETP (10 μM) and the fraction of cells retaining mitochondrial cytochrome c was determined at 30 hr. (B) Caspase-3-like activity was assayed in Puma+/+ and Puma-/- neurons 30 hr following exposure to NOC-12, BLM, TG, or ETP. Data represents the mean ± sem of cultures derived from individual Puma+/+ and Puma-/- mice (n=3-4 per genotype). * indicates significant difference between Puma+/+ and Puma-/- neurons following the same treatment as determined by one-tailed t-test ($p<0.05$). (C) Representative images of Puma+/+ and Puma-/- neurons treated with NOC-12 (200 μM) for 30 hr and immunostained for cytochrome c (green) and counterstained with Hoechst 33258 (pseudo-colored orange), scale bar = 25 μm. Note that, unlike Puma+/+ neurons (arrowhead), the majority of Puma-/- neurons (arrow) retain punctate cytochrome c staining following NOC-12 treatment.

3.3-Neuronal Apoptosis is Regulated by cdks

The activity of cdk 4/6, normally involved in cell cycle transition from the G_0 to G_1 phase, was previously identified as a key component of neuronal cell death in response to such diverse stimuli as NGF deprivation, UV raditation, and araC-mediated DNA damage (Park et al., 1998b). In order to initially test the effect that cdk 4/6 might have on neuronal apoptosis induced by ER- and oxidative- stress, cortical neurons were treated in the presence or absence of the small molecular cdk inhibitor flavopiridol.

As illustrated in figures 8 A, B, and C, the oxidative stress treatments, NOC-12, TBH, and BLM induced 57 ± 2%, 54 ± 5%, and 58 ± 4% of the cortical neurons to undergo apoptosis after 30 hours, respectively. The addition of flavopiridol significantly reduced the levels of apoptosis induced by NOC-12 to 23 ± 1%, by TBH to 16 ± 3%, and by BLM to 13 ± 2%, nearly to control levels. Similar results were observed following treatment with the DNA damaging agent ETP. As shown in figure 8D, ETP had induced apoptosis in 71 ± 4% of neurons by 24 hours. However, in the presence of flavopiridol ETP-induced apoptosis was significantly decreased to 19 ± 1%.

Figures 8 E and F illustrates the levels of apoptosis in cortical neurons for 30 hour treatments with the ER stressors, TU and TG, which induced 43 ± 4% and 49 ± 3% of the cells to die by apoptosis, respectively. Once again, the addition of flavopiridol significantly decreased the levels of apoptosis by both ER stressors to approximately 17 ± 3%. These data suggest that cyclin dependent kinase activity promotes stress-induced neuronal apoptosis.

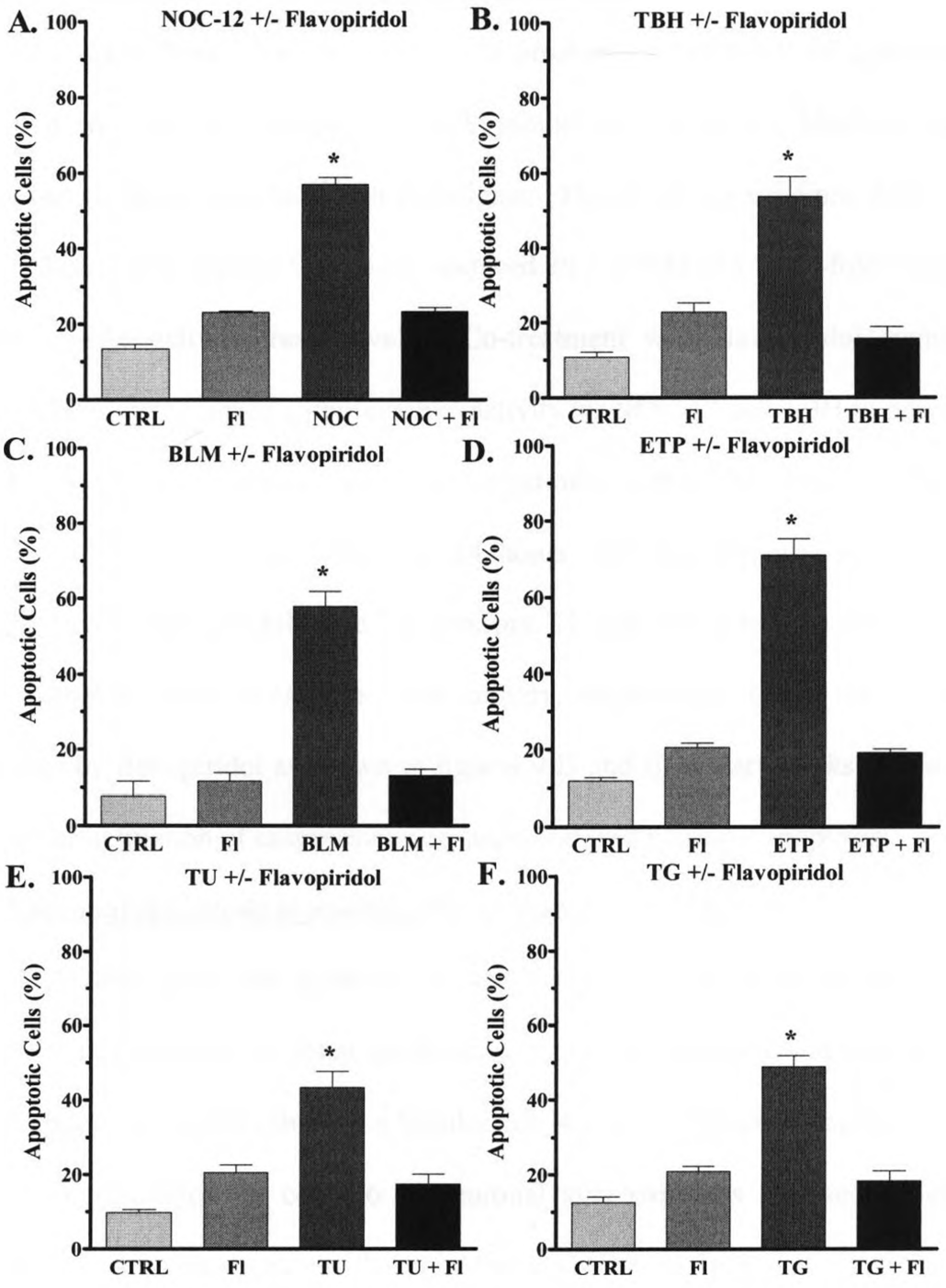


Figure 8. Neuronal Apoptosis is Promoted by cdks in Response to Oxidative Stress, DNA damage, and ER Stress. The fraction of apoptotic cortical neurons were counted by Hoechst 33258 staining following 24 hr induction of oxidative stress: (A) NOC-12 (200 mM, n=5), (B) TBH (200 mM, n=4), (C) BLM (0.05 U/mL, n=3), DNA damage: (D) ETP (10 mM, n=7), or ER stress: (E) TU (2 mM, n=4), (F) TG (2 mM, n=5) in the presence (+) or absence (-) of the cki Flavopiridol (Fl, 1mM). For all data sets: mean ± sem. Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase in apoptosis only in the death stimulus treated neurons as compared to all other groups (*$p<0.001$).

3.4-Caspase-3-Like Activation is Regulated by cdk

Caspase-3-like activity, which is a biochemical hallmark of apoptosis, was assessed in order to determine if cdk inhibition specifically blocked apoptotic processes in these neuronal death paradigms. The oxidative stressors, NOC-12 and TBH illustrated in figures 9 A and B, induced 10 ± 2-fold and 7 ± 1-fold increases in caspase-3-like activity, respectively. Co-treatment with flavopiridol significantly inhibited the induction of caspase-3-like activity by NOC-12 and TBH to near control levels. Similarly, figure 9 C shows that treatment with ETP induced a 14 ± 2-fold increase in caspase-3-like activity at 24 hours, and that this was blocked by co-treatment with flavopiridol. The ER stressors, TU and TG, induced a 10 ± 1-fold and 23 ± 4-fold increase in caspase-3-like activity, respectively, which was completely inhibited by flavopiridol as shown in figures 9 D and E. Clearly, cdks play a crucial role in the activation of caspases during stress-induced neuronal apoptosis.

3.5-Neuronal Apoptosis is Specifically Mediated by cdk 4/6

At this point, the question of which cdks are important in the signalling pathway for activating neuronal apoptosis could not be definitively answered because flavopiridol can inhibit other cdks besides cdk 4 and 6. Therefore, evaluation of the specific contribution by cdk 4/6 to neuronal apoptosis was assessed by enforced expression of the endogenous cdk 4/6 inhibitor p16INK4A (p16).

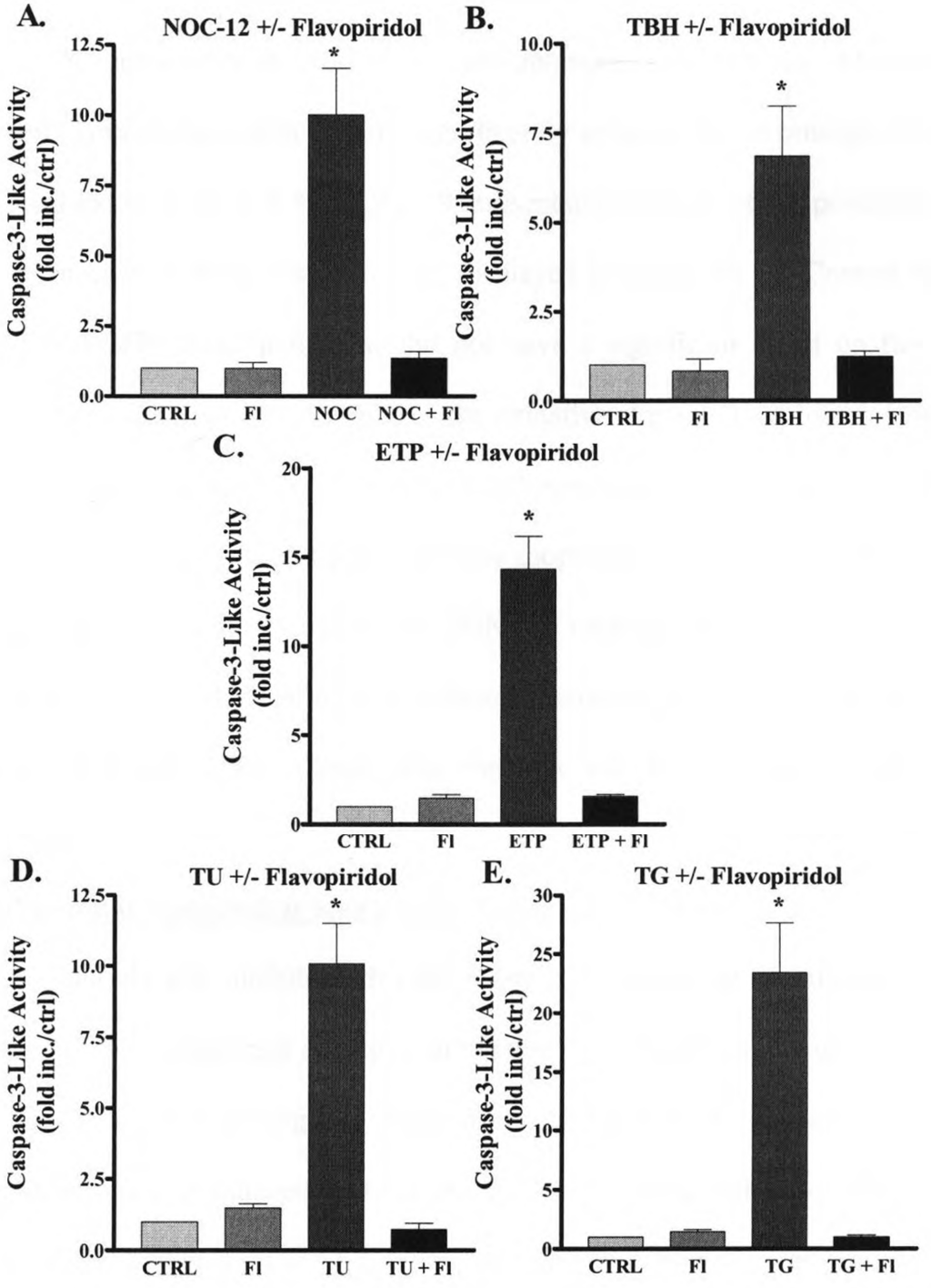


Figure 9. Caspase-3-Like Activity is Promoted by cdks in Response to Oxidative Stress, DNA damage, and ER Stress. Caspase-3-like activity was assayed from protein extracts of E15 CD1 murine cortical neuron cultures subjected to oxidative stress (30hr): (A) NOC-12 (200 mM, n=5), (B) TBH (200 mM, n=4), DNA damage (24hr): (C) ETP (10 mM, n=5), or ER stress (30hr): (D) TU (2 mM, n=6), (E) TG (2 mM, n=3) in the presence (+) or absence (-) of the cki Flavopiridol (Fl, 1mM). For all data sets: mean ± sem. Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase in caspase-3-like activity only in the death stimulus treated neurons as compared to all other groups (*$p<0.001$).

As shown in figure 10A, after 24 hours of treatment with ETP, 15.5 ± 0.9% of the CGNs infected with Ad-EGFP were apoptotic, a significant difference from control. Transduction with Ad-p16 significantly reduced the percentage of apoptotic cells caused by ETP to 1.3 ± 0.3%. Representative images of the positively-stained nuclei in each of these treatments are displayed in figure 10 B. Control infections with Ad-EGFP or Ad-p16 alone did not have a significant effect on the level of apoptosis in these neurons. Similarly, the oxidative stressor BLM induced significant levels of apoptosis in CGNs (52 ± 4% at 30 hours), and this was markedly reduced in neurons transduced with Ad-p16 (15 ± 2% apoptosis). Likewise the ER-stressor TG caused approximately 53 ± 11% of CGNs to undergo apoptosis within 30 hours, whereas only 1.8 ± 0.3% of Ad-p16 infected neurons exposed to TG were apoptotic at this point (Figure 10D). These data implicate cdk 4/6 in stress-induced neuronal apoptosis.

3.6-Neuronal Apoptosis is Specifically Mediated by cdk 4

The cki p16 inhibits both cdks 4 and 6, therefore, to specifically assess the role of cdk4 we examined apoptosis in neurons transduced with an adenoviral vector expressing a dominant-negative form of ck4 (DN-cdk4). As shown in figure 11A, treatment with ETP induced 17.4 ± 1.9% of CGNs infected with Ad-EGFP to undergo apoptosis by 24 hours. The CGNs infected with DN-cdk4 were significantly resistant to ETP treatment as only 4.7 ± 0.4% of these cells were apoptotic. The effects of these treatments on nuclear morphology are shown in figure 11 B.

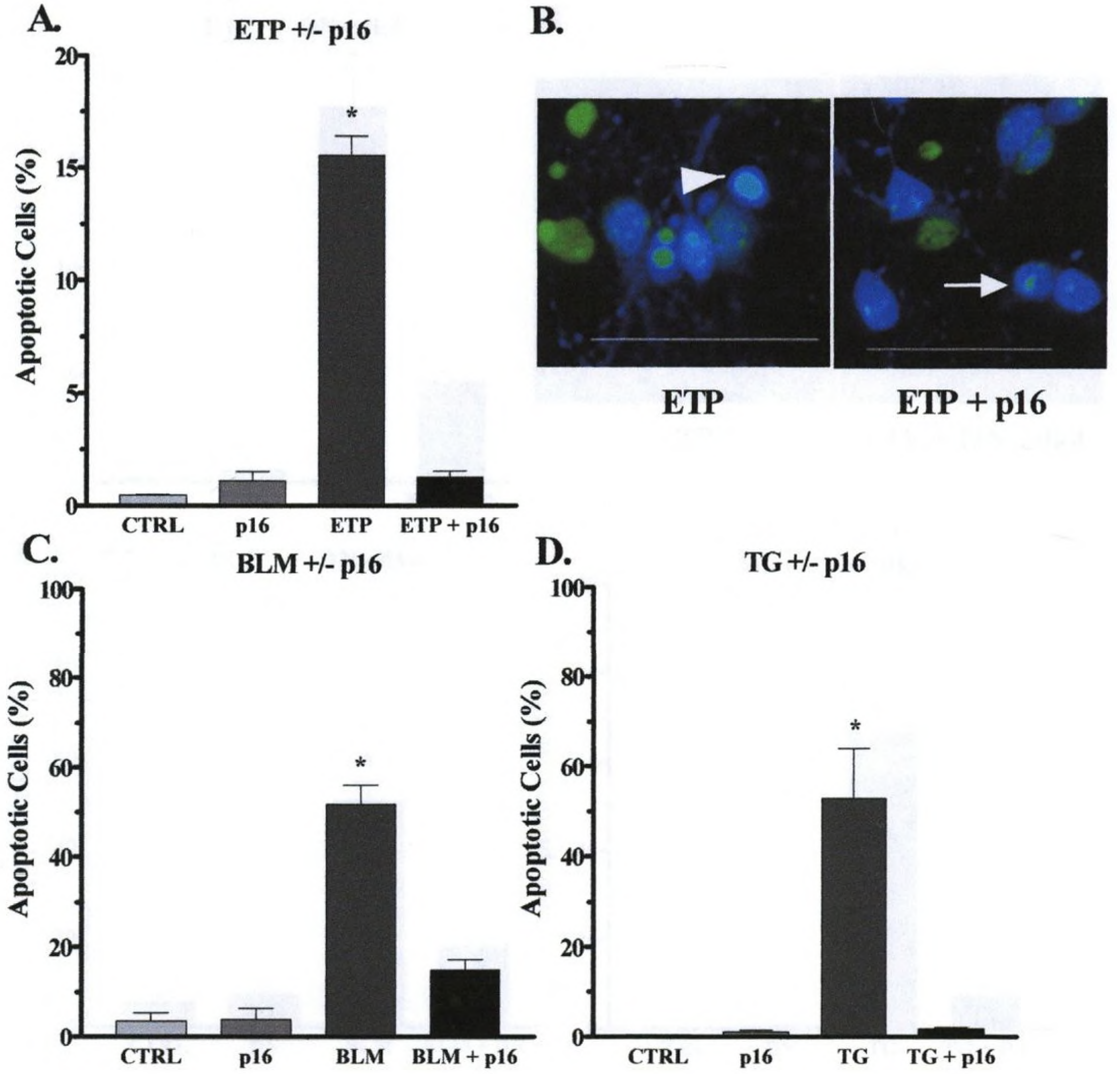


Figure 10. Neuronal Apoptosis is Promoted by cdk 4/6 in Response to Oxidative Stress, DNA damage, and ER Stress. The fraction of apoptotic cerebellar granule neurons cultured from 8 day-old CD1 mice were counted by Hoechst 33258 staining following 48 hr infection with 20 M.O.I. Ad-GFP (CTRL) or Ad-p16 (cdk 4/6 inhibitor) and then induction of DNA damage (24hr): (A) & (B) ETP (10 mM), oxidative stress (30hr): (C) BLM (0.05 U/mL), or ER stress (30hr): (D) TG (2 mM). For all data sets: mean ± sem, n=3. Representative image scale bar = 50 mm. The blue pseudo-colour staining is indicative of EGFP or Alexa-488 immunostained p16 expression, while the green pseudo-colour represents Hoechst 33258 nuclear counterstaining. Note the presence of GFP+ apoptotic nuclei, characterized by condensed and fragmented DNA, found primarily in the ETP-treated neurons (arrowhead), while the ETP-treated p16-stained nuclei appear healthy (arrow). Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase in apoptosis only in death stimulus treated neurons as compared to all other groups (*$p<0.001$).

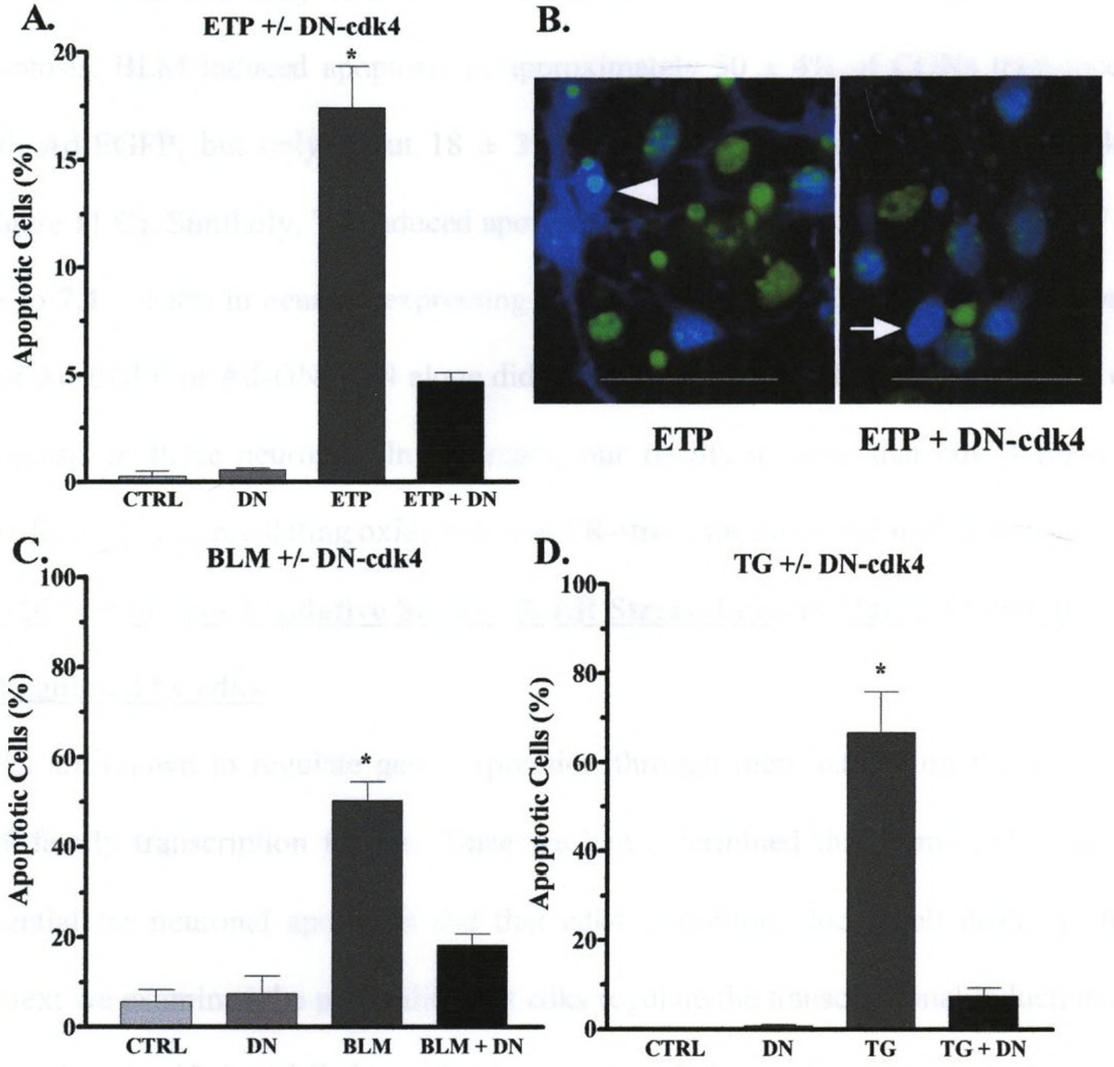


Figure 11. Neuronal Apoptosis is Promoted by cdk 4 in Response to Oxidative Stress, DNA damage, and ER Stress. The fraction of apoptotic cerebellar granule neurons cultured from 8 day-old CD1 mice were counted by Hoechst 33258 staining following 48 hr infection with 25 M.O.I. Ad-GFP (CTRL) or Ad-DN-cdk4 (DN) and then induction of DNA damage (24hr): (A) & (B) ETP (10 mM), oxidative stress (30hr): (C) BLM (0.05 U/mL), or ER stress (30hr): (D) TG (2 mM). For all data sets: mean ± sem, n=3. Representative image scale bar = 50 mm. The blue pseudo-colour staining is indicative of EGFP or GFP-tagged DN-cdk4 expression, while the green pseudo-colour represents Hoechst 33258 nuclear counterstaining. Note the presence of GFP+ apoptotic nuclei, characterized by condensed and fragmented DNA, found primarily in the ETP-treated neurons (arrowhead), while the ETP-treated DN-cdk4-stained nuclei appear healthy (arrow). Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase in apoptosis only in the death stimulus treated neurons as compared to all other groups (*$p<0.001$).

DN-cdk4 also markedly reduced the level of oxidative- and ER-stress induced apoptosis. BLM induced apoptosis in approximately 50 ± 4% of CGNs transduced with Ad-EGFP, but only about 18 ± 3% in CGNs transduced with Ad-DN-cdk4 (Figure 11 C). Similarly, TG induced apoptosis was reduced from approximately 67 ± 9% to 7.4 ± 1.8% in neurons expressing DN-cdk4 (Figure 11D). Control infections with Ad-EGFP or Ad-DN-cdk4 alone did not have a significant effect on the level of apoptosis in these neurons. In summary, our results indicate that cdk 4 plays a significant role in regulating oxidative- and ER-stress-induced neuronal apoptosis.

3.7-DNA damage-, Oxidative Stress-, & ER Stress- induced Puma Transcription is Regulated by cdks

Cdks are known to regulate gene expression through their actions on the Rb- and E2F-family transcription factors. Since we had determined that Puma induction is essential for neuronal apoptosis and that cdk4 inhibition blocks cell death in this context we examined the possibility that cdks regulate the transcriptional induction of Puma. Figures 12 A and B shows that treatment with the oxidative stressors, NOC-12 and TBH, significantly increased Puma mRNA to 5.6 ± 1.5-fold and 5.3 ± 0.7-fold over control levels, respectively. Importantly, the increase in Puma mRNA following NOC-12 and TBH treatments was abolished in the presence of flavopiridol. Similarly, flavopiridol prevented the transcriptional induction of Puma in response to the DNA damaging agent ETP and the ER-stressors TG and TU (Figures 12C-E). These results suggest that cdks promote Puma transcription in response to death stimuli in cortical neurons.

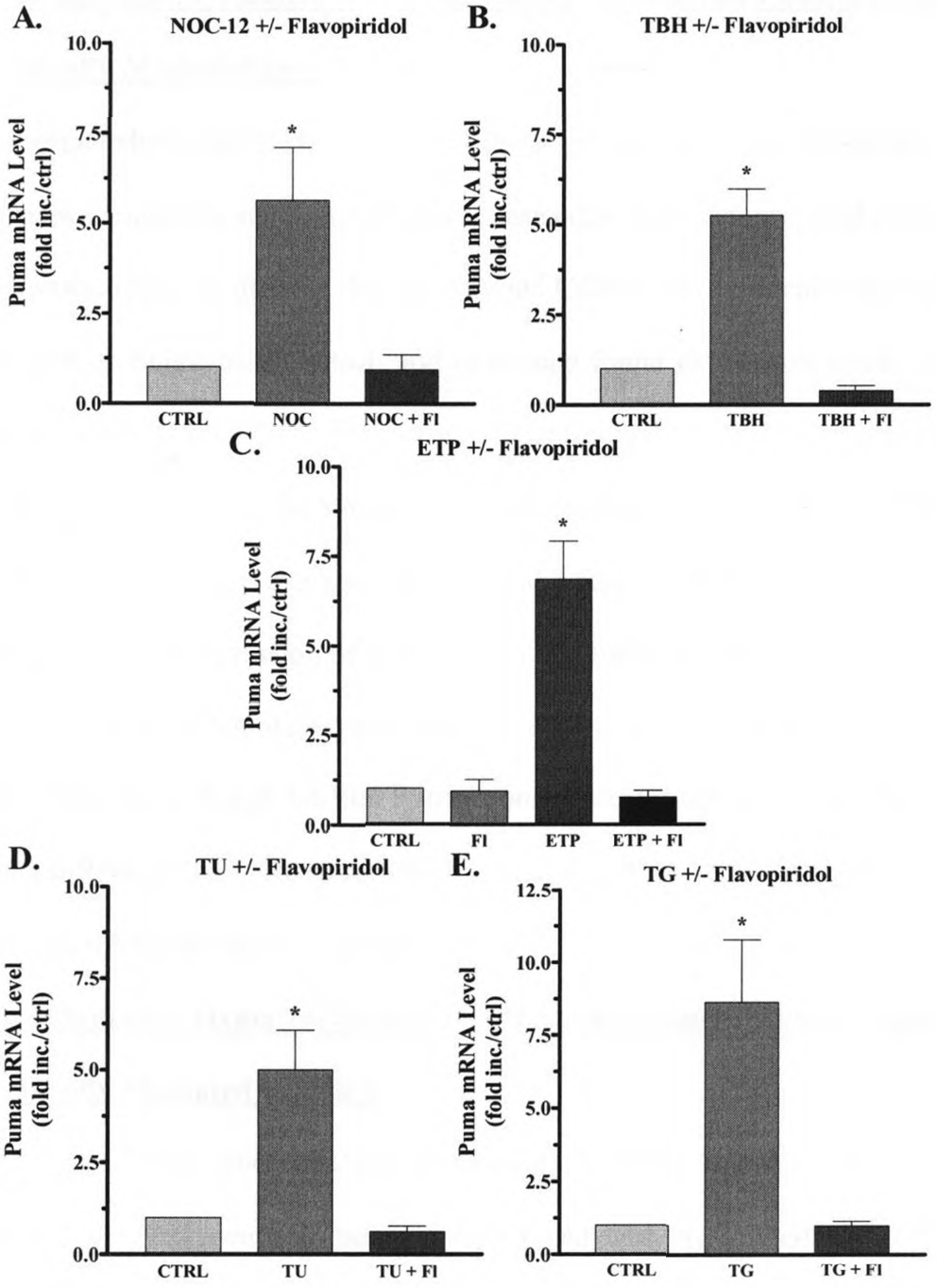


Figure 12. Puma Transcription is Promoted by cdks in Response to Oxidative Stress, DNA damage, and ER Stress. Puma mRNA levels were quantified by qRT-PCR from total RNA extracts of E15 CD1 murine cortical neuron cultures subjected to 10 hrs of oxidative stress : (A) NOC-12 (200 mM), (B) TBH (200 mM), DNA damage: (C) ETP (10 mM), or ER stress: (D) TU (2 mM), (E) TG (2 mM) in the presence (+) or absence (-) of the cki flavopiridol (Fl, 1mM). All data sets are normalized to S12 mRNA and reported as fold increase over control (mean ± sem, All n=3, except TG n=4). Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase of Puma mRNA only in the death stimulus treated neurons as compared to all other groups (*$p<0.05$).

3.8-DNA damage-, Oxidative Stress-, & ER Stress- induced Puma Transcription is Specifically Mediated by cdks 4/6

Having established that G_0-G_1 cdks contribute to oxidative- and ER-stress induced apoptosis we sought to determine whether these cdks were also involved in regulating Puma transcription. To address this we infected CGNs with an adenovirus expressing the cki p16 or EGFP as a control, and examined Puma expression levels following treatment with BLM, ETP or TG. As shown in figure 13A, BLM stimulated a 7.3 ± 1.2-fold increase in Puma mRNA over untreated control levels in CGNs infected with Ad-EGFP, but only an increase of 2.4 ± 0.3-fold in Ad-p16 infected neurons. Similarly, enforced expression of p16 dramatically reduced Puma induction following treatment with the DNA damaging agent ETP (Figure 13B) or the ER-stressor TG (Figure 13C). Ad-p16 and Ad-EGFP infections alone did not have a significant effect on Puma mRNA levels. These results, further suggest that cdk 4/6 activity promotes Puma transcription in response to neuronal death stimuli.

3.9-DNA damage-, Oxidative Stress-, & ER Stress- induced Puma Transcription is Specifically Mediated by cdk 4

To determine whether Cdk4 in particular contributes to Puma induction we examined Puma expression in neurons transduced with Ad-DN-cdk4 or the control vector Ad-EGFP. BLM treatment stimulated a 7.5 ± 1.2-fold increase in Puma mRNA over untreated control levels in neurons transduced with Ad-EGFP, but only a 2.8 ± 0.5-fold increase in neurons transduced with Ad-DN-cdk4 (Figure 14 A).

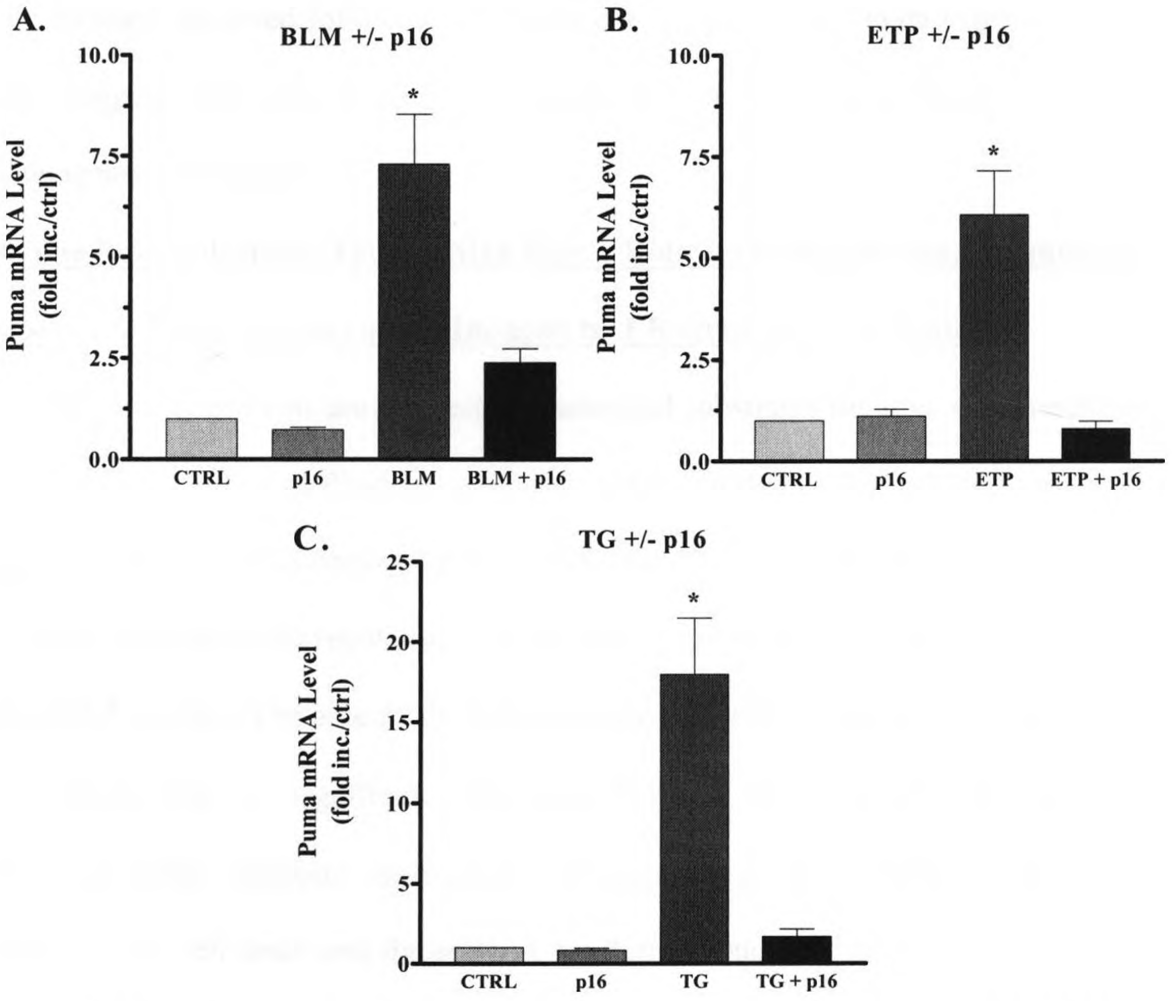


Figure 13. Puma Transcription is Promoted by cdk 4/6 in Response to Oxidative Stress, DNA damage, and ER Stress. Puma mRNA levels were quantified by qRT-PCR from total RNA extracts of 8 day-old CD1 murine cerebellar granule neuron cultures following 48 hr infection with 20 M.O.I. Ad-GFP (CTRL) or Ad-p16 (cdk 4/6 inhibitor) and then induction of 10 hrs of oxidative stress : (A) BLM (0.05 U/mL), DNA damage: (B) ETP (10 mM), or ER stress: (C) TG (2 mM). All data sets are normalized to S12 mRNA and reported as fold increase over control (mean ± sem, All n=3, except TG n=4). Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase in Puma mRNA only in the death stimulus treated CGNs as compared to all other groups (*$p<0.05$).

Similarly, DN-cdk4 expression blocked the 5.5 ± 1.3-fold increase in Puma expression observed following treatment with ETP (Figure 14B) and the nearly 11 ± 3-fold increase observed following TG treatment (Figure 14C). Taken together these results suggest that cdk4 plays a prominent role in regulating Puma induction following neuronal injury.

3.10-The Retinoblastoma Protein May Play a Role in the Repression of Neuronal Apoptosis & Puma Transcription Induced by ER stress & DNA damage

Rb family proteins are the best characterized substrates for cdks 4/6, therefore we examined the effect of Rb-deletion on Puma transcription and cell death in cortical neurons exposed to DNA damage or ER stress. We hypothesized that if Rb is the key cdk target involved in the regulation of Puma transcription and cell death, then the cki flavopiridol would not be able to block these processes in Rb-deficient neurons.

There was no significant difference between Rb-deficient and wildtype neurons for either endpoint measurement in response to DNA damage, shown in figure 15 A for cell death and figure 16 A for Puma induction. Also, the addition of flavopiridol protected the neurons from cell death and prevented Puma induction by CPT, regardless of Rb genotype.

However, it was interesting to note a small, but insignificant difference between the two genotypes in response to ER stress. Apoptosis occurred in 47.8 ± 6.5% of Rb wildtype neurons in response to TG treatment, whereas 56.2 ± 6.9% of Rb-null neurons underwent apoptosis (Figure 15 B).

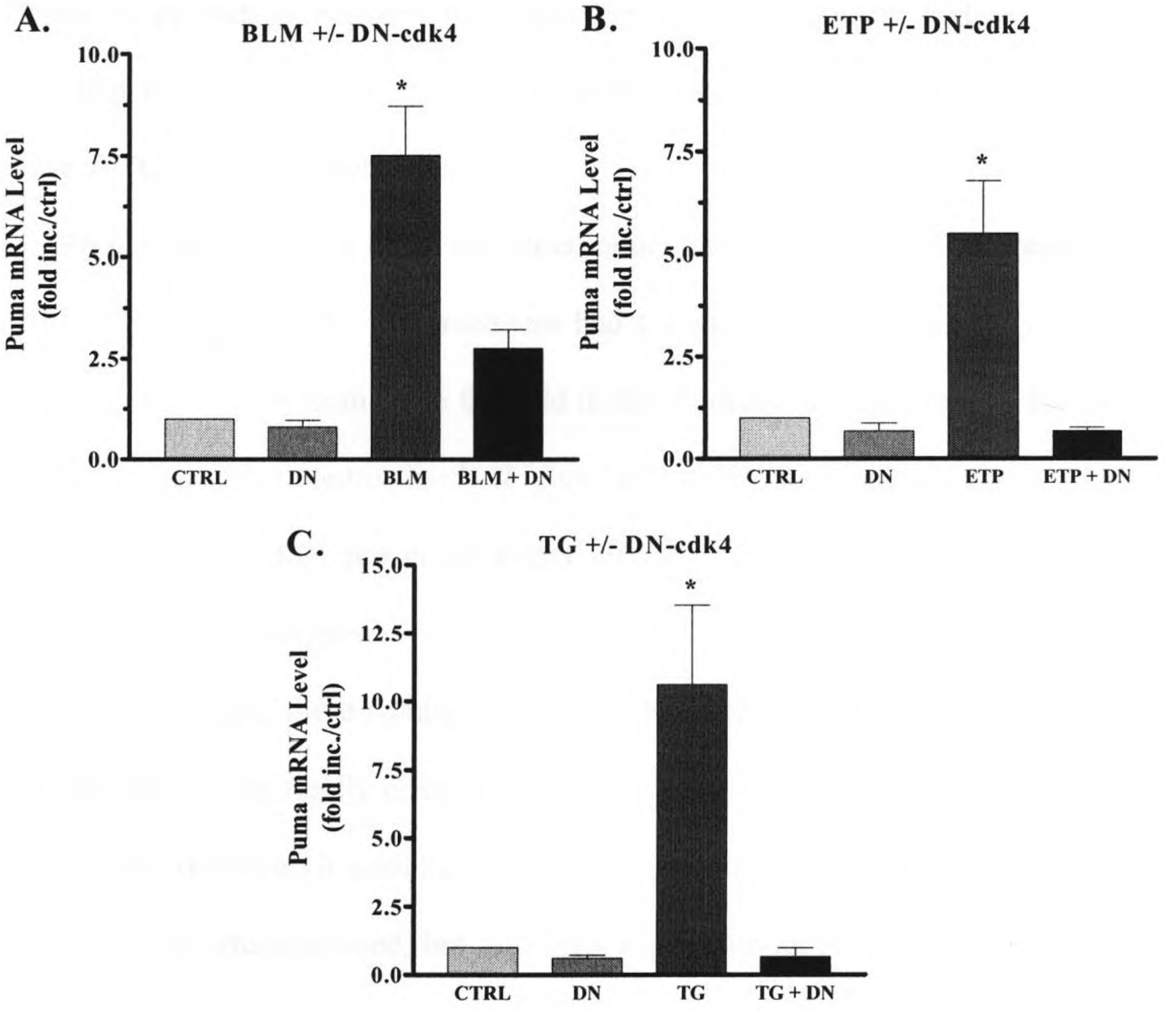


Figure 14. Puma Transcription is Promoted by cdk 4 in Response to Oxidative Stress, DNA damage, and ER Stress. Puma mRNA levels were quantified by qRT-PCR from total RNA extracts of 8 day-old CD1 murine cerebellar granule neuron cultures following 48 hr infection with 25 M.O.I. Ad-EGFP (CTRL) or Ad-DN-cdk4 (DN) and then induction of 10 hrs of oxidative stress : (A) BLM (0.05 U/mL), DNA damage: (B) ETP (10 mM), or ER stress: (C) TG (2 mM). All data sets are normalized to S12 mRNA and reported as fold increase over control (mean ± sem, All n=3, except TG n=4). Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant increase in Puma mRNA only in the death stimulus treated CGNs as compared to all other groups (*$p<0.05$).

Additionally, flavopiridol protected both Rb-null (15 ± 2% of cells) and wildtype (6 ± 1% of cells) cortical neurons from TG-induced apoptosis but the difference in protection between the genotypes was insignificant. These results indicate that Rb is not the crucial pocket protein repressing neuronal apoptosis in response to TG than CPT treatment.

Rb-deficiency had no effect on Puma induction in response to CPT treatment (Figure 16A). Interestingly, TG treatment had the effect of significantly ($p<0.01$) enhancing Puma mRNA from 4.5 ± 0.3-fold in the wildtype setting, to 8.5 ± 2.8-fold in Rb-null neurons over control levels (Figure 16 B). The addition of flavopiridol to TG treatments completely prevented Puma induction at 12 hours of exposure in neurons of both Rb genotypes.

Taken together, these results suggest that Rb is not the primary cdk target or that another Rb protein family member, such as p107 or p130, can compensate in the absence of Rb. However, it would appear that the Rb protein is significantly involved in preventing ER stress-induced, but not DNA damage-induced, Puma transcription and neuronal apoptosis.

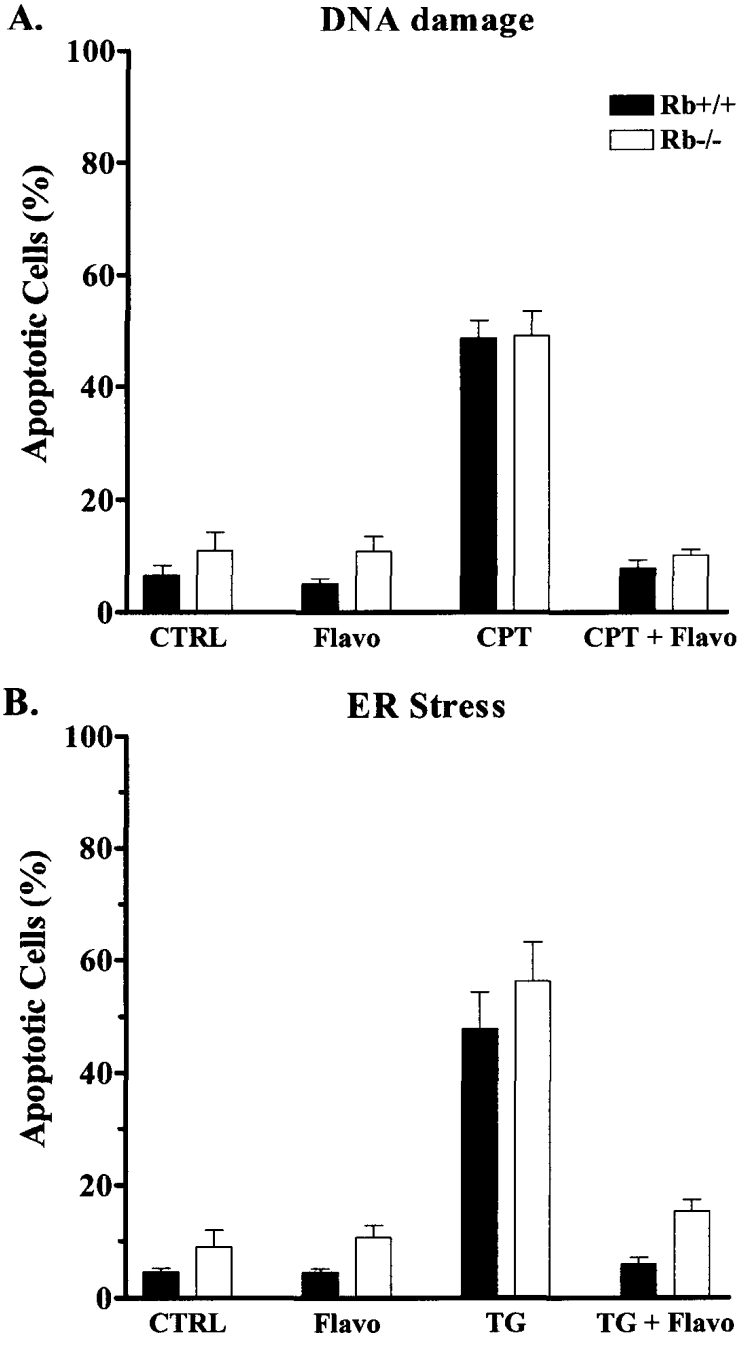


Figure 15. Deletion of the Rb Gene Has no Effect on the Level of Neuronal Apoptosis Promoted by cdks in Response to ER Stress or DNA damage.
The fraction of apoptotic cortical neurons cultured from E15 conditional Rb knockout mice (Solid bars = Rb+/+, Empty bars = Rb-/-) were counted by Hoechst 33258 staining following treatment with DNA damage (24hr): (A) CPT (10 mM, n=3), or ER stress (30hr): (B) TG (2 mM, n=4), in the presence (+) or absence (-) of the cki Flavopiridol (Flavo, 1 mM). For all data sets (mean ± sem). Statistical analysis by two-way ANOVA followed by Bonferroni's post-hoc test revealed no significant differences in apoptosis induced by DNA damage or ER stress with or without flavo in Rb-/- neurons as compared to respective treatments in Rb+/+ neurons.

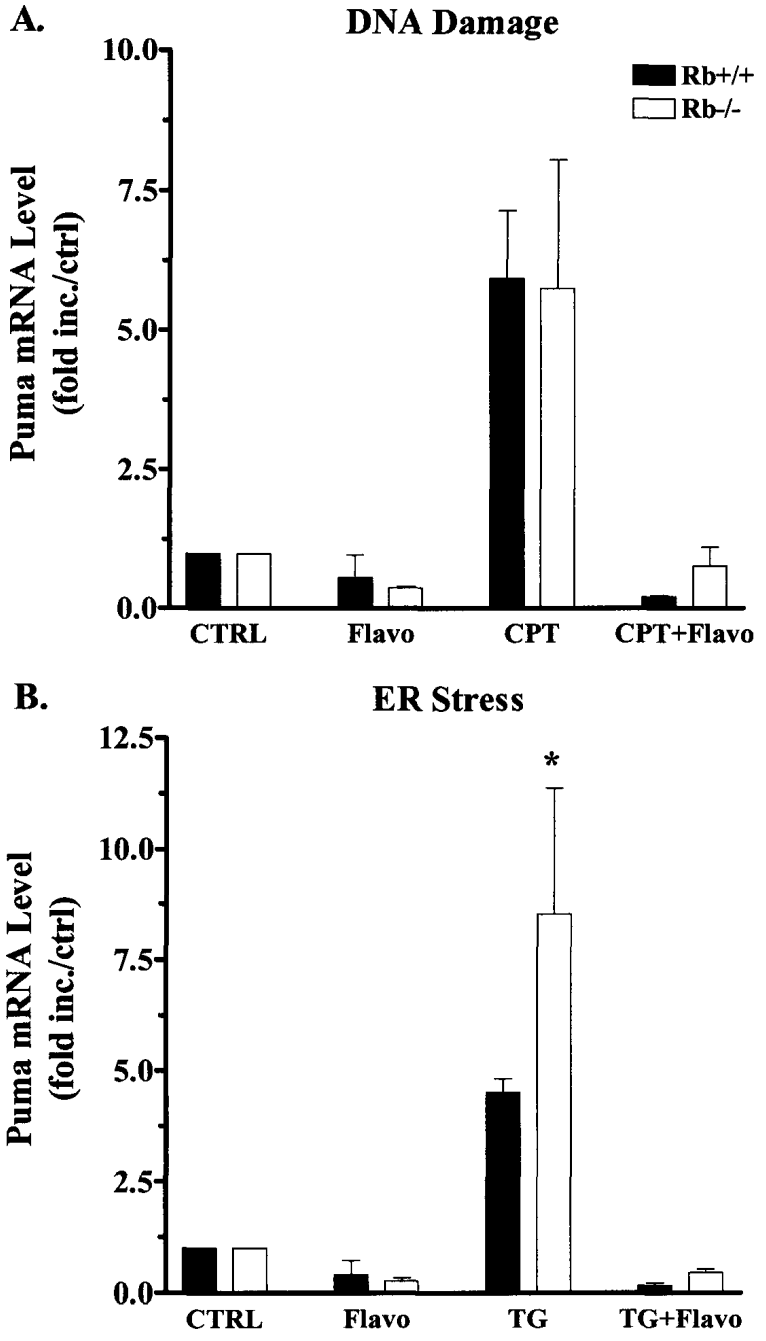


Figure 16. Deletion of the Rb Gene Enhances Puma Transcription Promoted by cdks in Response to ER Stress, but not DNA Damage.

Puma mRNA levels were quantified by qRT-PCR from total RNA extracts of E15 conditional Rb knockout murine cortical neuron cultures (Solid bars = Rb+/+, Empty bars = Rb-/-) subjected to 12 hrs of DNA damage: (A) CPT (10 mM, n=3), or ER stress: (B) TG (2 mM, n=4), in the presence (+) or absence of the cki Flavopiridol (Flavo, 1mM). All data sets are normalized to S12 mRNA and reported as fold increase over control (mean ± sem). Statistical analysis by two-way ANOVA followed by Bonferroni's post-hoc test revealed a significant increase in Puma mRNA for TG-treated Rb-/- neurons as compared to Rb+/+ neurons (*$p<0.05$).

CHAPTER 4- DISCUSSION

The mitochondrial pathway of apoptosis is a dynamic process which requires the activation of signalling pathways to downregulate anti-apoptotic factors and upregulate pro-apoptotic factors in order to mediate mitochondrial permeabilization and the subsequent induction of the caspase cascade (Tsujimoto, 2003). Previous studies by our lab and others have shown that one way in which this is accomplished in neurons is through the transcriptional induction of BH3-only genes in response to stress (Cregan et al., 2004a;Puthalakath and Strasser, 2002). We verified that new RNA and protein synthesis, through the co-addition of actinomycin D and cycloheximide, respectively, were required for oxidative stress- and ER stress-induced neuronal apoptosis (see Appendix A). Therefore, we initially examined the expression profiles of various pro-apoptotic Bcl-2 family genes in response to the neurodegenerative disease-associated stresses, DNA damage, oxidative stress, and ER stress. Although the levels of Bim and Noxa mRNA increased by at least two fold in response to most stresses, Puma was the only member that exhibited a sustained induction in response to all stresses.

This result prompted us to look at the necessity of PUMA in neuronal apoptosis through cell death treatments of primary cortical neurons from Puma knockout mice. Induction of apoptosis in response to all three types of stress was dramatically reduced in Puma-/- neurons compared to wildtype neurons, as measured by cytochrome c immunoreactivity, caspase-3 activity, and nuclear morphology. It is possible, however, that neuronal apoptosis is executed by a concerted effort between many BH3-only proteins and when only one protein is removed, the

response is impaired. For that reason, the same types of stress were tested by our lab in neurons from Bim and Noxa double knockout mice but no significant differences in the levels of apoptosis were detected compared to heterozygous littermates (see Appendix B). Taken together, these results show that PUMA is a necessary BH3-only protein for the execution of neuronal apoptosis in response to DNA damage, oxidative stress, and ER stress.

Recently, other groups have demonstrated similar findings of PUMA-mediated apoptosis in neurons responding to other death stimuli. For example, apoptosis induced in a cellular model of PD, 6-hydroxydopamine (6-OHDA) treatment of differentiated PC-12 cells, was inhibited through the use of small interfering RNA (siRNA) specific for Puma mRNA (Biswas et al., 2005b). Cortical neurons exposed *in vitro* to arsenite significantly upregulated the expression of Puma, Noxa, and Bim (Wong et al., 2005). However, only deletion of the Puma gene rescued the neurons from apoptosis. Similarly, cultured sympathetic neurons treated with the DNA damaging agent cytosine arabinoside (AraC) required the presence of the Puma gene, but not the Bim or Noxa genes, to undergo apoptosis (Wyttenbach and Tolkovsky, 2006). Thus, PUMA appears to have a dominant function in comparison with other BH3-only proteins.

Interestingly, our lab and the authors in the two previously discussed studies on PUMA-mediated neuronal apoptosis independently demonstrated that deletion of the multi-BH domain member Bax also rendered neurons resistant to these apoptotic stimuli (Cregan et al., 1999;Wong et al., 2005;Wyttenbach and Tolkovsky, 2006). In addition, neuronal cell death induced by PUMA overexpression was blocked in Bax-

deficient neurons suggesting that PUMA induces neuronal apoptosis through a BAX-dependent process.

The indirect mechanism by which PUMA and all of the other BH3-only proteins are thought to activate BAX involves their capacity to bind and inactivate the anti-apoptotic Bcl-2 protein family members, thus freeing BAX from inhibition (Bouillet and Strasser, 2002). Competitive binding assays for each of the five anti-apoptotic proteins between the immobilized BIM BH3 peptide and the conserved BH3 domain peptides from all eight different BH3-only proteins were used to calculate IC50 values (Chen et al., 2005). The results demonstrated that BH3 domains from each protein had differential binding affinities for each anti-apoptotic Bcl-2 protein. PUMA and BIM were the most promiscuous, binding all anti-apoptotic members with roughly the same high affinity, while NOXA was the most selective member as it was only able to bind Mcl-1 and A1.

Perhaps the potency of the PUMA protein in the activation of apoptosis is derived from its ability to sequester all of the anti-apoptotic proteins at once, allowing BAX to induce mitochondrial permeability without the need for direct activation (Willis et al., 2007). However, the fact that the BIM protein is just as potent as PUMA in this regard does not explain why only Puma and not Bim knockout neurons are rescued from apoptosis in our model.

A previous study comparing neuronal apoptosis through Bim induction versus the use of a BH3-only mimetic shown to antagonize Bcl-2 and Bcl-w, HA14-1, demonstrated that although both stimuli appeared to induce apoptosis by caspase activation, the mechanisms were quite different (Zimmermann et al., 2005).

Inhibitors of known transcriptional activation pathways, but not the anti-oxidants glutathione (GSH) and N-acetylcysteine (NAC), prevented apoptosis through Bim induction, which was the complete opposite for the BH3-only mimetic, HA14-1. This would suggest that HA14-1 was somehow inducing oxidative stress, shown to be Puma-responsive in our study.

Evidence for the direct mechanism of PUMA-mediated BAX activation is compelling. As previously mentioned, BAX seems to be the only multi-BH domain protein required for neuronal apoptosis so only its interactions with BH3-only proteins will be discussed here.

Protein interactions between PUMA-BAX and BID-BAX were previously identified by bacterial two-hybrid screens (Cartron et al., 2004). Analysis of immunoprecipitation assays with full-length and mutant peptides revealed that these protein interactions occurred with high affinity between the BH3 domains of PUMA or BID and the first alpha helix of the BAX protein.

BIM and BID have also been identified as direct activators of BAX in other studies (Harada et al., 2004;Walensky et al., 2006). One study showed that PUMA was one of the most potent indirect activators but a much weaker direct activator of BAX compared to BID and BIM in a cell-free liposome permeabilization assay and a cytochrome c release assay in co-transfected Hela cells (Kuwana et al., 2005). The ability of the PUMA protein to release cytochrome c was synergistically enhanced in the presence of BIM BH3 peptide or a constitutively active recombinant form of BID protein. These results clearly reflect the need for both functions of BH3-only proteins

in mediating mitochondrial permeabilization, as (indirect) 'sensitizers' and/or (direct) 'activators' of the mitochondrial pathway of apoptosis.

However, the possibility of a post-translational modification to PUMA that could make it a more effective 'activator' of apoptosis was not ruled out. A perfect example of this concept is the BID protein. The activated form of BID is truncated (tBid) by caspase 8 in response to death receptor activation and n-myristoylation of tBid is enough to promote its translocation to the mitochondria (Zha et al., 2000).

Another example of post-translational modification of a BH3-only protein is the phosphorylation of BAD. Normally this event is inhibitory (Harada et al., 1999) since it promotes the sequestration of BAD but, depending on the residue that is phosphorylated, it can actually promote the activation of this protein. This was shown to occur in the potassium withdrawal model of apoptosis in CGNs by the cyclin B-cdk1 cell cycle kinase complex, which mediated phosphorylation of BAD at serine residue 128 (Konishi et al., 2002).

The link between the cell cycle and apoptotic machinery described by those authors supports the existence of a transcriptional regulatory mechanism by cdks of the Puma gene, demonstrated in our study. Additionally, there have been many other reports of a connection between the activation of cell cycle molecules and apoptosis. For example, cyclin D1-associated kinase activity in neurons is upregulated in response to DNA damage, as shown by a steady increase in Rb phosphorylation in the first 4 hours following treatment (Park et al., 1998a). Furthermore, several different inhibitors of G1 phase cdk activity (e.g.- flavopiridol, p16(INK4a), p21(Waf1/Cip1), p27(Cip2), DN-cdks 2,4,6) are known to prevent neuronal apoptosis initiated by such

diverse stimuli as NGF withdrawal (Park et al., 1996;Park et al., 1998b), potassium withdrawal (Padmanabhan et al., 1999), various DNA damaging agents (e.g.- araC, camptothecin, and UV radiation) (Morris and Geller, 1996;Park et al., 1998b;Park et al., 2000), and proteasomal inhibition (Rideout et al., 2003).

In our study, we extended this list to include the inhibition of cdk-mediated neuronal apoptosis initiated by the neurodegenerative-associated stresses, ER stress and oxidative stress. Also, we provided a mechanism for cdk activity, in these stresses, to initiate the mitochondrial pathway of apoptosis: promoting transcriptional induction of the critical pro-apoptotic BH3-only gene Puma. Taken together, the critical function of cdk activity during ER and oxidative stress-induced neuronal apoptosis is the promotion of Puma transcription.

One possible way in which this may occur was demonstrated by a study of the transcriptional induction of another BH3-only gene, Bim, in a trophic factor withdrawal model of apoptosis (Biswas et al., 2005a). Upregulation of Bim transcription is first dependent on gene derepression of an Rb-E2F inhibitory complex. Derepression means relief from the active transcriptional repression of a gene. In the case of the Rb-E2F inhibitory complex, it involves the cdk-mediated phosphorylation and inactivation of the Rb protein, thus freeing it from binding and inhibiting the E2F transactivation domain. The authors found that in healthy, unstressed cells, the repression complex of p130-E2F4 in association with the chromatin modifiers HDAC1 and Suv39H1 actively inhibited the transcription of Mybs (Liu et al., 2005), transcription factors which can transactivate the Bim promoter during trophic factor withdrawal (Liu et al., 2004).

The question of which transcription factor, or combination of factors, is critically required for induction of the Puma gene in ER and oxidative stress remains to be answered. Since enhanced cdk activity promotes derepression of Rb-E2F inhibitory complexes, a lot of attention has been paid to this point in the signalling pathway in order to identify pro-apoptotic genes that are transactivated by the E2F activator proteins, E2F1-3.

In fact, several pro-apoptotic genes induced by E2F1 transactivation include ATM, family members of the apoptosis stimulating proteins of p53 (ASPP-1 & -2), JMY, tumor suppressor p53-induced nuclear protein 1 (TP53INP1) and even four members of the BH3-only gene family: Bim, Hrk/DP5, Noxa, and Puma (Hershko et al., 2005;Hershko and Ginsberg, 2004). This was determined through experiments performed in various cancer cell lines by E2F1 overexpression and artificial induction of E2F1 activity coupled to semi-quantitative RT-PCR and luciferase reporter assays. In analyzing the promoter regions for each of these genes, E2F consensus binding sites were uncovered in the DNA for every single one of them. Chromatin immunoprecipitation (ChIP) assays subsequently revealed that even endogenous levels of E2F1 were bound to the promoters of each gene, except ATM and TP53INP1.

These results are in agreement with our survival and qRT-PCR data in conditional Rb knockout neurons, which demonstrated significantly increased levels of apoptosis and Puma transcription in response to ER stress, but not DNA damage, compared to wildtype littermates. This could be because ER stress is a p53-independent apoptotic stimulus (Reimertz et al., 2003), at least in neurons, whereas

DNA damage-induced apoptosis is almost entirely dependent on p53 (Cregan et al., 2004a). Therefore, E2F1 could be playing a more prominent transactivational role in the ER stress setting.

Indeed, the E2F1 transcription factor is actively involved in apoptosis in both *in vitro* and *in vivo* models of neuronal cell death. For example, forced expression of E2F1 is sufficient to induce apoptosis in neurons (O'Hare et al., 2000). Neurons cultured from E2F1-deficient mice are resistant to amyloid-β peptide treatment and potassium withdrawal models of apoptosis, stimuli which are both dependent on BAX and caspase-3 (Giovanni et al., 2000;O'Hare et al., 2000). Furthermore, amyloid-β peptide treatment was shown to be a p53-independent stimulator of apoptosis (Giovanni et al., 2000). *In vivo* models of stroke in E2F1-deficient mice show decreased infarct volume and improved post-ischemic behaviour compared to their wildtype littermates (MacManus et al., 1999;MacManus et al., 2003).

In considering all of these different lines of evidence for the involvement of cdks and E2Fs in neuronal apoptosis, one could begin to construct a plausible model for the control of Puma transcription during ER and oxidative stress. These death stimuli promote cdk 4 activition which would phosphorylate pocket proteins bound at the Puma promoter. Both Rb and p130 are known to be expressed in postmitotic neurons and both can associate with E2F4 to form repression complexes on promoters of genes containing E2F binding elements. The phosphorylation of Rb or p130 would cause it to unbind E2F4 and inactivate the repressor complex. This would allow free E2F1 to bind the Puma promoter and activate its transcription. ChIP assays for endogenous E2F and Rb proteins at the Puma promoter will have to be performed in

healthy and stressed neurons in order to confirm this proposed mechanism. However, we have found that Rb-deletion has minimal effect on Puma induction and cell death. This suggests the possibility that p130 is a more prominent regulator of Puma induction or that it can at least compensate in the absence of Rb.

Although the cyclin-cdk-Rb-E2F pathway in the regulation of Puma transcription seems extremely promising in forging a link between the cell cycle and apoptosis in terminally differentiated and growth arrested cells such as neurons, it is quite hard to reconcile with the role for this pathway in actively proliferating cells. Clearly this pathway must be differentially regulated in both the proliferative and non-proliferative states in order to prevent the activation of apoptosis during the normal course of the cell cycle. Thus, it is likely that recruitment of other stress-induced transcriptional activators is also necessary for Puma induction. Indeed, we have previously shown that the transcriptional activator p53 is necessary for Puma induction in response to DNA damage. However, other members in our lab have demonstrated that p53 is dispensible for Puma induction and cell death induced by oxidative stress and ER-stress. This suggests that a different transactivator is involved in these death pathaways.

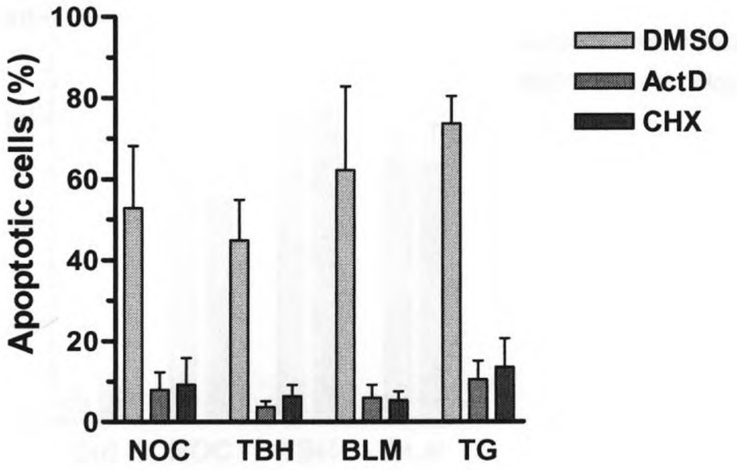
It is anticipated that further research into this regulatory mechanism of life and death in both neurons and actively dividing cells will provide us with insight in order to develop specific therapeutic interventions for the successful treatment of neurodegenerative diseases.

CHAPTER 5- SUMMARY & CONCLUSIONS

In this study, we demonstrated that the BH3-only genes Bim, Noxa, and Puma are transcriptionally upregulated in cortical neurons in response to DNA damage, oxidative stress, and ER stress. However, only induction of the Puma gene is sustained during exposure to each stimulus and only deletion of this gene was sufficient to rescue the neurons from undergoing apoptosis. In addition, use of the cyclin-dependent kinase inhibitors flavopiridol, p16, and DN-cdk4 in cortical and cerebellar granule neurons displayed near complete neuroprotective effects through inhibition of Puma induction and prevention of apoptosis.

In conclusion, PUMA is a critical protein required for execution of neuronal apoptosis and transcriptional induction of the Puma gene is mediated, *in vitro*, by cyclin-dependent kinases, likely cdks 4/6, in response to the neurodegenerative stresses: DNA damage, oxidative stress, & ER stress.

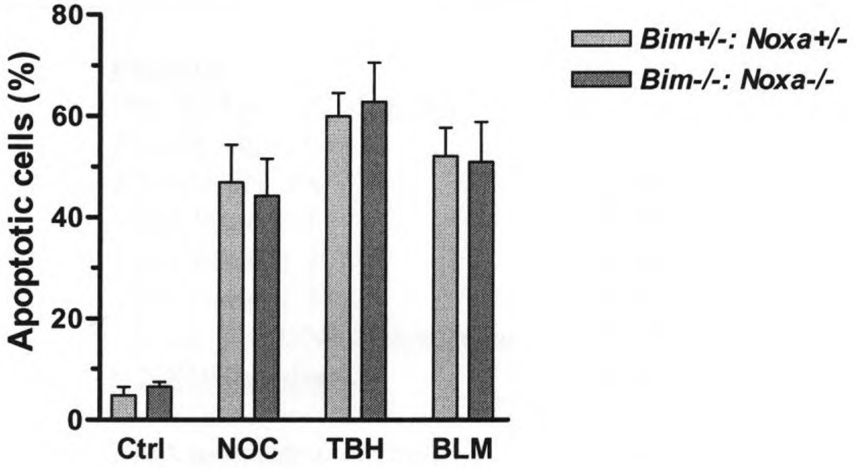
APPENDIX A- Survival studies in the presence of RNA & protein synthesis inhibitors



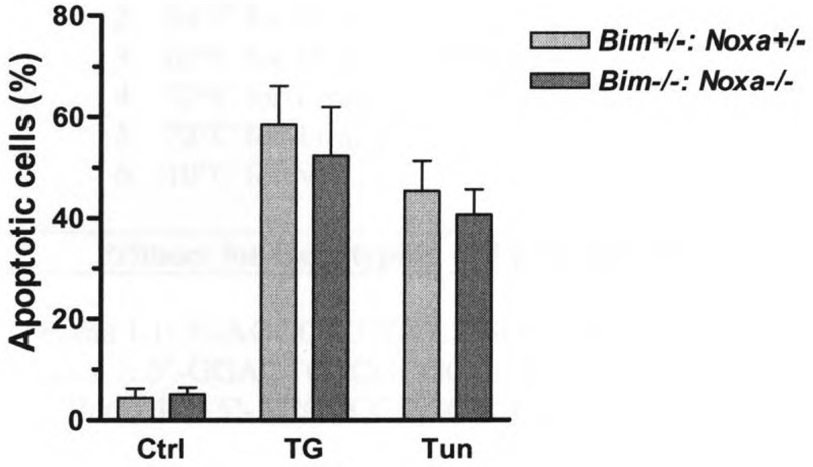
RNA synthesis and protein synthesis are required for the execution of neuronal apoptosis in response to oxidative stress and ER stress. CD1 cortical neurons were cultured and treated for 24 hours with oxidative stressors: 200 μM NOC-12, 200 μM TBH, or 0.05 U/ml BLM or the ER stressor: 2 μM TG in the presence or absence of the RNA synthesis inhibitor Actinomycin D (ActD) or the protein synthesis inhibitor cycloheximide (CHX) and then fixed and stained with Hoechst 33258 in order to assess nuclear morphology. The number of apoptotic neurons was recorded and expressed as a percentage of the total number of neurons counted for each treatment. Statistical analysis by one-way ANOVA followed by Tukey's post-hoc test revealed a significant difference between death treatments alone and those in the presence of ActD or CHX within each stimulus group.

APPENDIX B- Bim & Noxa double knockout survival studies

A.



B.



Bim and Noxa are not essential for neuronal apoptosis induced by oxidative stress and ER stress. Cortical neurons cultured from Bim:Noxa double heterozygotes and Bim:Noxa double knockouts were treated for 24 hours with (A) oxidative stressors: 200 μM NOC-12, 200 μM TBH, or 0.05 U/ml BLM or (B) ER stressors: 2 μM TG or 2 μM Tunicamycin and then fixed and stained with Hoechst 33258 in order to assess nuclear morphology. The number of apoptotic neurons was recorded and expressed as a percentage of the total number of neurons counted for each treatment. Statistical analysis by two-way ANOVA followed by Bonferroni's post-hoc test revealed no significant difference between the two genotypes in each treatment group.

APPENDIX C- Puma Genotyping

Fermentas PCR Reagents for Genotyping of Puma KO Mice

Reagent	Vol(µL)
10x Buffer(+KCl-$MgCl_2$)	2.50
25 mM MgCl2	1.50
2.5 mM dNTPs	2.00
5 µM Puma 1.1	4.00
5 µM Puma 1.2	4.00
5 µM Puma 1.3	4.00
1 U/µL Taq DNA polymerase	1.25
H2O(DNase-free)	3.75
	23.00
DNA template (~0.5ug/µL)	2.00
Total	25.00

PCR Cycling Conditions for Genotyping of Puma KO Mice

1. 94°C for 2 mins
2. 94°C for 45 secs
3. 60°C for 45 secs — 30 cycles
4. 72°C for 1 min
5. 72°C for 4 mins
6. 10°C forever

Primers for Genotyping of Puma KO Mice

Puma 1.1: 5'-AGGCTGTCCCTGGGGTCATCCC-3'
Puma 1.2: 5'-GGACTGTCGCGGGCTAGACCCTCTA-3'
Puma 1.3: 5'-ACCGCGGGCTCCGAGTAGC-3'

Expected PCR Products for Genotyping of Puma KO Mice

Wildtype band: 203 bp
Knockout band: 376 bp

APPENDIX D- Rb/Flox and Cre Genotyping

Fermentas PCR Reagents for Genotyping of Conditional Rb KO Mice

Rb-Flox		**Cre**	
Reagent	Vol(µL)	Reagent	Vol(µL)
10x Buffer(+KCl-$MgCl_2$)	2.50	10x Buffer(+KCl-$MgCl_2$)	2.50
25 mM MgCl2	2.50	25 mM MgCl2	2.00
2.5 mM dNTPs	2.00	2.5 mM dNTPs	2.00
5 µM Rb18 new	1.25	5 µM Cre5B	2.50
5 µM Rb19	1.25	5 µM Cre3B	2.50
1 U/µL Taq DNA polymerase	1.25	1 U/µL Taq DNA polymerase	1.50
DMSO	1.25	H2O(Dnase-free)	11.00
H2O(Dnase-free)	12.50		24.00
	24.50	DNA template (~0.5ug/µL)	1.00
DNA template (~0.5ug/µL)	0.50	Total	25.00
Total	25.00		

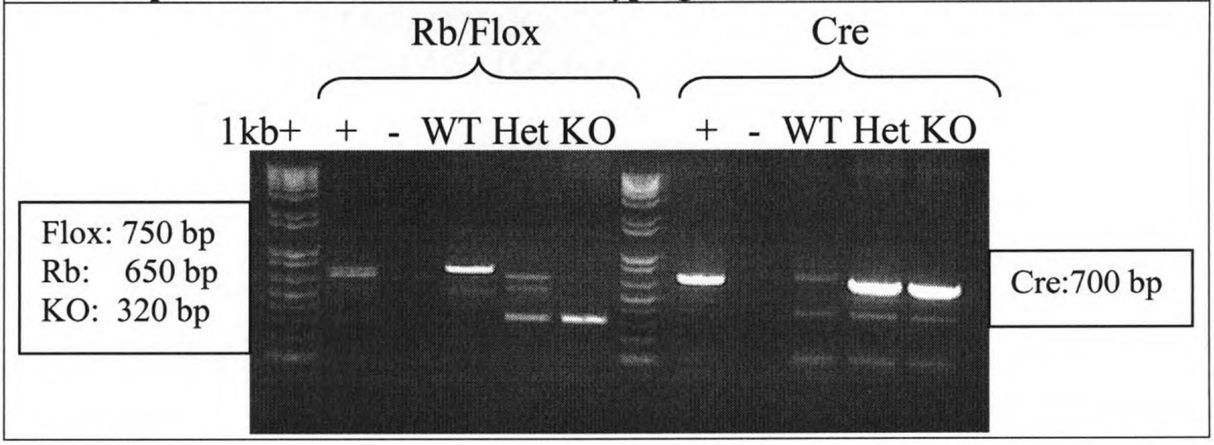
PCR Cycling Conditions for Genotyping of Conditional Rb KO Mice

Rb-Flox	**Cre**
1. 94°C for 5 mins	1. 95°C for 3 mins
2. 94°C for 45 secs	2. 95°C for 45 secs
3. 62°C for 45 secs (35 cycles)	3. 55°C for 45 secs (32 cycles)
4. 72°C for 1 min	4. 72°C for 1.5 mins
5. 72°C for 4 mins	5. 72°C for 5 mins
6. 10°C forever	6. 10°C forever

Primers for Genotyping of Conditional Rb KO Mice

Rb-Flox	**Cre**
Sense- Rb 18 new: 5'-GGCGTGTGCCATCAATG-3'	Sense- Cre5B: 5'-AATGCTTCTGTCCGTTTGCC-3'
Antisense- Rb 19: 5'-AACTCAAGGGAGACCTG-3'	Antisense- Cre3B: 5'-TGACCAGAGTCATCCTTAGCG-3'

Expected PCR Products for Genotyping of Conditional Rb KO Mice



APPENDIX E- qRT-PCR of Bcl-2 Family Genes

QIAGEN Reagents for qRT-PCR of Bcl-2 Family & S12 Genes

Reagent	Vol(µL)
2x QuantiTect SYBR Green RT-PCR Master Mix	12.50
5 µM Forward primer	3.00
5 µM Reverse primer	3.00
DNase, RNase-free H_2O	2.25
QuantiTect RT mix	0.25
	21.00
5 ng/µL RNA template	4.00
Total	25.00

Cycling Conditions for qRT-PCR of Bcl-2 Family & S12 Genes

1. 50°C for 30 mins
2. 95°C for 15 mins
3. 94°C for 25 secs
4. 58°C for 30 secs
5. 72°C for 40 secs — 36 cycles
6. Plate read
7. 72°C for 5 mins
8. Melting curve 65°C to 95°C, read every 0.2°C, hold 1 sec
9. END

Primers for qRT-PCR of Bcl-2 Family & S12 Genes

Gene	5'-Sense-3'	5'-Antisense-3'
Bad	AAGTCCGATCCCGGAATCC	GCTCACTCGGCTCAAACTCT
Bax	CCGGCGAATTGGAGATGAACT	CCAGCCCATGATGGTTCTGAT
Bid	GCCGAGCACATCACAGACC	TGGCAATGTTGTGGATGATTTCT
Bim	GGTAATCCCGACGGCGAAGGGGAC	AAGAGAAATACCCACTGGAGGACC
Noxa	AGCTCAACTCAGGAAGATCG	GCCGTAAATTCACTTTGTCTCC
Puma	AGCACTTAGAGTCGCCCGT	GAGGAGTCCCATGAAGAGATTG
S12	GGAAGGCATAGCTGCTGG	CCTCGATGACATCCTTGG

APPENDIX F- Animal S.O.P. Approval

Western

Feb. 10, 2005

"This is the 1st Renewal Approval of this protocol"
"A Full protocol submission will be required in 2008"

Dear Dr Cregan:

Your "Application to Use Animals for Research or Teaching" entitled:

"Mechanisms of Bax-Mediated Cell Death Following Neuronal Injury"
Funding Agency—CIHR Grant # MOP-68995

has been approved by the University Council on Animal Care. This approval is valid from February 1, 2005 to January 31, 2006. The number for this project remains as #2004-005-01.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR. PAIN LEVEL - B

Mice	-	CD1	Preg. F	-	80
	-	C57Bl6, Rbflox	M/F	-	100
	-	C57Bl6, p53+/-	M/F	-	100
	-	C57Bl6, Bax+/-	M/F	-	200
	-	C57Bl6, Puma+/-	M/F	-	200
	-	C57Bl6, Noxa+/-	M/F	-	100
	-	C57Bl6, Bim+/-	M/F	-	100

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies. SOP's are also available at http://www.uwo.ca/animal/acvs

310 Holding Period Post Admission
320 Euthanasia
321 Criteria for Early Euthanasia/Rodent

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

CHANGES: New staff added; animal numbers have increased.

c.c. Approved Protocol - S. Cregan, W. Lagerwerf
Approval Letter - W. Lagerwerf

APPENDIX G- Rb/Flox-Cre Breeding Scheme

Rb/Flox: BRF1+/+ x Rb/Rb: BRF1-Cre/+

F1: 25% Rb/Flox: BRF1-Cre/+

Rb/Flox: BRF1-Cre/+ x Rb/Flox: BRF1-Cre/+

F2: 8.33% Flox/Flox: BRF1+/+

33.33% Rb/Flox: BRF1-Cre/+

Rb/Flox: BRF1-Cre/+ x Flox/Flox: BRF1+/+

F3: 25% Rb/Flox: BRF1+/+

25% Rb/Flox: BRF1-Cre/+

25% Flox/Flox: BRF1+/+

25% Flox/Flox: BRF1-Cre/+

CHAPTER 6- REFERENCES

Adams,J.M., and Cory,S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene *26*, 1324-1337.

Alvarez,B., and Radi,R. (2003). Peroxynitrite reactivity with amino acids and proteins. Amino. Acids *25*, 295-311.

Antunes,F., Nunes,C., Laranjinha,J., and Cadenas,E. (2005). Redox interactions of nitric oxide with dopamine and its derivatives. Toxicology *208*, 207-212.

Attwooll,C., Lazzerini,D.E., and Helin,K. (2004). The E2F family: specific functions and overlapping interests. EMBO J. *23*, 4709-4716.

Barzilai,A., and Yamamoto,K. (2004). DNA damage responses to oxidative stress. DNA Repair (Amst) *3*, 1109-1115.

Biswas,S.C., Liu,D.X., and Greene,L.A. (2005a). Bim is a direct target of a neuronal E2F-dependent apoptotic pathway. J. Neurosci. *25*, 8349-8358.

Biswas,S.C., Ryu,E., Park,C., Malagelada,C., and Greene,L.A. (2005b). Puma and p53 play required roles in death evoked in a cellular model of Parkinson disease. Neurochem. Res. *30*, 839-845.

Biswas,S.C., Shi,Y., Vonsattel,J.P., Leung,C.L., Troy,C.M., and Greene,L.A. (2007). Bim is elevated in Alzheimer's disease neurons and is required for beta-amyloid-induced neuronal apoptosis. J. Neurosci. *27*, 893-900.

Blakely,W.F., Fuciarelli,A.F., Wegher,B.J., and Dizdaroglu,M. (1990). Hydrogen peroxide-induced base damage in deoxyribonucleic acid. Radiat. Res. *121*, 338-343.

Bouillet,P., Metcalf,D., Huang,D.C., Tarlinton,D.M., Kay,T.W., Kontgen,F., Adams,J.M., and Strasser,A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science *286*, 1735-1738.

Bouillet,P., Purton,J.F., Godfrey,D.I., Zhang,L.C., Coultas,L., Puthalakath,H., Pellegrini,M., Cory,S., Adams,J.M., and Strasser,A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature *415*, 922-926.

Bouillet,P., and Strasser,A. (2002). BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J. Cell Sci. *115*, 1567-1574.

Buck,L.T., and Pamenter,M.E. (2006). Adaptive responses of vertebrate neurons to anoxia--matching supply to demand. Respir. Physiol Neurobiol. *154*, 226-240.

Busser,J., Geldmacher,D.S., and Herrup,K. (1998). Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. J. Neurosci. *18*, 2801-2807.

Butterfield,D.A., and Boyd-Kimball,D. (2005). The critical role of methionine 35 in Alzheimer's amyloid beta-peptide (1-42)-induced oxidative stress and neurotoxicity. Biochim. Biophys. Acta *1703*, 149-156.

Butterworth,N.J., Williams,L., Bullock,J.Y., Love,D.R., Faull,R.L., and Dragunow,M. (1998). Trinucleotide (CAG) repeat length is positively correlated with the degree of DNA fragmentation in Huntington's disease striatum. Neuroscience *87*, 49-53.

Cartron,P.F., Gallenne,T., Bougras,G., Gautier,F., Manero,F., Vusio,P., Meflah,K., Vallette,F.M., and Juin,P. (2004). The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. Mol. Cell *16*, 807-818.

Chen,L., and Gao,X. (2002). Neuronal apoptosis induced by endoplasmic reticulum stress. Neurochem. Res. *27*, 891-898.

Chen,L., Willis,S.N., Wei,A., Smith,B.J., Fletcher,J.I., Hinds,M.G., Colman,P.M., Day,C.L., Adams,J.M., and Huang,D.C. (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol. Cell *17*, 393-403.

Cheng,E.H., Wei,M.C., Weiler,S., Flavell,R.A., Mak,T.W., Lindsten,T., and Korsmeyer,S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell *8*, 705-711.

Chinopoulos,C., and Adam-Vizi,V. (2006). Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme. FEBS J. *273*, 433-450.

Chiu,D.T., van den,B.J., Kuypers,F.A., Hung,I.J., Wei,J.S., and Liu,T.Z. (1996). Correlation of membrane lipid peroxidation with oxidation of hemoglobin variants: possibly related to the rates of hemin release. Free Radic. Biol. Med. *21*, 89-95.

Chong,Z.Z., Li,F., and Maiese,K. (2005). Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. Prog. Neurobiol. *75*, 207-246.

Cobrinik,D. (2005). Pocket proteins and cell cycle control. Oncogene *24*, 2796-2809.

Contestabile,A. (2001). Oxidative stress in neurodegeneration: mechanisms and therapeutic perspectives. Curr. Top. Med. Chem. *1*, 553-568.

Cregan,S.P., Arbour,N.A., Maclaurin,J.G., Callaghan,S.M., Fortin,A., Cheung,E.C., Guberman,D.S., Park,D.S., and Slack,R.S. (2004a). p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. J. Neurosci. *24*, 10003-10012.

Cregan,S.P., Dawson,V.L., and Slack,R.S. (2004b). Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene *23*, 2785-2796.

Cregan,S.P., Fortin,A., Maclaurin,J.G., Callaghan,S.M., Cecconi,F., Yu,S.W., Dawson,T.M., Dawson,V.L., Park,D.S., Kroemer,G., and Slack,R.S. (2002). Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. J. Cell Biol. *158*, 507-517.

Cregan,S.P., Maclaurin,J.G., Craig,C.G., Robertson,G.S., Nicholson,D.W., Park,D.S., and Slack,R.S. (1999). Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. J. Neurosci. *19*, 7860-7869.

Culmsee,C., and Landshamer,S. (2006). Molecular insights into mechanisms of the cell death program: role in the progression of neurodegenerative disorders. Curr. Alzheimer Res. *3*, 269-283.

Dalle-Donne,I., Rossi,R., Colombo,R., Giustarini,D., and Milzani,A. (2006). Biomarkers of oxidative damage in human disease. Clin. Chem. *52*, 601-623.

Dawson,T.M., Dawson,V.L., and Snyder,S.H. (1992). A novel neuronal messenger molecule in brain: the free radical, nitric oxide. Ann. Neurol. *32*, 297-311.

Degracia,D.J., and Montie,H.L. (2004). Cerebral ischemia and the unfolded protein response. J. Neurochem. *91*, 1-8.

Degregori,J., and Johnson,D.G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. Curr. Mol. Med. *6*, 739-748.

Deshmukh,M., and Johnson,E.M., Jr. (1998). Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome c. Neuron *21*, 695-705.

Dimova,D.K., and Dyson,N.J. (2005). The E2F transcriptional network: old acquaintances with new faces. Oncogene *24*, 2810-2826.

Dizdaroglu,M., Rao,G., Halliwell,B., and Gajewski,E. (1991). Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. Arch. Biochem. Biophys. *285*, 317-324.

Du,W., and Pogoriler,J. (2006). Retinoblastoma family genes. Oncogene *25*, 5190-5200.

Ekshyyan,O., and Aw,T.Y. (2004). Apoptosis: a key in neurodegenerative disorders. Curr. Neurovasc. Res. *1*, 355-371.

El-Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W., and Vogelstein,B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Gines,S., Ivanova,E., Seong,I.S., Saura,C.A., and MacDonald,M.E. (2003). Enhanced Akt signaling is an early pro-survival response that reflects N-methyl-D-aspartate receptor activation in Huntington's disease knock-in striatal cells. J. Biol. Chem. *278*, 50514-50522.

Giovanni,A., Keramaris,E., Morris,E.J., Hou,S.T., O'Hare,M., Dyson,N., Robertson,G.S., Slack,R.S., and Park,D.S. (2000). E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. J. Biol. Chem. *275*, 11553-11560.

Halliwell,B. (1978). Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: the key role of superoxide dismutase. Cell Biol. Int. Rep. *2*, 113-128.

Halliwell,B. (1992). Reactive oxygen species and the central nervous system. J. Neurochem. *59*, 1609-1623.

Halliwell,B. (2006). Oxidative stress and neurodegeneration: where are we now? J. Neurochem. *97*, 1634-1658.

Halliwell,B., Zhao,K., and Whiteman,M. (1999). Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. Free Radic. Res. *31*, 651-669.

Hannon,G.J., and Beach,D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature *371*, 257-261.

Hanrott,K., Gudmunsen,L., O'Neill,M.J., and Wonnacott,S. (2006). 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta. J. Biol. Chem. *281*, 5373-5382.

Harada,H., Becknell,B., Wilm,M., Mann,M., Huang,L.J., Taylor,S.S., Scott,J.D., and Korsmeyer,S.J. (1999). Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol. Cell *3*, 413-422.

Harada,H., Quearry,B., Ruiz-Vela,A., and Korsmeyer,S.J. (2004). Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proc. Natl. Acad. Sci. U. S. A *101*, 15313-15317.

Harris,C.A., and Johnson,E.M., Jr. (2001). BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J. Biol. Chem. *276*, 37754-37760.

Hartmann,A., Hunot,S., Michel,P.P., Muriel,M.P., Vyas,S., Faucheux,B.A., Mouatt-Prigent,A., Turmel,H., Srinivasan,A., Ruberg,M., Evan,G.I., Agid,Y., and Hirsch,E.C. (2000). Caspase-3: A vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. Proc. Natl. Acad. Sci. U. S. A *97*, 2875-2880.

Helin,K. (1998). Regulation of cell proliferation by the E2F transcription factors. Curr. Opin. Genet. Dev. *8*, 28-35.

Hershko,T., Chaussepied,M., Oren,M., and Ginsberg,D. (2005). Novel link between E2F and p53: proapoptotic cofactors of p53 are transcriptionally upregulated by E2F. Cell Death. Differ. *12*, 377-383.

Hershko,T., and Ginsberg,D. (2004). Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. J. Biol. Chem. *279*, 8627-8634.

Hong,S.J., Dawson,T.M., and Dawson,V.L. (2004). Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol. Sci. *25*, 259-264.

Horowitz,J.M., Pastor,D.M., Goyal,A., Kar,S., Ramdeen,N., Hallas,B.H., and Torres,G. (2003). BAX protein-immunoreactivity in midbrain neurons of Parkinson's disease patients. Brain Res. Bull. *62*, 55-61.

Huang,C.C., Faber,P.W., Persichetti,F., Mittal,V., Vonsattel,J.P., MacDonald,M.E., and Gusella,J.F. (1998). Amyloid formation by mutant huntingtin: threshold, progressivity and recruitment of normal polyglutamine proteins. Somat. Cell Mol. Genet. *24*, 217-233.

Jellinger,K.A. (2001). Cell death mechanisms in neurodegeneration. J. Cell Mol. Med. *5*, 1-17.

Jin,K., Mao,X.O., Eshoo,M.W., Nagayama,T., Minami,M., Simon,R.P., and Greenberg,D.A. (2001). Microarray analysis of hippocampal gene expression in global cerebral ischemia. Ann. Neurol. *50*, 93-103.

Jin,Z., and El-Deiry,W.S. (2005). Overview of cell death signaling pathways. Cancer Biol. Ther. *4*, 139-163.

Katayama,T., Imaizumi,K., Manabe,T., Hitomi,J., Kudo,T., and Tohyama,M. (2004). Induction of neuronal death by ER stress in Alzheimer's disease. J. Chem. Neuroanat. *28*, 67-78.

Ke,N., Godzik,A., and Reed,J.C. (2001). Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak. J. Biol. Chem. *276*, 12481-12484.

Keramaris,E., Stefanis,L., MacLaurin,J., Harada,N., Takaku,K., Ishikawa,T., Taketo,M.M., Robertson,G.S., Nicholson,D.W., Slack,R.S., and Park,D.S. (2000). Involvement of caspase 3 in apoptotic death of cortical neurons evoked by DNA damage. Mol. Cell Neurosci. *15*, 368-379.

Kerr,J.F., Winterford,C.M., and Harmon,B.V. (1994). Apoptosis. Its significance in cancer and cancer therapy. Cancer *73*, 2013-2026.

Kerr,J.F., Wyllie,A.H., and Currie,A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Kidd,P.M. (2005). Neurodegeneration from mitochondrial insufficiency: nutrients, stem cells, growth factors, and prospects for brain rebuilding using integrative management. Altern. Med. Rev. *10*, 268-293.

Kirkland,R.A., and Franklin,J.L. (2003). Bax, reactive oxygen, and cytochrome c release in neuronal apoptosis. Antioxid. Redox. Signal. *5*, 589-596.

Kitamura,Y., Shimohama,S., Kamoshima,W., Ota,T., Matsuoka,Y., Nomura,Y., Smith,M.A., Perry,G., Whitehouse,P.J., and Taniguchi,T. (1998). Alteration of proteins regulating apoptosis, Bcl-2, Bcl-x, Bax, Bak, Bad, ICH-1 and CPP32, in Alzheimer's disease. Brain Res. *780*, 260-269.

Konishi,Y., Lehtinen,M., Donovan,N., and Bonni,A. (2002). Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol. Cell *9*, 1005-1016.

Kranenburg,O., van der Eb,A.J., and Zantema,A. (1996). Cyclin D1 is an essential mediator of apoptotic neuronal cell death. EMBO J. *15*, 46-54.

Krieger,C., and Duchen,M.R. (2002). Mitochondria, Ca2+ and neurodegenerative disease. Eur. J. Pharmacol. *447*, 177-188.

Kudo,T. (2003). [Involvement of unfolded protein responses in neurodegeneration]. Nihon Shinkei Seishin Yakurigaku Zasshi *23*, 105-109.

Kurz,E.U., and Lees-Miller,S.P. (2004). DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amst) *3*, 889-900.

Kuwana,T., Bouchier-Hayes,L., Chipuk,J.E., Bonzon,C., Sullivan,B.A., Green,D.R., and Newmeyer,D.D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. Mol. Cell *17*, 525-535.

Lee,S.S., Kim,Y.M., Junn,E., Lee,G., Park,K.H., Tanaka,M., Ronchetti,R.D., Quezado,M.M., and Mouradian,M.M. (2003). Cell cycle aberrations by alpha-synuclein over-expression and cyclin B immunoreactivity in Lewy bodies. Neurobiol. Aging *24*, 687-696.

Liu,D.X., Biswas,S.C., and Greene,L.A. (2004). B-myb and C-myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage. J. Neurosci. *24*, 8720-8725.

Liu,D.X., Nath,N., Chellappan,S.P., and Greene,L.A. (2005). Regulation of neuron survival and death by p130 and associated chromatin modifiers. Genes Dev. *19*, 719-732.

Loo,D.T., Copani,A., Pike,C.J., Whittemore,E.R., Walencewicz,A.J., and Cotman,C.W. (1993). Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc. Natl. Acad. Sci. U. S. A *90*, 7951-7955.

Lossi,L., and Merighi,A. (2003). In vivo cellular and molecular mechanisms of neuronal apoptosis in the mammalian CNS. Prog. Neurobiol. *69*, 287-312.

Lu,G., Kwong,W.H., Li,Q., Wang,X., Feng,Z., and Yew,D.T. (2005). bcl2, bax, and nestin in the brains of patients with neurodegeneration and those of normal aging. J. Mol. Neurosci. *27*, 167-174.

Lyras,L., Cairns,N.J., Jenner,A., Jenner,P., and Halliwell,B. (1997). An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. J. Neurochem. *68*, 2061-2069.

Lyras,L., Perry,R.H., Perry,E.K., Ince,P.G., Jenner,A., Jenner,P., and Halliwell,B. (1998). Oxidative damage to proteins, lipids, and DNA in cortical brain regions from patients with dementia with Lewy bodies. J. Neurochem. *71*, 302-312.

MacManus,J.P., Jian,M., Preston,E., Rasquinha,I., Webster,J., and Zurakowski,B. (2003). Absence of the transcription factor E2F1 attenuates brain injury and improves behavior after focal ischemia in mice. J. Cereb. Blood Flow Metab *23*, 1020-1028.

MacManus,J.P., Koch,C.J., Jian,M., Walker,T., and Zurakowski,B. (1999). Decreased brain infarct following focal ischemia in mice lacking the transcription factor E2F1. Neuroreport *10*, 2711-2714.

Marzo,I., Brenner,C., Zamzami,N., Jurgensmeier,J.M., Susin,S.A., Vieira,H.L., Prevost,M.C., Xie,Z., Matsuyama,S., Reed,J.C., and Kroemer,G. (1998). Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. Science *281*, 2027-2031.

Mattson,M.P. (2000). Apoptosis in neurodegenerative disorders. Nat. Rev. Mol. Cell Biol. *1*, 120-129.

Mello Filho,A.C., and Meneghini,R. (1984). In vivo formation of single-strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss reaction. Biochim. Biophys. Acta *781*, 56-63.

Misiti,F., Martorana,G.E., Nocca,G., Di,S.E., Giardina,B., and Clementi,M.E. (2004). Methionine 35 oxidation reduces toxic and pro-apoptotic effects of the amyloid beta-protein fragment (31-35) on isolated brain mitochondria. Neuroscience *126*, 297-303.

Moro,M.A., Almeida,A., Bolanos,J.P., and Lizasoain,I. (2005). Mitochondrial respiratory chain and free radical generation in stroke. Free Radic. Biol. Med. *39*, 1291-1304.

Morris,E.J., and Geller,H.M. (1996). Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. J. Cell Biol. *134*, 757-770.

Nakano,K., and Vousden,K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol. Cell *7*, 683-694.

O'Hare,M.J., Hou,S.T., Morris,E.J., Cregan,S.P., Xu,Q., Slack,R.S., and Park,D.S. (2000). Induction and modulation of cerebellar granule neuron death by E2F-1. J. Biol. Chem. *275*, 25358-25364.

Oda,E., Ohki,R., Murasawa,H., Nemoto,J., Shibue,T., Yamashita,T., Tokino,T., Taniguchi,T., and Tanaka,N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Overmyer,M., Kraszpulski,M., Helisalmi,S., Soininen,H., and Alafuzoff,I. (2000). DNA fragmentation, gliosis and histological hallmarks of Alzheimer's disease. Acta Neuropathol. (Berl) *100*, 681-687.

Padmanabhan,J., Park,D.S., Greene,L.A., and Shelanski,M.L. (1999). Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis. J. Neurosci. *19*, 8747-8756.

Park,D.S., Farinelli,S.E., and Greene,L.A. (1996). Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. J. Biol. Chem. *271*, 8161-8169.

Park,D.S., Morris,E.J., Bremner,R., Keramaris,E., Padmanabhan,J., Rosenbaum,M., Shelanski,M.L., Geller,H.M., and Greene,L.A. (2000). Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. J. Neurosci. *20*, 3104-3114.

Park,D.S., Morris,E.J., Padmanabhan,J., Shelanski,M.L., Geller,H.M., and Greene,L.A. (1998a). Cyclin-dependent kinases participate in death of neurons evoked by DNA-damaging agents. J. Cell Biol. *143*, 457-467.

Park,D.S., Morris,E.J., Stefanis,L., Troy,C.M., Shelanski,M.L., Geller,H.M., and Greene,L.A. (1998b). Multiple pathways of neuronal death induced by DNA-damaging agents, NGF deprivation, and oxidative stress. J. Neurosci. *18*, 830-840.

Pavlov,E.V., Priault,M., Pietkiewicz,D., Cheng,E.H., Antonsson,B., Manon,S., Korsmeyer,S.J., Mannella,C.A., and Kinnally,K.W. (2001). A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast. J. Cell Biol. *155*, 725-731.

Pines,J. (1995). Cyclins and cyclin-dependent kinases: a biochemical view. Biochem. J. *308 (Pt 3)*, 697-711.

Puthalakath,H., and Strasser,A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death. Differ. *9*, 505-512.

Reimertz,C., Kogel,D., Rami,A., Chittenden,T., and Prehn,J.H. (2003). Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. J. Cell Biol. *162*, 587-597.

Reynisdottir,I., Polyak,K., Iavarone,A., and Massague,J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. *9*, 1831-1845.

Rideout,H.J., Wang,Q., Park,D.S., and Stefanis,L. (2003). Cyclin-dependent kinase activity is required for apoptotic death but not inclusion formation in cortical neurons after proteasomal inhibition. J. Neurosci. *23*, 1237-1245.

Roy,M., and Sapolsky,R. (1999). Neuronal apoptosis in acute necrotic insults: why is this subject such a mess? Trends Neurosci. *22*, 419-422.

Sapirstein,A., and Bonventre,J.V. (2000). Phospholipases A2 in ischemic and toxic brain injury. Neurochem. Res. *25*, 745-753.

Scorrano,L., and Korsmeyer,S.J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem. Biophys. Res. Commun. *304*, 437-444.

Scorrano,L., Oakes,S.A., Opferman,J.T., Cheng,E.H., Sorcinelli,M.D., Pozzan,T., and Korsmeyer,S.J. (2003). BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science *300*, 135-139.

Sedarous,M., Keramaris,E., O'Hare,M., Melloni,E., Slack,R.S., Elce,J.S., Greer,P.A., and Park,D.S. (2003). Calpains mediate p53 activation and neuronal death evoked by DNA damage. J. Biol. Chem. *278*, 26031-26038.

Shackelford,R.E., Kaufmann,W.K., and Paules,R.S. (2000). Oxidative stress and cell cycle checkpoint function. Free Radic. Biol. Med. *28*, 1387-1404.

Shen,X., Zhang,K., and Kaufman,R.J. (2004). The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. J. Chem. Neuroanat. *28*, 79-92.

Shimizu,S., Konishi,A., Kodama,T., and Tsujimoto,Y. (2000). BH4 domain of antiapoptotic Bcl-2 family members closes voltage-dependent anion channel and inhibits apoptotic mitochondrial changes and cell death. Proc. Natl. Acad. Sci. U. S. A *97*, 3100-3105.

Shimizu,S., Narita,M., and Tsujimoto,Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature *399*, 483-487.

Skulachev,V.P. (1997). Aging is a specific biological function rather than the result of a disorder in complex living systems: biochemical evidence in support of Weismann's hypothesis. Biochemistry (Mosc.) *62*, 1191-1195.

Smith,F.M., Raghupathi,R., MacKinnon,M.A., McIntosh,T.K., Saatman,K.E., Meaney,D.F., and Graham,D.I. (2000). TUNEL-positive staining of surface contusions after fatal head injury in man. Acta Neuropathol. (Berl) *100*, 537-545.

Stefanis,L. (2005). Caspase-dependent and -independent neuronal death: two distinct pathways to neuronal injury. Neuroscientist. *11*, 50-62.

Tao,Y., Kassatly,R.F., Cress,W.D., and Horowitz,J.M. (1997). Subunit composition determines E2F DNA-binding site specificity. Mol. Cell Biol. *17*, 6994-7007.

Tsujimoto,Y. (2003). Cell death regulation by the Bcl-2 protein family in the mitochondria. J. Cell Physiol *195*, 158-167.

van Gurp,M., Festjens,N., van,L.G., Saelens,X., and Vandenabeele,P. (2003). Mitochondrial intermembrane proteins in cell death. Biochem. Biophys. Res. Commun. *304*, 487-497.

Verkhratsky,A., and Toescu,E.C. (2003). Endoplasmic reticulum Ca(2+) homeostasis and neuronal death. J. Cell Mol. Med. *7*, 351-361.

Vermeulen,K., Van Bockstaele,D.R., and Berneman,Z.N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. *36*, 131-149.

Walensky,L.D., Pitter,K., Morash,J., Oh,K.J., Barbuto,S., Fisher,J., Smith,E., Verdine,G.L., and Korsmeyer,S.J. (2006). A stapled BID BH3 helix directly binds and activates BAX. Mol. Cell *24*, 199-210.

Wek,R.C., Jiang,H.Y., and Anthony,T.G. (2006). Coping with stress: eIF2 kinases and translational control. Biochem. Soc. Trans. *34*, 7-11.

White,R.J., and Reynolds,I.J. (1997). Mitochondria accumulate Ca2+ following intense glutamate stimulation of cultured rat forebrain neurones. J. Physiol *498 (Pt 1)*, 31-47.

Willis,S.N., Fletcher,J.I., Kaufmann,T., van Delft,M.F., Chen,L., Czabotar,P.E., Ierino,H., Lee,E.F., Fairlie,W.D., Bouillet,P., Strasser,A., Kluck,R.M., Adams,J.M., and Huang,D.C. (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science *315*, 856-859.

Won,S.J., Kim,D.Y., and Gwag,B.J. (2002). Cellular and molecular pathways of ischemic neuronal death. J. Biochem. Mol. Biol. *35*, 67-86.

Wong,H.K., Fricker,M., Wyttenbach,A., Villunger,A., Michalak,E.M., Strasser,A., and Tolkovsky,A.M. (2005). Mutually exclusive subsets of BH3-only proteins are activated by the p53 and c-Jun N-terminal kinase/c-Jun signaling pathways during cortical neuron apoptosis induced by arsenite. Mol. Cell Biol. *25*, 8732-8747.

Wyttenbach,A., and Tolkovsky,A.M. (2006). The BH3-only protein Puma is both necessary and sufficient for neuronal apoptosis induced by DNA damage in sympathetic neurons. J. Neurochem. *96*, 1213-1226.

Xu,C., Bailly-Maitre,B., and Reed,J.C. (2005). Endoplasmic reticulum stress: cell life and death decisions. J. Clin. Invest *115*, 2656-2664.

Yin,X.M., Wang,K., Gross,A., Zhao,Y., Zinkel,S., Klocke,B., Roth,K.A., and Korsmeyer,S.J. (1999). Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature *400*, 886-891.

Yu,J., Zhang,L., Hwang,P.M., Kinzler,K.W., and Vogelstein,B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell *7*, 673-682.

Yuan,J., Lipinski,M., and Degterev,A. (2003). Diversity in the mechanisms of neuronal cell death. Neuron *40*, 401-413.

Zarkowska,T., and Mittnacht,S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J. Biol. Chem. *272*, 12738-12746.

Zha,J., Weiler,S., Oh,K.J., Wei,M.C., and Korsmeyer,S.J. (2000). Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science *290*, 1761-1765.

Zimmermann,A.K., Loucks,F.A., Le,S.S., Butts,B.D., Florez-McClure,M.L., Bouchard,R.J., Heidenreich,K.A., and Linseman,D.A. (2005). Distinct mechanisms of

neuronal apoptosis are triggered by antagonism of Bcl-2/Bcl-x(L) versus induction of the BH3-only protein Bim. J. Neurochem. *94*, 22-36.