Role of ATF4 in neuronal death mediated by DNA damage, endoplasmic reticulum stress and ischemia-hypoxia

Zohreh Galehdar

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Department of Cellular and Molecular Medicine, Faculty of Medicine
University of Ottawa

"The important thing is not to stop questioning;
***curiosity** has its own reason for existing."*

Albert Einstein.

*"Nothing in life is to be feared, it is only **to be understood**.*
*Now is the time **to understand more**, so that we may **fear less**."*

Marie Curie

Abstract.

An increasing body of evidence points to a key role of endoplasmic reticulum (ER) stress in chronic and acute neurodegenerative diseases. Indeed, markers of ER stress are common features of neurons destined to die in these conditions. In the present study we demonstrate that PUMA, a BH3-only member of the Bcl-2 family is essential for ER stress-induced cell death. PUMA is known to be a key transcriptional target of p53, however we have found that ER stress triggers PUMA induction and cell death through a p53-independent mechanism involving instead the ER stress inducible transcription factor ATF4. Specifically, we demonstrate that ectopic expression of ATF4 sensitizes neurons to ER stress induced apoptosis, and that ATF4-deficient neurons exhibit markedly reduced levels of PUMA expression and cell death. However, chromatin immunoprecipitation experiments suggest that ATF4 does not directly regulate the PUMA promoter. Rather, we found that ATF4 induces expression of the transcription factor CHOP, and that CHOP in turn directly activates PUMA induction. Specifically, we demonstrate that CHOP binds to the PUMA promoter during ER stress and that CHOP knockdown attenuates PUMA induction and neuronal apoptosis. In summary, we have identified a key signaling pathway in ER stress induced neuronal death involving ATF4-CHOP mediated transactivation of the pro-apoptotic Bcl-2 family member PUMA.

Protein aggregates and markers of ER stress response have also been observed in dying neurons in several animal models of cerebral ischemia. Therefore, to decipher the significance of the ER stress apoptotic response, we investigate the role of ATF4-CHOP signaling pathway in ischemic neuronal injury. Ischemic stroke results from a transient or permanent reduction in cerebral blood flow in the brain. In spite of much research in trying

to develop therapeutic strategies, most clinical trials have failed. These failures demonstrate that effective treatments require a more complete understanding of molecular signals that lead to neuronal death. However, stroke is a complex scenario since distinct mechanisms may involve in rapid and/or delayed neuronal death. The signaling pathways regulating these mechanisms however are not fully defined. Previous studies had suggested that ER stress playing a pivotal role in post-ischemic neuronal death. Yet, the relevance of ER stress signals was not fully known in ischemic neuronal injury. Accordingly, this thesis research attempts to explore the functional role of ER stress -inducible pathway, ATF4-CHOP axis, in different models of neuronal death (delayed and excitotoxic cell death) evoked by ischemia. The data indicates that ATF4 is essential in delayed type of death in vitro. In focal ischemia model (tMCAO) ATF4 also plays a role as a mediator of death signal *in vivo*. However, CHOP function looks more complex, and our data did not support the role of CHOP in ischemic neuronal death.

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List of Manuscripts

I. **Galehdar Z**, Swan P, Fuerth B, Callaghan SM, Park DS, Cregan SP. (2010) Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA. **Journal of Neuroscience.** 30(50): 16938-4810.

II. **Galehdar Z**, Safarpour F, Callaghan SM, Cregan SP, Park DS. The role of ATF4-CHOP pathway in neuronal damage-induced by cerebral ischemic injury. ***(Manuscript in preparation)***

Appended Articles

I. Bouman L, Schlierf A, Lutz AK, Shan J, Deinlein A, Kast J, **Galehdar Z**, Palmisano V, Patenge N, Berg D, Gasser T, Augustin R, Trümbach D, Irrcher I, Park DS, Wurst W, Kilberg MS, Tatzelt J, Winklhofer KF. (2011) Parkin is transcriptionally regulated by ATF4: evidence for an interconnection between mitochondrial stress and ER stress. **Cell Death Differntiation.** 18(5):769-82.

II. Iyirhiaro GO, Brust TB, Rashidian J, **Galehdar Z**, Osman A, Phillips M, Slack RS, Macvicar BA, Park DS. (2008) Delayed combinatorial treatment with flavopiridol and minocycline provides longer term protection for neuronal soma but not dendrites following global ischemia. **Journal of Neurochemistry**. 105(3):703-13.

List of figures & Tables

List of Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ANOVA	Analysis of Variance
Apaf1	Apoptotic protease activating factor 1
Apo-1	Apoptosis antigen 1
ASK1	Apoptosis-signal-regulating kinase 1
ATF	Activating Transcription Factor
Bad	Bcl-2 Associated Death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2–associated X protein
Bbc3	Bcl-2 binding component 3
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BH	Bcl-2 homology
Bid	BH3 interacting-domain death agonist
BiP	Bax-inhibiting peptide
53BP1	Tumor suppressor p53-binding protein 1
bZIP	Basic Leucine Zipper Domain
Caspase	Cysteine aspartyle protease
CD95	Cluster of differentiation 95
cDNA	Complementary DNA
C/EBP	CCAAT-enhancer-binding proteins
CED-3	Cell death protein-3
CGN	Cerebellar Granule Neuron
ChIP	Chromatin immunoprecipitation
CHOP	C/EBP-Homologous Protein
CNS	Central Nervous System
CPT	Camptothecin
CREB	cAMP response element-binding protein
CTRL	Control
DD	Death domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DNA	Deoxyribonucleic acid
DR4	Death receptor 4
DR5	Death receptor 5
DSB	DNA double-strand break
DTT	Dithiothreitol

ER	Endoplasmic reticulum
Egl-1	Egg laying defective-1
eIF2α	Eukaryotic Initiation Factor 2 alpha
FADD	Fas-associated death domain protein
FAS	Fatty acid synthase
FASL	FAS ligand
FDA	Food and Drug Administration
Foxa3a	Forkhead box, class O, 3a
GADD153	Growth arrest- and DNA damage-inducible gene 153
GDP	Guanosine diphosphate
GFP	Green Fluorescent Protein
GRP78	Glucose-regulated protein 78
GTP	Guanosine triphosphate
HD	Huntington's disease
IV	Intravenous
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
KO	Knockout
LC3	Light chain 3
MCAO	Middle Cerebral Artery Occlusion
MEF	Mouse embryonic fibroblast
Met-tRNAiMet	Methionyl-initiator tRNA
ml	Milliliter
MPTP	1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine
n	Sample size
NF-κB	Nuclear Factor Kappa Beta
OMM	Outer mitochondrial membrane
OMMP	Outer mitochondrial membrane permeabilization
P	Probability
PARP1	Poly ADP-ribose polymerase 1
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PERK	Pancreatic ER kinase (PKR)- like ER kinase
P58 (IPK)	Protein 58-inhibitor of PKR (Protein Kinase R)
pMCAO	permanent Middle Cerebral Artery Occlusion
PolyQ	Polyglutamine
PUMA	P53 up-regulated modulator of apoptosis
ROS	Reactive Oxygen species
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean

SERCA	Sarco-endoplasmic reticulum Ca^{2+}-ATPases
SQSTM1	Sequestosome 1
sXBP1	Spliced XBP1
TBI	Traumatic brain injury
tBid	Truncated Bid
TG	Thapsigargin
TM	Tunicamycin
tMCAO	transient Middle Cerebral Artery Occlusion
TNFα	Tumor Necrosis Factor alpha
Topo	Topoisomerase
tPA	Tissue Plasminogen Activator
TRADD	TNF Receptor Associated Death Domain
Trail	TNF-related apoptosis- inducing ligand
TRB3	Tribbles homolog 3
ULK1	Autophagy-initiating kinase 1
UPR	Unfolded protein response
5'UTR	Five prime untranslated region
uORF	Upstream open reading frame
WT	Wild Type
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
XBP1	X-Box binding protein-1
µg	micro gram
µm	micro Molar

Thesis format

This thesis has been prepared based on the format conformed to the guidelines of the Departments of Neuroscience and Cellular and Molecular Medicine. It has been written as a compendium of 4 chapters including: an introduction followed by two manuscripts and a general discussion as it relates to current knowledge in this field of research.

Chapter one, the introductory section, is a summarized review of the scientific literature relevant to the research presented in chapters two and three. This chapter emphasizes on programmed cell death and apoptosis, and general section dedicated to neuronal death-induced by DNA damage, and ER stress. A brief overview of the ischemic injury in neuronal death will also be presented, highlighting the role of transcription factor ATF4.

Chapter two presents an article entitled: "Neuronal Apoptosis-Induced by ER Stress is regulated by ATF4-CHOP Mediated Induction of the BH3-only Member PUMA". This work was published on the Journal of Neuroscience in the year 2010 and it has been presented here exactly as published. This paper, for the first time, provided downstream ER stress gene targets involving in neuronal apoptosis pathway.

Chapter three presents a manuscript entitled: "The role of ATF4-CHOP pathway in neuronal-damage induced by cerebral ischemic injury". This manuscript is in preparation. In this chapter the roles of ATF4 and its target gene, CHOP, in ischemic neuronal death has been investigated.

Finally, An overview of the major findings of this thesis is presented in **Chapter four**. A discussion on the potential direction that each of the projects could take in the future is included as well.

Chapter 1.

General Introduction

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."

Sir William Lawrence Bragg (1890 - 1971)
(Nobel Prize for Physics, 1915)

1. General Introduction

How we sense, perceive and behave depends on the organization and number of elements that make up our nervous system. Although the brain represents only 2% of human body weight, it is considered the most remarkable aspect of nervous system. The fundamental unit in the brain is the neuron. Neurons connect to each other through complex networks. While neurons are known as the longest living cells in mammals, near 50% of neurons will be eliminated during the embryonic period (Franklin, 2003). This natural elimination ensures proper and precise neuronal connection (Yuan et al., 2003). Conversely, the mammalian adult brain has a limited capacity to repair damage. In fact, the lack of proper self-repair system in the adult brain makes it incapable to regenerate neurons following damage. Neuronal death is an important topic in neuroscience research. Many therapeutic attempts have been made to protect neurons. Unfortunately most efforts have failed to provide positive long-term therapeutic benefit. To overcome this problem, it is critical to better understand the key molecular events that orchestrate the fate of injured neurons. The main focus of this thesis, therefore, is to identify key molecular elements involved in the fate of damaged neurons.

1.1. Programmed Cell Death or Apoptosis

The term of *Apoptosis* was coined in the early 70s in developing hepatocytes, the chief functional cells in the liver, to describe a form of cellular death with distinct

morphology (Kerr et al., 1972). This term was originally taken from Greek literature meaning "falling off", similar to leaves dropping off from trees. This term stresses the fact that cell death is essential during the life cycle of the organisms.[1] Apoptotic cells undergo a series of changes. Morphologically, apoptosis manifests with DNA fragmentation, chromatin condensation, and reduction in cellular volume. After death, cells are fragmented to apoptotic bodies. Generally these morphological changes are recognized as the common features in apoptotic cell death (Kerr et al., 1972, Saraste and Pulkki, 2000). Apoptosis is commonly used to signify programmed cell death, meaning that cell death by apoptosis requires an intracellular 'program' (Savill, 1994, Hacker, 2000).

1.1.A. Apoptotic Neuronal Cell Death

Apoptosis, or programmed cell death, is viewed as an evolutionarily conserved biological process involved in tissue remodeling for maintaining the proper number of cells. In addition, as an active cell-deletion pathway, it is implicated in eliminating damaged cells, without any concomitant inflammatory reaction. Consequently, other cells in neighboring tissue will be protected (Kerr et al., 1972, Pallet and Hebert, 2011). This aspect of apoptosis is in contrast to necrotic cell death. Apoptosis and necrosis are two basic forms of cell death. During necrotic cell death, an inflammatory reaction as a result of cell swelling and rupturing causes secondary cell damage in nearby cells (Padosch et al., 2001, Kermer et al., 2004, Elmore, 2007) **(Table 1.1).**

The concept of programmed cell death, as an essential event in developing nervous system, has been well studied (Burek and Oppenheim, 1996). In mature brain, most

[1] http://www.ncbi.nlm.nih.gov/books/NBK21475

neurons are post mitotic and cannot be replaced. Hence, neuronal death is tightly regulated in adulthood. Indeed, aberrant cell death is considered a catastrophic problem in the adult brain. Inappropriate or defective apoptosis can result in many human disorders from cancers to acute and chronic neurodegenerative diseases (Desjardins and Ledoux, 1998, Kaufmann and Gores, 2000, Lowe and Lin, 2000, Kermer et al., 2004, Sairanen et al., 2006, Broughton et al., 2009). Current research implicates the critical role of apoptosis in many of these disorders. Therefore, the field of apoptosis research continues to focus on the elucidation of molecular mechanisms, which regulate apoptosis. The desired outcome of these studies in the CNS is to discover effective therapies that can trigger apoptosis and potentially ameliorate in conditions of degeneration or injury.

Table 1.1. Morphological, and biochemical differences between apoptosis and necrosis

Apoptosis	Necrosis
Cells shrink	Cells swell
Cells detach from each other	Cells remain attached
Plasma membrane remains intact	Plasma membrane is disrupted
Nucleus is condensed, and fragmented	Nucleus swells, autolysis happens
Apoptotic bodies formation	Not apoptotic body formation
Nuclear DNA fragmentation	Nuclear DNA fragmentation
Phagocytosis of cellular fragments	Cellular lysis
No inflammation	Inflammation

*Diseases of Kidney & Urinary Tract, Robert W. Schrier, Volume II, 8th Edition, 2007

1.2. Mechanism of Apoptosis

Apoptosis is recognized as highly regulated suicide cell death program (Stefanis, 2005). The mechanism of apoptosis is complex. Current evidence emphasizes caspase activation as a central regulator of apoptosis associated with many of neurodegenerative disorders (Hartmann et al., 2000, Sweeney and Barton, 2000, Hartmann et al., 2001, Sanchez Mejia and Friedlander, 2001, Rohn and Head, 2008). In many of these diseases, the caspase-cascade system precedes the execution of cell death rather than being the consequence of cell demise. Caspases are specialized cysteine proteases family proteins responsible for apoptosis. They have homology with CED-3, the gene that commits neurons in *C. elegans* to apoptosis.

Apoptosis, a gene-directed cellular self-destruction program, is a critical component of development in *C. elegans.* Among all cells that are generated to form this nematode worm, certain numbers of cells undergo apoptosis. The critical involvement of caspases in apoptosis was discovered in this nematode. More than 20 cell-death genes have been identified in *C. elegans*. Several genes have been identified which serve as the foundation of developmental death. These include CED-3, 9, 4 and egl-1. Identifying the homology of these genes helped scientists to understand how the apoptotic machinery worked in other species including human. Mammalian apoptotic protease-activating factor-1 (APAF-1) has been found as CED-4 homology. CED-9 and egl-1 encode members of anti-and pro-apoptotic members of BCL-2 family, respectively. Ced-9 encodes a functional homolog of the mammalian anti-apoptotic bcl-2, while egl-1 encodes a protein that contains a region

similar to the BH3 (Bcl-2 homology region 3) domain of mammalian cell death activators[2] (Hengartner and Horvitz, 1994).

Among all caspases known in mammals, caspase-3 has the closest homology with CED-3 (Johnson and Bridgham, 2000). This gene is found as the most indispensable developmental death gene in *C. elegans*. It is the only apoptotic caspase in this nematode worm (Riedl and Shi, 2004, Lettre and Hengartner, 2006). Activation of CED-3 caspase requires CED-4. On the other hand, CED-4 is constitutively inhibited by anti-apoptotic gene, CED-9. Egl-1 functions as an upstream activator in programmed cell death pathway in *C.elegans.* It interacts with CED-9 and release CED-4 from CED-4/CED-9 complexes to activate the CED-3 caspase and ultimately apoptosis (Conradt and Horvitz, 1998, Yan et al., 2005).

Caspases are primarily produced as inactive zymogens. Following some specific proteolytic cleavage, they are converted to the active form. Apoptosis is primarily executed by active caspase (Donepudi and Grutter, 2002). Apoptotic caspases fall into two types, initiators or executioners, based on their point of entry into the apoptotic cascade (Kadirvel et al., 2010). Caspase-3 is recognized as the most notable executioner caspases (Boatright and Salvesen, 2003). The proteolytic activity of caspase-3 results in the morphological changes that occur during apoptosis (Liu et al., 1997). In mammalian systems, there are at least two major apoptosis-biochemical signaling pathways termed the extrinsic pathway and the intrinsic pathway. These two pathways are not completely separate from each other. Increasing evidence indicates that there is cross talk between these two pathways. Both of these signaling pathways converge to the activation of executioner caspases, the critical

[2] http://deathbase.org/protein_report.php?id=c_elegans_f23b12.9

elements in apoptosis, and the consequent recruitment of cell death (Stefanis, 2005, Tait and Green, 2010).

The *extrinsic or receptor-mediated death pathway* begins outside of the cell. This pathway mediates by binding the ligand to the variety of death receptors including Tumor Necrosis Factor (TNF), Fas, and Trail family receptors. The death receptors contain an intracellular interaction death domain (DD). Upon binding the ligands to extracellular domain of the death receptors at the cell surface, the receptors are aggregated. This leads to the recruitment of the death adaptor molecules to the intracellular aggregated death domains (DDs). One of the major adaptor proteins that is received the death signal is Fas Associated protein with Death Domain (FADD). FADD interacts with DD domain of death receptors either directly or through the other adaptor protein termed TRADD (TNF Receptor Associated Death Domain). In addition, FADD binds to procaspase-8 through its Death Effector Domain (DED) to assemble a multi–protein complex termed Death Inducing Signaling Complex (DISC), resulting in the activation of caspase-8. Active caspase-8 triggers other executioner caspases such as caspase-3 to provoke apoptosis (Putcha et al., 2002, Khosravi-Far and Esposti, 2004).

A variety of non-receptor-mediated stimuli can induce the intracellular signals and activate the second apoptotic pathway, the *intrinsic pathway* (Kroemer, 2003, Elmore, 2007). In this signaling pathway, mitochondria play a crucial role in the regulation of cell death events. Therefore, this pathway is recognized as the *mitochondrial pathway*. Most mammalian apoptotic cell death proceeds via disruption of the outer mitochondrial membrane (OMM) in this pathway. Outer mitochondrial membrane permeabilization (OMMP) depends on the activation, translocation and oligomerization of Bcl-2 family

proteins (Er et al., 2006). Bcl-2 family proteins are the major regulators of the mitochondrial death events. Overall 25 members of this family have been identified. Based on the presence and number of Bcl-2 Homology domain (BH domain), this family is composed of three classes: the anti-apoptotic proteins, such as Bcl-2 and Bcl-XL have BH1 through BH4 domains. The second class is the pro-apoptotic members, which can be divided into two categories: members with BH1 through BH3 domains, such as Bax and Bak, and those with only BH3 domains, such as Bad, and Bim (Yang et al., 1995, Wang et al., 1996a, Kelekar and Thompson, 1998, O'Connor et al., 1998). Pro- and anti-apoptotic members of Bcl-2 family proteins govern the mitochondrial outer membrane permeability through regulation of cytochrome C release from mitochondria (Elmore, 2007).

Upon stress stimuli, the Bad protein is dephosphorylated and associated with Bcl-2/Bcl-XL complex, inhibiting anti-apoptotic activity of these proteins. Following this inhibition, the pro-apoptotic regulator Bax protein translocates to the mitochondrial membrane and changes the membrane permeability. Altering mitochondrial permeability triggers cytochrome C release from the mitochondrial intermembrane space into the cytosol. In the cytosol, cytochrome C interacts with an adaptor protein, apoptotic protease activating factor 1 (Apaf1), to promote proteolytic protein caspase-9 activation. The cytochrome C, Apaf1 and procaspase-9 form a macromolecular complex called apoptosome. Apoptosome activates initiator caspase-9, which in turn activates caspase-3, caspase-6, and caspase-7. Activations of these effector caspases execute apoptosis death program (Haupt et al., 2003, Nicholson and Thornberry, 2003) **(Fig.1.1)**.

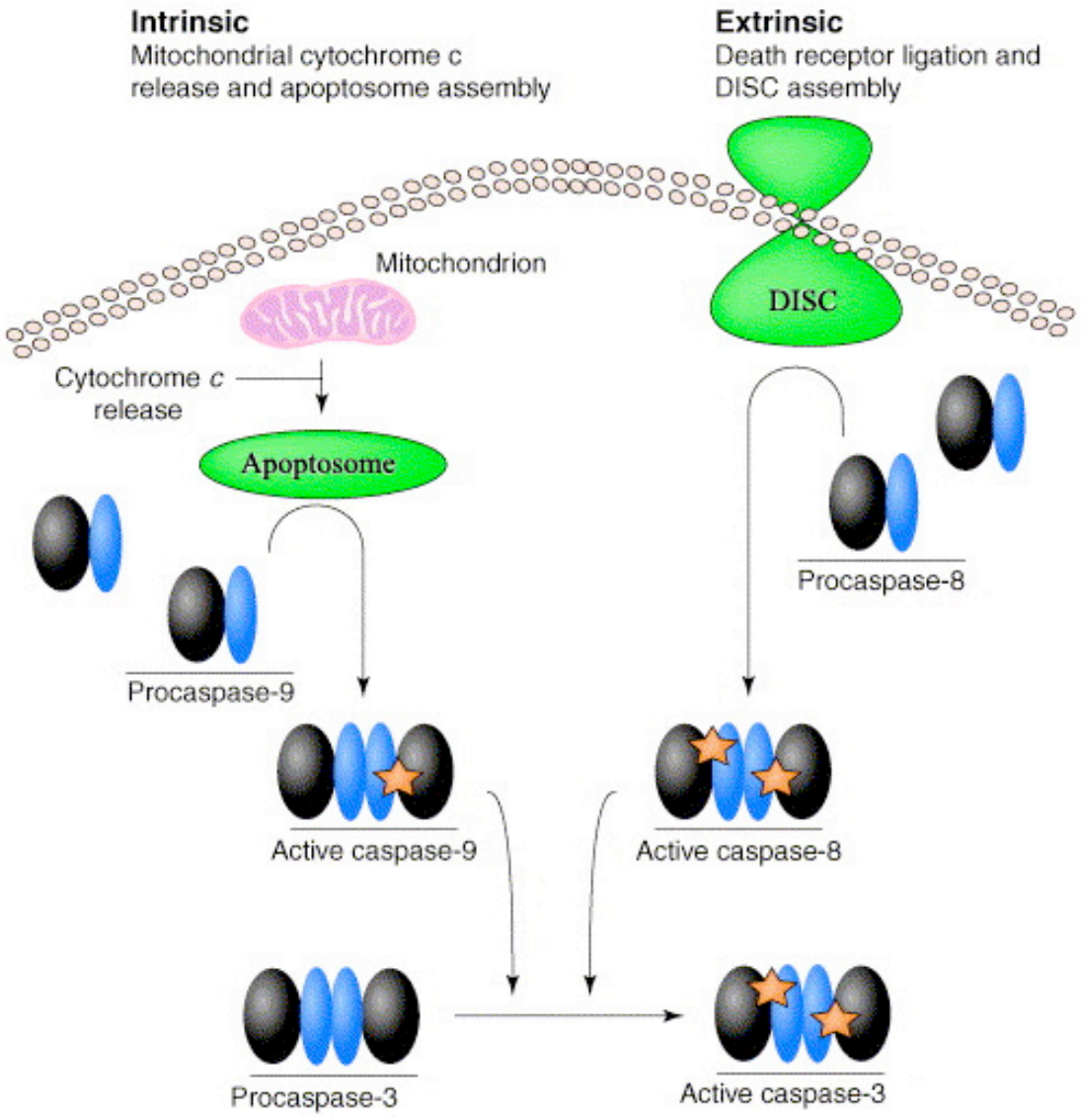

Intrinsic
Mitochondrial cytochrome c release and apoptosome assembly
Extrinsic
Death receptor ligation and DISC assembly
Mitochondrion
DISC
Cytochrome c release
Apoptosome
Procaspase-9
Procaspase-8
Active caspase-9
Active caspase-8
Procaspase-3
Active caspase-3

Figure 1.1.

Figure 1. 1. Schematic overview of the apoptotic pathways. The apoptotic caspases are classified as initiators or executioners, depending on their point of entry into the apoptotic cascade. Two pathways can initiate apoptosis: death receptors pathway (extrinsic pathway) and mitochondria pathway (intrinsic pathway). Engagement of either the extrinsic or the intrinsic death pathways leads to the activation of the initiator caspases. The initiator caspases constitute the first step in a minimal two-step death cascade by activating the executioner caspases. In the extrinsic and intrinsic apoptotic pathways activation of initiator caspases, caspase-8 and caspase-9 respectively, leads to activation of the executioner caspase-3. Activation of caspase-3 eventually results in apoptosis (Boatright and Salvesen, 2003).

Sometimes the extrinsic signaling cascade is not strong enough to trigger cell execution. Therefore the signal needs to be amplified through the mitochondrial-dependent pathway[3]. Mitochondrial active caspase-8 can attach to full-length Bid in cytosol, the pro-apoptotic BH3-only family member, and cleaves it. Truncated form of Bid (tBid) translocates to the mitochondria, and transduces signals from the cytoplasmic membrane to mitochondria. Following translocation, tBid interacts with pro-apoptotic Bax/Bak to induce the release of cytochrome C from the mitochondria. Cytochrome C activates caspase-9, which triggers caspase-3 to initiate execution cell death. This is one example of cross talk between receptor-mediated and mitochondrial apoptotic pathways (Li et al., 1998, Luo et al., 1998, Igney and Krammer, 2002).

Furthermore, these two apoptotic pathways can converge through p53 protein (Haupt et al., 2003). The tumor suppressor p53 protein is a pivotal molecule that acts as a cellular sensor. It can be activated in response to variety of external and internal stress stimuli. P53 can function as a central node of either viable cell cycle arrest or activation of apoptosis (Jin and Levine, 2001). This protein can bind to DNA as a homo-tetramer complex, and can thereby regulate a large number of genes. Although p53 is considered as a key regulatory element of the mitochondrial apoptosis pathway, its contribution in the regulation of the essential regulatory components in the extrinsic death pathway has also been documented (Michalak et al., 2005) **(Table 1.2)**. The role of p53 in mediating apoptosis will be discussed in greater detail below.

[3] http://www.celldeath.de/encyclo/aporev/aporev.htm

Table 1.2. Genes essential for p53-dependent apoptosis, linking between the extrinsic and intrinsic apoptotic pathways

Gene	Function	Reference
APAF1	Activates pro-caspase 9	(Fortin et al., 2001, Robles et al., 2001)
BAX	Pro-apoptotic BCL2 family member	(Miyashita and Reed, 1995, Cregan et al., 1999)
BID	Pro-apoptotic BCL2 family member (BH3-only)	(Sax et al., 2002)
DR4	Death receptor for TRAIL	(Guan et al., 2001)
DR5/KILLER	Death receptor for TRAIL	(Wu et al., 1997)
FAS/APO-1	Death receptor	(Owen-Schaub et al., 1995, Martin et al., 2005)
NOXA	Pro-apoptotic BCL2 family member (BH3-only)	(Oda et al., 2000, Shibue et al., 2006)
PUMA	Pro-apoptotic BCL2 family member (BH3-only)	(Han et al., 2001, Nakano and Vousden, 2001)
TNFα	Pro-inflammatory cytokine, belongs to the tumor necrosis factor family proteins	(Rokhlin et al., 2000, Campbell et al., 2008, Pastor et al., 2010)

This table shows some of the target genes essential for p53-mediated apoptosis. It has been demonstrated that most of these genes contain p53 response elements and they are activated in a p53-dependent manner in response to cellular stress [Modified from (Michalak et al., 2005)].

1.2.A. P53 - Dependent Apoptosis

The p53 tumor suppressor gene is a crucial transcriptional activator protein, protecting an organism from damaged cells. This protein has been named " *The Guardian of the Cell*" (Goodsell, 1999). As a gatekeeper, p53 plays its protection role either through halting the cell cycle or initiating apoptosis. As a transcriptional activator, p53 can bind to DNA and regulate the expression of large number of genes. P53 functions as a cellular stress sensor. Normally, this protein is maintained at a low level in most cells. Some cellular stresses such as DNA damage and hypoxia can trigger p53 production. Disruption of this important gene can cause dire consequences. P53 is the most commonly mutated gene, implicated in several human diseases. For instance, p53 mutation occurs in more than 50% of human cancers (Hollstein et al., 1991). Moreover, the implication of p53 in human neurodegenerative disorders has been elucidated (de la Monte et al., 1997, Mogi et al., 2007).

Predominantly, p53-transduced apoptosis is mediated through the intrinsic/mitochondrial pathway. It is believed that p53 plays its role in mediating apoptosis mostly through transcriptional activation of its target genes (Bates and Vousden, 1999). A pro-apoptotic Bcl-2 family member Bax is the first p53 primary response gene, implicated in apoptosis mitochondrial pathway (Miyashita and Reed, 1995). In addition, following stress stimuli, other Bcl-2 family members encoding BH3-only proteins Noxa and Puma are activated in the p53-dependent apoptosis pathway (Oda et al., 2000, Nakano and Vousden, 2001). P53 also regulates Apaf1, one of the pivotal elements in apoptosome formation (Fortin et al., 2001).

P53 can also regulate other genes that may contribute to the second apoptosis

pathway. Other p53-related pro-apoptotic genes are components of the extrinsic/death receptor-mediated pathway. In this death pathway, p53 exerts its effects on apoptosis through activation of the two death receptor proteins, Fas/Apo-1, and DR5/KILLER. The cell surface receptor Fas (CD95/Apo-1) is a key component of the extrinsic pathway. The outcome of Fas engagement by its ligand (FasL) is apoptosis. Upon cellular stress, such as γ-irradiation, p53 induces Fas mRNA expression in a tissue specific manner (Embree-Ku et al., 2002). In addition to stimulating Fas transcription, there is a strong correlation between p53 and DR5 expression. DR5/KILLER is a member of the TRAIL (TNF-related apoptosis inducing ligand) receptor family. It is a DNA-damage inducible death receptor gene, regulated by p53 tumor suppressor (Wu et al., 1997, Wu et al., 2000). Association of both Fas and DR5 in inducing apoptosis occurs through caspase activation, cytochrome release, and finally mitochondrial cascade engagement. In summary, while the extrinsic and intrinsic death pathways seem to have primarily distinct mechanisms by which the death signal is transduced, they can cooperate in common p53-dependent apoptosis (Ozoren and El-Deiry, 2003).

1.2.A.1. P53 Mediates Neuronal Apoptosis in Response to DNA Damage

The p53-dependent apoptosis pathway has essential implications in the mature nervous system. The transcription factor p53, as an apoptosis regulator, plays a key role in the regulation of neuronal death following injury. Evidence for pivotal function of p53 in neuronal apoptosis has been documented in the data available from in vivo and in vitro studies. Analysis of brain tissue samples in patients with neurodegenerative diseases has shown the elevation of p53 expression (de la Monte et al., 1997, de la Monte et al., 1998). In addition, data obtained from experimental animal models with neurodegenerative states

have revealed the activation of p53 in affected neurons following injuries. For instance, these studies have implicated p53 involvement in the progression of various neurological disorders such as stroke, AD, and PD (Morrison and Kinoshita, 2000) (**Table 1.3)**. Cell culture studies have further established that the enhanced expression of p53 protein by itself, using a viral vector, is sufficient to induce neuronal apoptosis (Slack et al., 1996). These results were further confirmed with p53 loss-of function data. Extensive research has confirmed that the absence of the p53 tumor suppressor gene protects neurons from neurotoxicity effects of certain toxins including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), glutamate, and DNA damaging agents (Enokido et al., 1996, Trimmer et al., 1996, Uberti et al., 1998). Among all cellular stresses, the accumulation of DNA damage in the cells has emerged as one of the potential candidates responsible for cell dysfunction and death (Lombard et al., 2005, Hoeijmakers, 2009). The genotoxic stress evoked by DNA damage is considered as a fundamental problem in neuronal injury. DNA damage is a common feature underlying certain neuropathological states including cerebral ischemia, Alzheimer's and Parkinson's diseases, among others (Chen et al., 1997a, Adamec et al., 1999, Hoang et al., 2009). Indeed, a marker of DNA damage, a G2/M checkpoint protein 53BP1, has been observed in brain regions with neurodegenerative conditions such as Alzheimer's disease (Chen et al., 2010a). 53BP1 is a p53 binding protein, reliable for tracking DNA damage (Wang et al., 2002). Substantial efforts have been made to elucidate the DNA damage-apoptotic molecular pathway. Elucidating the molecular basis of DNA damage response, a hallmark of apoptosis, particularly in nervous system may constitute effective therapeutic strategies in these neurodegenerative diseases.

Table 1.3. *In vivo* and *in vitro* studies in the link between p53 and neuronal injury

Neurodegenerative Conditions	References
In vivo	
Alzheimer's disease	(de la Monte et al., 1997, Kitamura et al., 1997, de la Monte et al., 1998)
Aβ transgenic mouse	(LaFerla et al., 1996)
Parkinsonism induced by MPTP	(Duan et al., 2002)
Amyotrophic lateral sclerosis	(de la Monte et al., 1998)
Ionizing radiation	(Herzog et al., 1998)
Ischemia	(Chopp et al., 1992, Li et al., 1994)
Seizures/Excitotoxiciy	(Nakai et al., 2000)
Traumatic brain injury	(Kaya et al., 1999, Muir et al., 1999, Napieralski et al., 1999)
In vitro	
Cytosine arabinoside	(Anderson and Tolkovsky, 1999)
Glutamate	(Uberti et al., 1998, Chen and Chuang, 1999)
Hypoxia	(Banasiak and Haddad, 1998)
Ionizing radiation	(Jordan et al., 1997)

Alterations in p53 mRNA and protein expression have been associated with neuronal damage in a variety of *in vivo* and *in vitro* model systems [Modified from (Morrison and Kinoshita, 2000)].

1.3. DNA Damage and Neurodegenerative Diseases

The involvement of DNA damage to the mechanism of neurodegenerative disorders has been reported for a few decades. For example, in vitro studies in non-neuronal cells such as fibroblasts and lymphocytes derived from AD patients showed higher sensitivity to the DNA damage inducers compared to normal cells (Mullaart et al., 1990). In addition, further studies on DNA damage in AD confirmed the 2-fold increase of DNA strand breaks in the AD cerebral cortex isolated from the patients compared with the control[4] (Mullaart et al., 1990). DNA lesions have also been detected in the brain samples of PD patients compared with age-matched controls (Zhang et al., 1999). DNA damage has equally been implicated in the death of motor neurons in ALS. DNA strand breaks have been found in the vulnerable neurons of ALS patients (Martin, 2001). Moreover, neuronal DNA damage has been observed, as an early event, following induction of transient ischemia in experimental animal models (Chen et al., 1997a). All these results highlight the possibility of DNA damage implicated with several human neurological disorders. Although the signaling pathway mediating DNA damage in neuronal death is starting to be appreciated, the complexity of this signaling network needs to be fully elucidated.

1.3.A. DNA Damage in Neurons

Neurons are relatively unique; they are post-mitotic cells that cannot be replaced following damage. DNA damage has emerged as one of the key initiators of neuronal death, rather than a consequence of cellular deterioration (Keramaris et al., 2003). Evidence obtained from neuronal death in certain knockout mice models suggests a close

[4] J.H. Robbins. B.A. Bridges, E.C. Friedberg (Eds.), Cellular Responses to DNA Damage, Alan R. Liss, Inc, New York (1983), pp. 671–700

link between DNA damage and neurodegenerative conditions. For example, under endogenous cellular stress, certain mouce models deficient of DNA repair genes showed higher level of apoptosis, including mice deficient in non-homologous end-joining genes (Ku proteins, DNA ligase IV and XRCC4) (Barnes et al., 1998, Gao et al., 1998, Gu et al., 2000, Laposa and Cleaver, 2001). Moreover, cortical neuronal cultures obtained from mice lacking the gene for the DNA damage-sensing enzyme, poly ADP-ribose polymerase 1 (PARP1), were resistant to exposure to a range of cellular stressors (Wang et al., 2004). PARP1 is one of the substrates for caspase-3. PARP1 activation appears to be associated with neuronal apoptosis, following ischemic brain injury (Endres et al., 1997, Chaitanya et al., 2010). Additionally, the p53 transcription factor appears to sense the DNA damage. A variety of potentially damaging stimuli can modify the p53 gene, transcriptionally or post-translationally, leading to the activation of p53 pro-death target genes (Harms et al., 2004, Tedeschi and Di Giovanni, 2009).

Both environmental genotoxins such as ionizing radiation and intracellular reactive metabolic by-products such as ROS can attack DNA and cause structural and chemical alteration in the DNA. DNA damaging agents can induce DNA lesion in diverse spectrum such as DNA double-strand break (DSB) (Robison and Bradley, 1984, Rao, 1993). A DNA double-strand lesion is considered the most severe type of damage leading to detrimental consequence such as apoptosis (Jackson, 2001, Merlo et al., 2005). In vitro studies of neurons have indicated that DNA strand break inducers, such as camptothecin and etoposide, can induce apoptosis through distinct pathways (Morris et al., 2001). Camptothecin and etoposide are anticancer agents that can target DNA topoisomerases, the nuclease enzymes essential in cellular translations. Topoisomerases manipulate DNA to

solve the topological problems of DNA during DNA replication, and transcription (Wang, 1996).

1.3.A.1. Camptothecin DNA Damage Model

Camptothecin (CPT) is an antitumor drug that specifically inhibits topoisomerase-I (Topo-I) to induce DNA single-strand break (SSB) (Staker et al., 2002, Yamauchi et al., 2011). Topo-I is a remarkable nuclease enzyme that controls the relaxation process of supercoiled DNA, to optimize DNA replication and transcription. Inhibition of Topo-1 can lead to neuronal apoptosis (Morris and Geller, 1996).

Research has demonstrated that active transcription and translation are required for camptothecin neurotoxicity and neuronal death (Morris and Geller, 1996). Hence, CPT toxicity is utilized as a powerful in vitro model to study the mechanism of neuronal death.

1.3.A.2 Etoposide DNA Damage Model

The other valuable tool used as an in vitro model in neuronal death is DNA damage induced by etoposide cytotoxicity (Nakajima et al., 1994). *Etoposide* is one of the widely used chemotherapeutic drugs that can inhibit DNA topoisomerase II and induce apoptosis. Topo-II converts topological forms of DNA by making transient double-stranded breaks (DSBs) (Hande, 2003). Etoposide inhibits the function of Topo-II enzyme through increasing the steady-state concentration of the enzyme-DNA cleavage complex. This introduces high levels of transient DNA strand breaks in the cell leading to DNA damage. (Hande, 1998, Coultas and Strasser, 2000).

1.4. The Endoplasmic Reticulum (ER) Stress and Cell Death

In addition to the role of DNA damage in cell death, there is evidence indicating that disturbances in the normal functions of an intracellular organelle such as the endoplasmic reticulum can lead to a cellular stress response. Endoplasmic reticulum or ER is one of the organelles that belong to the endomembrane system within the cell and it is essential for various cellular activities. It is an organelle that facilitates the folding process of newly synthesized secretory and transmembrane proteins. Almost one-third of all cellular proteins are translocated to the ER for final modifications (Chen et al., 2010b). Proteins have many different functions which means that they can be found in many parts of cells. Newly synthesized proteins must be folded into proper conformation to exhibit proper targeting and function[5]. Protein homeostasis is essential for cell viability, thus an improper folding processing can affect functions of cells and cell's fate. Under physiological conditions, cells produce some misfolded proteins. It has been estimated almost 30% of newly synthesized proteins are degraded due to lack of appropriate folding (Schubert et al., 2000). Thus any acute increase in the translation levels can be problematic due to a potential buildup of misfolded proteins (Lai et al., 2007). The endoplasmic reticulum possesses a quality control system to ensure that proper folded proteins are transported. Proper functioning of ER delimits the frontier between survival and cellular demise. Excessive production of protein synthesis or an accumulation of misfolded proteins disturbs the physiological functions of the ER and cause the condition called ER stress (Breckenridge

[5] Molecular Biology of the Cell, 4th edition. Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. 2002

et al., 2003). Various stimuli such as alteration in ER Ca^{2+} homeostasis, alteration of ER redox state, hypoxia, and glucose deprivation can elicit the ER stress condition, among others (Reiling et al., 2011).

In response to ER stress, certain adaptive signaling pathways are activated to alleviate the toxic effects of the accumulated misfolded proteins. The primary goal of these pathways is reestablishing ER function and protecting cells from further damage. However, ER stress can evolve into apoptosis. Prolonged ER stress, due to the excessive buildup of these aggregated proteins, impairs these protective effects leading to cell death (Xu et al., 2005).

As it was mentioned earlier in this chapter, apoptosis proceeds through two general pathways: extrinsic and intrinsic. In the former case, death ligand-binding receptors cause the activation of caspases. While in the latter case, releasing the cytochrome C from mitochondria into the cytosol recruits the caspase activation that leads to death (Tait and Green, 2010). Persistent ER stress can also initiate the apoptotic program through activation of these pathways. Endoplasmic reticulum stress is a repository for activation of several pro and anti-apoptotic molecules such as pro-apoptotic caspase-12 and transcription factor CHOP, and anti-apoptotic ER chaperone protein GRP78 (Nakka et al., 2010). For instance, ER related transcription factor CHOP has been reported to correlate with cell death through regulation of anti-apoptotic Bcl-2 and death receptor DR5 (McCullough et al., 2001, Yamaguchi and Wang, 2004). Conversely, the anti-apoptotic Bcl-2 protein can inhibit apoptosis induced by ER stress (Annis et al., 2001). Indeed, the lack of pro-apoptotic Bcl-2 family proteins, Bax and Bak, are protective against ER stress (Zong et al., 2003). Results obtained from mouse embryonic fibroblasts (MEFs) show that

localization of Bax and Bak in both mitochondria and ER is required for the activation of apoptotic signals (Scorrano et al., 2003). Yet, there is evidence showing that ER stress can activate the caspase cascade independent of the mitochondrial apoptotic pathway. For example, ER-resident caspase 12 activates cytoplasmic caspase 3 in response to ER stressor treatment (Hitomi et al., 2004). Additionally, the lack of mitochondrial cytochrome C in the Apaf1 null cells does not show a protective effect against apoptosis induced by ER stressors (Rao et al., 2004). All these results suggest that biochemical death pathway activated by ER stress can act as an alternative mechanism involved in apoptosis.

Several studies have indicated the accumulation of misfolded proteins and the central initiating role of ER stress in pathogenesis of some neurodegenerative diseases (Paschen and Mengesdorf, 2005). However, the mechanistic link between ER stress and neuronal death are only partially demonstrated. Thus, dissecting the ER stress apoptotic pathway will help better understanding the pathogenesis of these neurological disorders.

1.4.A. ER stress and Neurodegenerative disease

Emerging evidence indicates that diverse neurodegenerative diseases have a common feature, which is the aggregation of misfolded proteins in the brain (Irvine et al., 2008). Indeed, the molecular markers of ER stress have been identified in the neurons destined to die in several neurological conditions (Kim et al., 2008). In vivo studies in postmortem AD brain patients indicated the ER stress in the neurons. Similarly, ER stress signaling molecules have been observed in neurons in models of Parkinson's disease. Additional studies of cellular models of Huntington's disease also delineated the involvement of ER stress in this disease. Moreover, the inhibition of ER stress reduced neuronal loss in animal models of stroke (Ryu et al., 2002, DeGracia and Montie, 2004, Tajiri et al., 2004,

Unterberger et al., 2006, Reijonen et al., 2008, Hoozemans et al., 2009, Vidal et al., 2011). All these data strongly suggest that an accumulation of abnormal proteins resulting in ER stress might participate as a critical factor in the pathogenesis of these devastating disorders. Consequently, understanding the ER stress molecular pathways may provide insight into these diseases' mechanisms for efficient therapies. In order to elucidate the underlying mechanisms involved in ER stress-mediated neuronal death, pharmacological agents such as tunicamycin (TM) and thapsigargin (TG) are generally used to experimentally induce ER stress in neurons. The common effect of these drugs is to disrupt the ER functions and thereby produce ER protein misfolding. Obviously, to induce ER stress, the amount of these drugs and time of their application are stated based on the cells and system being studied.

1.4.A.1. Tunicamycin ER Stress Model

Tunicamycin is a drug that acts as a potent protein glycosylation inhibitor. Therefore, it is known as a valuable research tool for studying cellular events involving protein modification. Glycosylation or adding sugars to proteins is a fundamental post-translational modification, which takes place in different organelles including the ER. Tunicamycin inhibits glycosylation completely in the ER leading to extensive protein misfolding and an enhanced endoplasmic reticulum stress response.

1.4.A.2. Thapsigargin ER Stress Model

Application of thapsigargin (TG) proved to be a valuable tool to investigate ER stress pathways. *Thapsigargin* is the most widely used inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCA) in mammalian cells (Thastrup et al., 1990). SERCA is a

class of transporter proteins that pumps cytoplasmic calcium ions (Ca^{2+}) into the ER (Treiman et al., 1998). Thapsigargin increases the cytosolic Ca^{2+} concentration by highly specific inhibition of the ER Ca^{2+} pump (Thastrup et al., 1990). Inhibition of these pumps depletes the ER calcium stores leading to an accumulation of unfolded proteins and eventually apoptosis (Wuytack et al., 2002).

1.5. ER Stress signaling pathways or Unfolded Protein Response (UPR)

The ER is an organelle extremely sensitive to alteration in homeostasis. The accumulation of incorrectly folded proteins has detrimental effects on approximately one-third of cellular proteins that translocate to the ER (Mori, 2000). Efficiency of protein-folding reactions depends on appropriate environmental, genetic, and metabolic conditions. As a consequence, unpleasant environmental insults, gene mutations, or energy deprivation can alter ER luminal environment and protein-folding reaction, initiating misfolding proteins and ER stress (Malhotra and Kaufman, 2007). The ability of cells to respond to ER stress is a fundamental factor, important for cell survival.

In response to the accumulation of misfolded proteins in the lumen of ER or ER stress, the set of pro-survival signaling pathways are activated. These intricate adaptive pathways are collectively termed unfolded protein response or UPR. The UPR is defined as an intracellular transient adaptation response that sense the ER stress. This pathway assists cells to deal with improper folded proteins and cope with stress.

In mammals, the UPR pathway leads to the activation of several adaptive mechanisms for dealing with stress. During these mechanisms, protein translation is generally attenuated in response to ER stress, to decrease protein loading to the ER. The ER stress signals are also transmitted to the nucleus to induce transcription of the ER

localized molecular chaperones and folding enzymes to enhance protein-folding capacity. In addition, protein degradation is enhanced to protect the cells from the toxic effect arising from the accumulation of aberrant proteins in the ER. However, if recovery is not achieved due to excessive stress, UPR will initiate apoptosis. Thus, the principal purposes of activation of the UPR pathway are adaptation for survival or induction of apoptosis. Over the past years, significant effort has been made to identify the molecular signaling of the UPR that emanate from the ER.

Following ER stress, three major branches in the UPR pathway are initiating by ER stress sensor proteins. They are IRE1 (Inositol-requiring enzyme 1), PERK (pancreatic ER kinase (PKR)- like ER kinase), and ATF6 (activating transcription factor 6). All these molecular sensors are transmembrane receptor proteins with an ER luminal domain sensing the ER stress and a cytosolic domain activating UPR downstream molecules. In non-stressed cells, these ER receptors are maintained in an inactive mode by binding to the ER chaperone protein, GRP78/BiP. The ER contains a number of molecular chaperones that assist in protein folding. GRP78 (Glucose regulated protein) also called BiP (Binding immunoglubin protein) is a major ER chaperone protein with the ability to control the ER stress sensor proteins. This protein is also used as a marker of ER stress (Lee, 2005). UPR is initiated by the activation of three transmembrane receptors. Upon induction of ER stress, the UPR branches, IRE1α, PERK, and ATF6 are released from GRP78 and activated **(Fig. 1.2)**. The initial outcome of these activations is to combat the detrimental effects of stress. However, the behavior of these pathways has varying with time course. In vitro studies of ER stress in a human cell line and mouse embryonic fibroblast (MEF) showed that the duration of ER stress switched the effect of UPR branches from the

protective phase to the apoptotic mode (Lin et al., 2007, Li et al., 2010). If the problem related to protein aggregation is persistent, all these UPR sensor proteins can stimulate expression of pro-apoptotic molecules. Hence, the signals emanating from the stressed ER can play a fundamental role in induction of apoptosis. The molecular mechanisms, through which UPR signals switch cell fate to apoptosis, are discussed in the following section in more details.

1.5.A. IRE1 Pathway

The oldest evolutionary branch of UPR is activation of IRE1 (Tabas and Ron, 2011). IRE1 is a transmembrane protein, localized in the ER. It is a bi-functional kinase/endoribonuclease enzyme with an N-terminal serine/threonine kinase luminal domain and a C-terminal cytosolic endoribonuclease (RNAse) domain. There are two mammalian IRE1 proteins; IRE1α that is expressed in all cell types, and IRE1β whoes expression is restricted to certain cell types including epithelial cells in the gastrointestinal tract (Tirasophon et al., 1998, Wang et al., 1998). During ER stress, the association between luminal domain of IRE1α and the ER chaperone protein, BiP/GRP78 is disrupted leading to IRE1α oligomerization. Subsequently, IRE1α is autophosphorylated and its RNase domain is activated. Activated IRE1α splices the mRNA of the transcription factor X-Box binding protein-1 (XBP1). Spliced XBP1 (sXBP1) is an active and stable transcription factor that regulates subsets of genes such as a heat shock protein, p58 (IPK), to enhance protein folding and quality control (Lee et al., 2003). The p58 (IPK) binds and inhibits another ER stress sensor protein, PERK, leading to the reduction of PERK-mediated translational block (Yan et al., 2002). It is possible that the effect of p58 (IPK) on protein translational block represents the termination of UPR. Hence, it seems that

activation of IRE1α-XBP1 axis is an adaptive response in ER stress condition. If this adaptive function of UPR works successfully, the ER will gain its normal function and the cell will survive. Although IRE1α pathway has initially pro-survival effects, in a strong ER stress condition, it can initiate apoptosis. It has been demonstrated that active IRE1α recruits the adaptor protein TNF-receptor-associated factor 2 (TRAF2). Subsequently, the IRE1-TRAF2 complex recruits the apoptosis-signal-regulating kinases (ASK1). It has been shown that overexpression of ASK1 can induce apoptosis. ASK1 stimulates c-Jun N-terminal kinase (JNK) activation, resulting in cell death. It has been suggested that activated JNK mediates cell death through the regulation of Bcl-2 family protein (Lei and Davis, 2003, Bassik et al., 2004).

1.5.B. PERK Pathway

A second branch of UPR is triggered by the activation of the PERK protein. Like IRE1, PERK is a transmembrane protein with the luminal sensing domain and the cytosolic domain; but the cytosolic domain has only serine/threonine kinase activity. As for IRE1α, in non-stressed cells, PERK is kept inactive by binding the BiP/GRP78 chaperone protein to its luminal domain. Upon ER stress, PERK is dissociated from BiP, autophosphorylated and its conformation is changed. Following that, PERK phosphorylates the α-subunit of its downstream target substrate, eukaryotic initiation factor 2alpha (eIF2α) to attenuate general protein translation. Translation is a process that occurs in ribosomes with the help of tRNA. The eIF2α is a subunit of the ternary complex consisting of eIF2, GTP and methionyl-initiator tRNA (Met-tRNAiMet) that deliver tRNA to the ribosome. During initiation of translation, as GTP is hydrolyzed, eIF2 needs to be activated following each round of initiation. GDP-GTP exchange is responsible for this

activation. The phosphorylation of eIF2α prevents this exchange, thereby reducing the active ternary complex required for translation initiation. Ultimately, general translation will be attenuated (Holcik and Sonenberg, 2005). Inhibition of translation decreases the load of protein at the ER, alleviates ER stress and aids cell survival. Study on PERK deficient mouse embryonic fibroblasts (MEF) showed impaired attenuation of protein translation and increased level of cell death. These results confirmed the essential role of PERK in cell survival (Harding et al., 2000b). The involvement of the PERK-eIF2α pathway as an immediate response to ER stress has been reported in several human neuronal diseases (Kumar et al., 2001, Chang et al., 2002, Hoozemans et al., 2007).

Although phosphorylation of eIF2α by activation of PERK attenuates general translation, translation of a few mRNAs is selectively increased. The expression of these genes attempts to ameliorate the cellular stress. Among them, one of the most studied genes is activating transcription factor 4 (ATF4) (Northover, 1975, Vattem and Wek, 2004). ATF4 is a transcription factor that plays a role in the regulation of several genes, following stress. It is thought that the regulation of these genes by ATF4 can recover the cell from stress that induces eIF2α phosphorylation (Whitney et al., 2009). In conclusion, PERK activation is initially considered as a protective pathway. However, induction of CHOP, a gene encoding a C/EBP-homologous protein, following PERK activation may switch this pathway from pro-survival to pro-death. CHOP, also known as growth arrest and DNA damage inducible gene 153 (GADD153), is a pro-apoptotic transcription factor. Despite its name, it is more responsive to ER stress than DNA damage (Wang et al., 1996b, Tjalve, 1997). The role and function of this transcription factor will be detailed later in this chapter.

1.5.C. ATF6 Pathway

Activating transcription factor 6 (ATF6) is another regulatory protein involved in the UPR pathway. This transcription factor is synthesized as an ER transmembrane protein. Like IRE1 and PERK proteins, following ER stress, ATF6 is dissociated from the GRP78 chaperone protein. Upon dissociation, it translocated to the Golgi apparatus, where it is cleaved into its cytosolic active form. The active form of ATF6 migrates to the nucleus to induce genes with ER stress response element (ERSE) in their promoter. The ERSE is present in the promoters regions of various UPR target genes including transcription factors CHOP and XBP1 (Yoshida et al., 2000). ATF6 can induce CHOP mRNA, however there are no reports available whether the induction of CHOP by ATF6 plays a role in ER stress-induced apoptosis or not. This suggests that signaling through ATF6 work mainly as a pro-survival pathway. Active ATF6 can also increase XBP1 transcription (Yoshida et al., 2001, Kaufman, 2002, Schroder and Kaufman, 2005). On the other hand, IRE1 pathway also induces XBP1 transcription. Thus, signaling through ATF6 and IRE1 may merge. However, the lack of either IRE1 or ATF6 individually does not suppress XBP1 mRNA induction, suggesting that these two signaling pathways may work in parallel (Lee et al., 2002). In addition, it has been shown that PERK-eIF2α pathway facilitates ATF6 expression during ER stress, in ATF4-dependent manner. Based on a large amount of evidence, it is suggested that the signaling from downstream effectors of IRE1, PERK and ATF6 merges to help cells to cope to the stress (Liu and Kaufman, 2003, Teske et al., 2011). However, in severe conditions of ER stress, the signaling through these pathways may push the cell down the path of death. This may be done through downstream molecules, which are activated by these pathways. The following section will discuss a

number of potential downstream targets that may drive the cell to the execution phase. In this part, the role of ATF family proteins and Bcl-2 family members will be dissected, focusing on ATF4 and Puma, respectively.

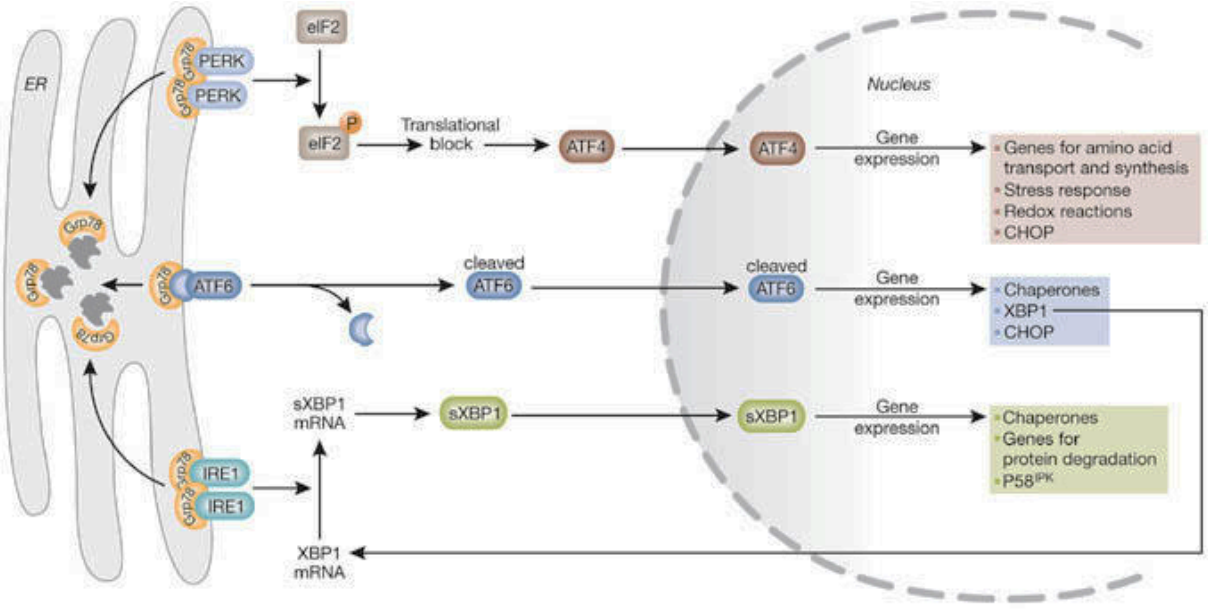

ER
PERK
PERK
GRP78
eIF2
P
eIF2
Translational block
ATF4
ATF4
Nucleus
Gene expression
Genes for amino acid transport and synthesis
Stress response
Redox reactions
CHOP
ATF6
cleaved ATF6
cleaved ATF6
Gene expression
Chaperones
XBP1
CHOP
sXBP1 mRNA
sXBP1
sXBP1
Gene expression
Chaperones
Genes for protein degradation
P58IPK
IRE1
IRE1
XBP1 mRNA

Figure 1.2

Figure 1.2. The unfolded protein response. On aggregation of unfolded proteins, GRP78 dissociates from the three ER stress receptors, PERK, ATF6, and IRE1, allowing their activation. Activated PERK blocks general protein synthesis by phosphorylating eIF2α. This phosphorylation enables an alternative translation of ATF4. ATF4, as a transcription factor, translocates to the nucleus and induces the transcription of genes required to restore ER homeostasis. ATF6 is activated by limited proteolysis after its translocation from the ER to the Golgi apparatus. Active ATF6 regulates the expression of ER chaperones and transcription factor, XBP1. To achieve its active form, XBP1 must undergo mRNA splicing, which is carried out by IRE1. Spliced XBP1 protein (sXBP1) translocates to the nucleus and controls the transcription of chaperones, the co-chaperone and PERK-inhibitor $P58^{IPK}$, as well as genes involved in protein degradation. This concerted action aims to restore ER function by blocking further build-up of client proteins, enhancing the folding capacity and initiating degradation of protein aggregates (Szegezdi et al., 2006).

1.6 ATF Family Proteins and ER Stress

The activating transcription factor (ATF)/cAMP responsive element binding protein (CREB) family proteins represents an extensive group of basic-region leucine zipper (bZIP) transcription factors with high similarity in the leucine zipper motif. The bZIP is the critical domain in these proteins; the Leucine zipper domain facilitates interaction between proteins and heterodimer formation, while the basic region is a DNA binding domain for protein-DNA complex configuration. Members of ATF family protein can selectively form heterodimers to bind DNA (Hai et al., 1989, Deppmann et al., 2006). Hence, they can play critical roles in many processes necessary for proper functioning of the cell. Thus far, more than 10 different ATF member proteins have been isolated (Hai et al., 1999). A common theme among members of this protein family is ability to respond to cellular stress.[6] For example, it has been proposed that various ER stressors including thapsigargin and tunicamycin induce the transcription of ATF6 (Namba et al., 2007). It was also found that ATF2 was implicated in the regulation of ER stress chaperon protein, Grp78 (Chen et al., 1997b, Bhoumik et al., 2007). Further evidence showed the induction of other ATF family members, ATF3 and ATF5, by stress signals. Increasing of ATF3 and ATF5 expression has been reported in mouse embryonic fibroblasts (MEFs), in response to ER stress in an eIF2α - dependent manner (Jiang et al., 2004). However, central to the eIF2α phosphorylation, is the preferential translation of ATF4 mRNA (Zhou et al., 2008). ATF4 is one of the most studied genes whose its mRNAs is selectively regulated in response to UPR activation, presumably to help the cell to cope with the stress.

[6] Transcription Factors/ATF. Hai T., Lu D., &Wolford C.C., Encyclopedia of Respiratory Medicine, 2006, Pages 257-260

In addition, among all members of this protein family, ATF4 is a protein that plays a protective role in ER stress remediation; In particular, its protective role has been well documented in cancer cells. Nevertheless, there are some studies describing a pro-death role for this protein during some stress conditions (Ameri and Harris, 2008). In the next section, the role of ATF4 in response to ER stress is described in further detail.

1.6.A. ATF4 and ER Stress, The Pro-Survival/Death Functions

The ATF4, also referred to as cyclic AMP-responsive element DNA-binding protein (CREB2), is a member of the ATF protein family and its mRNA is paradoxically translated in response to ER stress. ATF4 translation is regulated by two open reading frames (uORFs), uORF1 and uORF2, located in its five prime untranslated region (5'UTR) (Vattem and Wek, 2004). The uORF1 is a positive-acting element that promotes ribosome scanning, while the uORF2 is an inhibitory element that blocks ATF4 expression. In unstressed cells, in the presence of abundant eIF2α-GTP and low level of phosphorylated eIF2α, uORF1 directs the reinitiation of ribosomes to the inhibitory uORF2, which represses ATF4 translation. In stress conditions, a high level of phosphorylated eIF2α facilitates reinitiation at ATF4 over the uORF2. Therefore, a ribosome small subunit can bypass this inhibitory ORF and start translation of ATF4 and increase its protein levels (Harding et al., 2000a, Lu et al., 2004, Vattem and Wek, 2004). There are also reports about transcriptional regulation of ATF4; however it is the translational regulation of this gene that has been considerably studied in several stress conditions (Dey et al., 2010, Miyamoto et al., 2011).

As a bZIP transcription factor, ATF4 can bind to a specific binding site in a promoter of various genes as homodimers or heterodimers. In vitro studies have shown that ATF4

can bind to all C/EBP bZIP proteins to form a heterodimer. The C/EBP family proteins represent a group of transcription factors, essential in the regulation of several cellular functions such as cellular homeostasis and apoptosis (Newman and Keating, 2003, Tsukada et al., 2011). Several studies have indicated the role of ATF4 in the regulation of the promoter of various genes implicated in UPR. For example, following up-regulation of ATF4 translation resulting from ER stress, this protein binds to ATF/CRE-like site in the Grp78 promoter. The ATF/CRE-like site is a consensus ATF4 binding site defined as (5′-TGACGTGA-3′) (Hai and Hartman, 2001, Ameri and Harris, 2008). In this way, ATF4 contributes to the ER stress induction of GRP78 (Luo et al., 2003). Grp78, a 78 glucose-regulated protein, is a major ER molecular chaperone with anti-apoptotic activity (Lee, 2005).

Cellular stress conditions such as hypoxia, oxidative stress, and nutrient deprivation can lead to ER stress, and can culminate in the upregulation of ATF4 translation (Siu et al., 2002, Blais et al., 2004, Malhotra and Kaufman, 2007). It has been shown that the ATF4 translation plays an important role in cell survival. For example, Fibroblasts derived from ATF4 knockout mice are more sensitive to prolonged hypoxia-induced apoptosis and oxidative stress, compared to wild type MEFs (Harding et al., 2003, Bi et al., 2005). Moreover, knocking down ATF4 significantly increased apoptosis in several tumor cell lines following UPR activation in amino acid deprivation, or proteosome inhibition (Ye et al., 2010, Hu et al., 2012). ATF4 deficiency also causes a defect in resistance to arsenite-induced DNA damage. Arsenite is a carcinogen, inducing DNA damage and causing various human cancers. ATF4-deficient cells have higher apoptotic rate upon arsenite toxicity compared to controls (Fung et al., 2007). Nevertheless, under certain conditions,

ATF4 expression may exert lethal effects on cells, such as death induction in neuroectodermal tumor cells during ER stress (Armstrong et al., 2010). So, although the pro-survival role of ATF4 in these studies is well documented, there are also a few studies relevant to the pro-death role of ATF4 [reviewed in Ref (Ameri and Harris, 2008)]. Thus, it seems that the effect of ATF4 in cell fate is likely to be context-dependent.

As it was mentioned previously in this chapter, the PERK-eIF2α pathway is an immediate response to ER stress, which is involved in several human neuronal diseases (Kumar et al., 2001, Chang et al., 2002, Hoozemans et al., 2007). Consequently, activation of the downstream target of this pathway, ATF4, may be an important factor in these disorders. The importance of ATF4 function has been reported in neurons in response to various cellular stresses. For instance, silencing of ATF4 enhances neuronal death in PD models. This effect can be rescued by overexpression of this protein (Sun et al., 2013). On the other hand, ATF4 has deleterious effects on neuronal survival throughout oxidative stress (Lange et al., 2008). These results are indicating that the effect of ATF4 in neuronal death is likely to be controversial and more complex.

The mechanism(s) by which ATF4 regulates neuronal fate is not well known. In addition, there are no convincing data available for the role of ATF4 in ER stress and DNA damage in neurons. Accordingly, identifying the role of this protein in response to these cellular stresses in neurons and precise molecular cascade downstream of it are essential. Further study of the expression of ATF4 target genes that lead to neuronal death may provide insight into the ATF4 functions in human neuronal diseases.

1.6.B. The Mechanism(s) of ATF4 - Mediated Apoptosis

As stated above, the molecular mechanisms involved in ATF4-related cell death are

not fully identified. Studies on human cancer cell lines suggest that ATF4 may play its role in apoptosis through the activation of Noxa, a pro-apoptotic BH3-only Bcl-2 protein, following ER stress (Wang et al., 2009b, Zhu et al., 2012). BH3-only proteins are able to initiate apoptosis through the activation of other pro-apoptotic Bcl-2 family proteins such as Bax and Bak (Youle and Strasser, 2008). Similarly, studies have shown that overexpression of Noxa in MEFs can induce apoptosis through the activation of Bak. In addition, MEFs deficient in Noxa showed reduction in ER stress-induced apoptosis. Therefore, Noxa may be an apoptosis mediator that contributes in ER stress-induced apoptosis pathway (Li et al., 2006).

CHOP is another potential downstream gene of ATF4 during ER stress. CHOP, also is a transcription factor that can be highly induced by range of various cellular stresses (Marciniak et al., 2004). CHOP is better known as ER stress-induced apoptotic gene. *CHOP*$^{-/-}$ mice cells are resistant to apoptosis mediated by severe ER stress [reviewed in Ref. (Oyadomari and Mori, 2004)].

CHOP expression has been also reported in neurons following ER stress. Gene expression microarray analysis of tunicamycin-treated human neuroblastoma cells exhibited high expression of CHOP transcription factor (Reimertz et al., 2003). In addition, CHOP was activated during neurotrophic factor deprivation in neurons. Deprivation of neurotrophic factors activated ER stress pathway that resulted in increased CHOP expression. Expression of CHOP induced apoptosis in this paradigm. This effect was suppressed in *CHOP*$^{-/-}$ mice[7]. Various studies have shown that following ER stress ATF4 binds directly to the CHOP promoter to induce the CHOP protein (Fawcett et al., 1999,

[7] Tajiri S. et al. 2006, FEBS Letters. 580 (14), pp. 3462-3468.

Harding et al., 2000a, Ma et al., 2002). Although these studies have correlated CHOP expression with ATF4, a mechanistic link between CHOP regulation by ATF4 and neuronal apoptosis has never been demonstrated.

1.7 Bcl-2 Proteins and ER Stress

As stated several times in this chapter, chronic ER stress leads to apoptosis. Although the precise mechanism that triggers apoptosis is not clear, some proteins have been identified in this cellular stress paradigm. One of the apoptotic arms in ER stress is Bcl-2 family protein. For a long time, the Bcl-2 family members have been known exclusively as the mediators of the mitochondrial apoptotic pathway. Recent evidence has delineated the importance of this family in the ER stress mediating apoptosis (Distelhorst and McCormick, 1996, Wei et al., 2001). Subsequently, localization of Bcl-2 family members in the ER and its significance to apoptosis have been studied (Krajewski et al., 1993, Lithgow et al., 1994, Zong et al., 2003, Hurt et al., 2004).

This protein family contains both anti- and pro-apoptotic members. Both pro and anti-apoptotic members of Bcl-2 family are involved in apoptosis mediated by ER stress. For example, overexpression of anti-apoptotic ER-targeted Bcl-2 protects cells from ER apoptotic stimuli (Annis et al., 2001, Rudner et al., 2001). On the other hand, localization of pro-apoptotic Bax and Bak in the ER can initiate apoptosis. Consequently, cells deficient in both Bax and Bak are resistant to death induced by prolonged or severe ER stress (Zong et al., 2003, Oakes et al., 2005). In addition, expression of Bak can induce swelling and restructuring of the ER (Klee and Pimentel-Muinos, 2005). Evidence merged from different studies has also demonstrated the involvement of pro-apoptotic BH3-only members of Bcl-2 family including Bim, Noxa, and Puma, in ER stress-induced apoptosis

(Morishima et al., 2004, Luo et al., 2005, Li et al., 2006, Puthalakath et al., 2007). Some of these BH3-only proteins, such as Puma, are able to interact with the entire anti-apoptotic Bcl-2 repertoire when an apoptotic stimulus presents. In this way, they inactivate the protective functions of the anti-apoptotic proteins (Kuwana et al., 2005, Chipuk et al., 2008). In response to stress-signals such as ER stress, these proteins act as sentinels to sense the damage in the cell and trigger apoptosis (Bouillet and Strasser, 2002). Among them, Puma is a BH3-only protein that elicits the most potent cell death response (Yu and Zhang, 2008).

1.7.A. PUMA and ER Stress

PUMA encodes a BH3-only protein with high pro-apoptotic ability. In 2001, three different groups of scientists cloned this gene independently with different nomenclature, p53 upregulated modulator of apoptosis (PUMA) or Bcl-2 binding component 3 (Bbc3), as a p53 target gene or Bcl-2 binding protein (Han et al., 2001, Nakano and Vousden, 2001, Yu et al., 2001). Studies with transgenic mice, carrying the null mutation of PUMA, have illustrated the role of this protein as a principled mediator in cell death in response to diverse apoptotic stimuli (Jeffers et al., 2003, Villunger et al., 2003) For example, target deletion of PUMA in cancer cells resulted in a reduction in apoptosis induced by various stresses such as DNA damage and hypoxia (Yu et al., 2003). Involvement of PUMA in ER stress can also lead to apoptosis. Increases in mRNA and protein levels of PUMA during ER dysfunctions have been reported in various human cancer cell lines and mouse embryonic fibroblasts (MEFs). For example, MEFs deficient in PUMA are more resistant against ER stress-induced apoptosis (Reimertz et al., 2003, Li et al., 2006, Jiang et al., 2008, Cazanave et al., 2010).

It has been suggested that PUMA is involved in various pathological processes including neurodegenerative conditions and strokes. For instance, PUMA was implicated in neuronal apoptosis mediated by Parkinson-related toxins. This effect was reversed in PUMA-deficient neurons (Biswas et al., 2005b, Steckley et al., 2007). Results achieved from the genetic deletion of PUMA also indicated improved neuronal survival in ALS transgenic mice in response to ER stress (Kieran et al., 2007). Furthermore, PUMA expression has been upregulated in regions of the hippocampus that exhibited apoptosis following ischemic injury (Reimertz et al., 2003). These data suggested the significant contribution of PUMA in these neuronal disorders. Inhibition of PUMA as an important target may be a useful way for curbing apoptosis in the neurodegenerative disorders. However, to achieve this goal, it is necessary to understand how this molecule is involved in ER stress-mediated neuronal apoptosis. In addition, its regulation in this death paradigm needs to be clearly identified.

1.7.B. Regulation of PUMA-Mediated Apoptosis

To the best of our knowledge, with more than 300 articles published about PUMA, this protein has been classically ascribed as a p53-inducible apoptotic target gene (Yu and Zhang, 2005, Wang et al., 2007, Yu and Zhang, 2008). As it was previously mentioned, tumor suppressor p53 can trigger apoptosis in different cells including neurons. Results obtained from study on sympathetic neurons in Bax null mice showed that PUMA could bind directly to Bax and link p53 with Bax to induce DNA damage-apoptosis. Linking of p53 and Bax through PUMA was also documented by further study in oxidative stress-induced neuronal death. These studies illustrated that PUMA was an essential downstream target of p53 to induce neuronal death (Wyttenbach and Tolkovsky, 2006, Steckley et al.,

2007). However, there is no evidence supporting this regulatory pathway in neuronal apoptosis-induced by ER stress. A number of studies have suggested that the regulation of PUMA expression might be orchestrated by other proteins rather than by tumor suppressor p53. Several transcription factors have been demonstrated to regulate PUMA activity in response to stress (Hikisz and Kilianska, 2012). For example, loss of function studies showed that transcription factor CHOP was necessary for PUMA up-regulation in liver cells in response to ER stress (Cazanave et al., 2010). Together these studies suggest that the regulation of PUMA expression likely depends on cell type and stress stimuli. Thus far, there is no report demonstrating the main regulator of PUMA in neuronal apoptosis-induced by ER stress. Whether p53 or CHOP regulates PUMA expression in ER stress-mediated apoptosis in neurons is an important subject that needs to be elucidated.

1.8. Ischemic Neuronal Death

Stroke is an acute neurological problem worldwide. On average, every 10 minutes one stroke case happens in Canada[8]. Stroke is resulting from a transient or permanent inhibition of energy metabolism due to interruption of blood flow to the brain. Following the interruption, oxygen and glucose deprivation occurs and triggers neuronal death in the affected regions of the brain. Damage to the brain can be due to the rupture of blood vessels (hemorrhagic stroke) or from the blockage of blood vessels (ischemic stroke). While hemorrhagic stroke covers less than 20% of these cases, ischemic stroke accounts for more than 80% of them (Thrift et al., 2001). In the ischemic type, also known as cerebral ischemia, blood vessels are blocked by a local thrombosis (occlusion of the vessel

[8] www.canadianstrokenetwork.ca

by a blood clot formed in the brain), or an embolic particle (a blood clot formed somewhere outside of the brain such as heart that has traveled through the blood vessel to the brain). The thrombotic cause, also known as cerebral thrombosis, occurs in almost 50% of all ischemic stroke cases (Jeyaseelan et al., 2008). In the ischemic stroke, an irreversible damaged region with the lowest blood flow and rapid death (core) is surrounded with penumbra, a region that is hypoperfused by collateral vessels and has less damage in its structure and metabolism (Astrup et al., 1981). However, depending on the severity and the duration of the ischemic insult; eventually apoptotic cell death happens in the penumbra with a long delay (Miles and Knuckey, 1998, Lambertsen et al., 2012) **(Fig.1.3)**. Because of the delayed cell death, the stroke therapy focuses on salvaging neurons in the penumbra region. Yet, efficiency of the current available drugs is very limited.

1.8.A. Tissue Plasminogen Activator, a Stroke Therapeutic Agent

Following ischemic stroke, to avoid damage to brain, the blood clot must be quickly removed. Activase, also known as tissue-type plasminogen activator (tPA), is the most effective therapeutic agent in ischemic stroke. The tPA is a secreted protease, naturally found in the wall of blood vessels. Activation of this enzyme in blood turns inactive plasminogen into active protease plasmin. Plasmin breakdowns fibrin and dissolves blood clot. As the clot is dissolved, the blood flow is restored, and viable tissues are re-perfused (Gravanis and Tsirka, 2008). The tPA is the only drug approved by FDA for treatment of ischemic stroke. In order to improve neurological recovery and reduce disability after stroke, intravenous administration of tPA (IV tPA) has been used since 1995[9].

[9] http://www.ninds.nih.gov

Although the efficacy of tPA has been approved, it is used only in a few cases of stroke. For example, the most effective time to receive tPA is within the 3 hours following the onset stroke attack. A narrow time frame window for receiving tPA after stroke attack as well as risk of intracerebral hemorrhage, and low efficiency to dissolve the large clots, limit the usage of this drug (Collen et al., 1986, Gardell, 1993, Clark et al., 1999). Currently, there are significant researches directed to overcome these problems and discover new targets, as possible therapies in stroke.

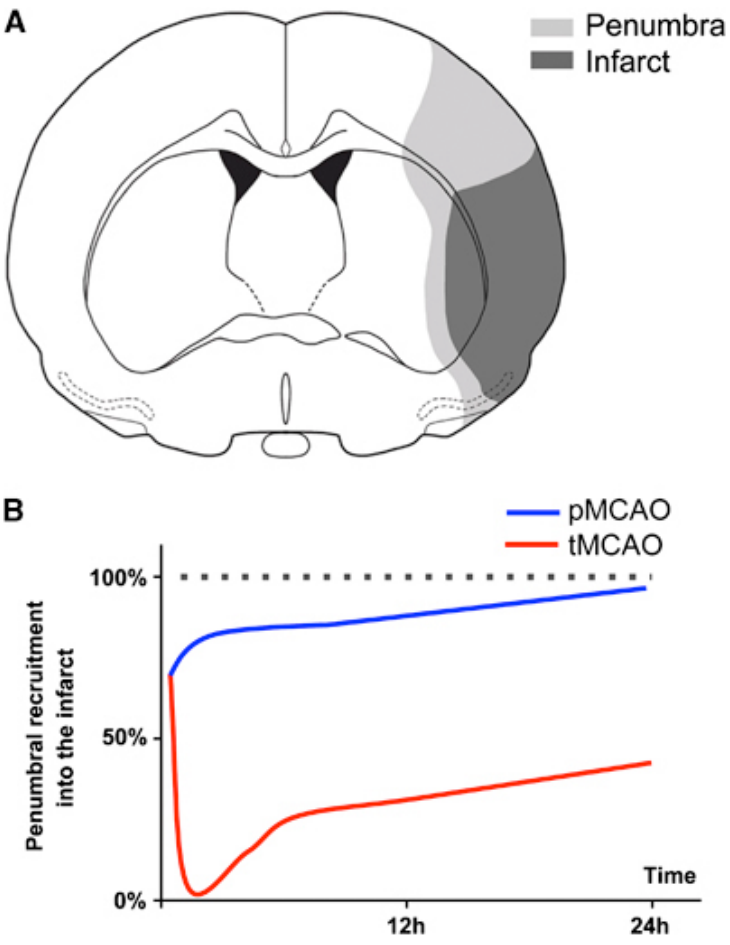

A
Penumbra
Infarct
B
pMCAO
tMCAO
100%
50%
0%
Penumbral recruitment into the infarct
Time
12h
24h

Figure 1.3.

Figure 1.3. The penumbra and infarct (core) in coronal brain section and dynamics of the recruitment of the penumbra into the infarct in experimental stroke (**A**) Schematic presentation of the penumbra and infarct early within the acute phase, after middle cerebral artery occlusion (MCAO) in a C57Bl/6 mouse, when the penumbra may still be recruited into the infarct, if the ischemic process is not counteracted. (**B**) Graphic presentation illustrating the difference in the recruitment of the penumbra into the infarct after transient MCAO (tMCAO) and permanent MCAO (pMCAO) in the mouse. The dotted line represents the total infarct volume. The vertical distance between the dotted line, and the blue and red line, respectively, represents the size of the penumbra after pMCAO and tMCAO. At 24 hours, the penumbra has been completely recruited into the infarct after pMCAO, but not after tMCAO, in which case the penumbra initially expands due to the reperfusion, whereafter it gradually regresses (Lambertsen et al., 2012).

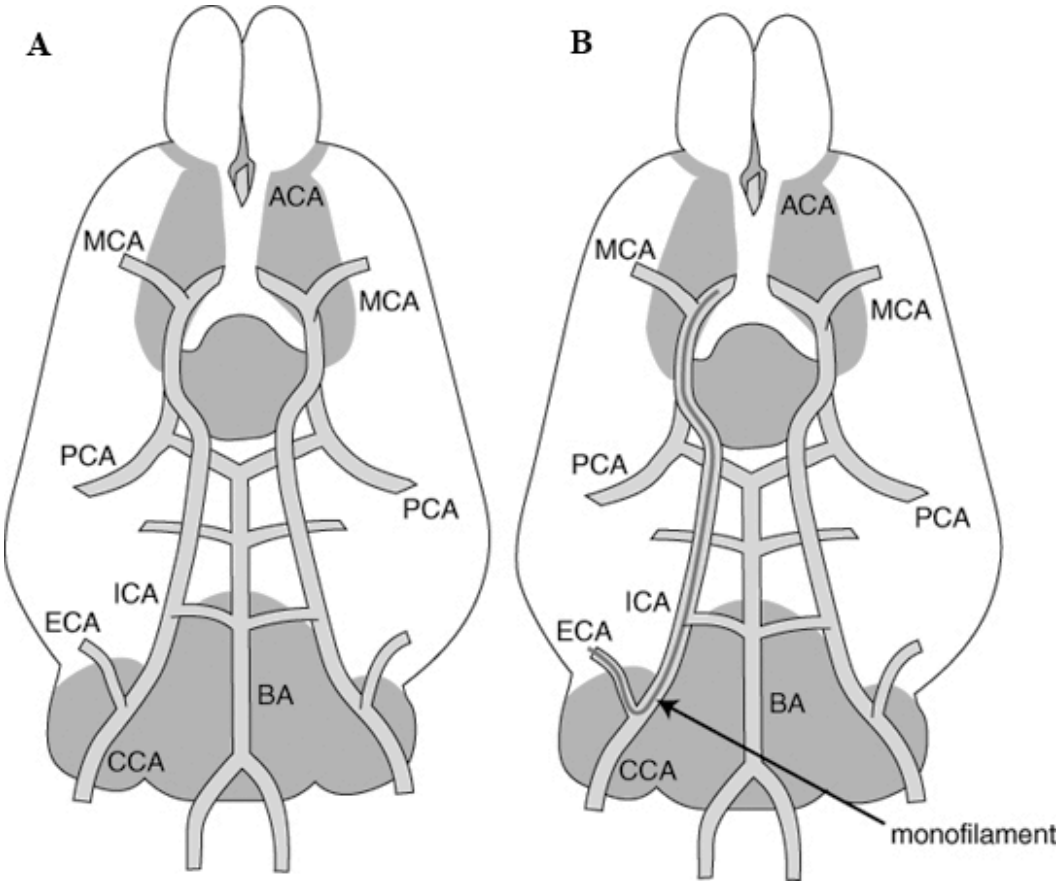
1.8.B. Animal Models Used to Study Ischemia in Neurons

To study the effect of human stroke and the mechanisms in ischemic injury, several experimental models have been developed, mostly in small rodents (Carmichael, 2005). Animal models of stroke in neuroscience research have substantially changed our understanding of the pathogenesis of stroke and have triggered research for stroke treatment. Based on the type of ischemic injury induced in animals and infarct distributions, these models can be classified in different categories: global, focal, and multifocal cerebral ischemia. There is no single animal model able to cover all aspects of human ischemic stroke. Yet, focal cerebral ischemia is known as the most common animal model category, representing the human stroke (Howells et al., 2010). The focal ischemic models have been extremely useful in understanding mechanisms of human stroke. This type of ischemic injury is usually due to transient or permanent occlusion of a major cerebral artery, such as the middle cerebral artery. It has been suggested that up to 80% of all strokes result from ischemic damage in middle cerebral artery (MCA) in the brain[10] (Rousselet et al., 2012). As a result, blood flow in a specific region of the brain is significantly reduced and ischemic injury occurs (Braeuninger and Kleinschnitz, 2009). Therefore, among all focal ischemic models, middle cerebral artery occlusion (MCAO) is the most commonly used rodent models worldwide (Braeuninger et al., 2012) **(Fig 1.4)**. This technique does not require craniotomy (surgical removal of part of the bone from the skull), and can produce focal occlusion of a large cerebral artery as seen in human stroke (Carmichael, 2005). In addition, duration of occlusion can be controlled in order to investigate the events that

[10] Mohr, J.P., Gautier, J.C., Hier, D., and Stein, R.W. 1986. Middle Cerebral Artery. *In* Stroke, Vol. 1, Pathophysiology, Diagnosis and Management. (H.J.M. Barnett, B.M. Stein, J.P. Mohr, and F.M. Yatsu, eds.) pp. 377-450. Churchill Livingstone, New York.

occur after reperfusion (O'Neill and Clemens, 2001).

Sensitivity of neurons to the ischemic insults can also be investigated in *in vitro* model of ischemia. In vitro model of hypoxia-reoxygenation have provided useful tool to study the molecular pathways that regulate neuronal apoptosis. In addition, by changing the duration of the insults, this model can resemble delayed or rapid neuronal death seen in vivo. Previous studies have indicated that reoxygenation after hypoxia induces neuronal death in vivo and in vitro (Negishi et al., 2001). Both hypoxia (lack of oxygen) and reoxygenation (restoration of oxygen after hypoxia) occur in a wide variety of human diseases (Li and Jackson, 2002). Following hypoxic/reoxygenation episodes, several genes are activated to promote or inhibit neuronal apoptosis. This gene expression likely determines neuronal fate after stroke (Banasiak et al., 2000, Jin et al., 2002).


A
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MCA
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PCA
ICA
ECA
BA
CCA
monofilament

Figure 1.4.

Figure 1.4. Middle Cerebral Artery Occlusion (MCAO). In this figure, MCAO has been illustrated as an intraluminal monofilament model of focal cerebral ischemia. Carotid and cerebral arteries have been shown before **(A)** and after **(B)** insertion of the intraluminal monofilament. ACA, anterior cerebral artery; BA, basilar artery; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCA, middle cerebral artery; PCA, posterior cerebral artery (O'Neill and Clemens, 2001).

1.8.C. Ischemic Neuronal Death and ER stress

Molecular mechanisms involved in ischemic neuronal death are complex and the signaling pathways, which regulate ischemic death, are still not completely understood. Recently, there is a growing body of evidence highlighting the role of ER stress in ischemic injury. However, the molecular mechanisms of ER damage in ischemic neurons are not clear yet (Berridge, 2002). Recent research has shown that selective inhibition of eIF2α phosphorylation led to the reduction of brain damage after ischemia/reperfusion injury in rats. As it was stated before, the PERK-eIF2α pathway is an immediate response to ER stress. In addition, the enhanced expression of ER stress protein marker, GRP78 in the affected ischemic brain regions, indicated the activation of unfolded protein response. These data suggested the potential involvement of ER stress in mediating neuronal death-induced by ischemic injury (Nakka et al., 2010). On the other hand, an in vitro study has shown that hypoxia could induce ER stress target genes such as CHOP, which played as a protective factor against the ischemic injury (Halterman et al., 2010). Although substantial progress has been made to understand the role of ER stress target genes in ischemic neuronal death, the idea about this role is still debatable. Consequently, further understanding the cellular and molecular mechanisms underlying the role of ER stress target genes in ischemic neuronal damage is essential.

1.9. Statement of Research Problem, Rationale and Objectives

The research content of this thesis is part of the general objective of exploring the effect of DNA damage and ER stress in neuronal death with emphasis on the role of transcription factor ATF4. In addition, given the obvious link between p53 and

neurodegenerative disorders, the role of the p53 target protein, PUMA, was studied in mediating neuronal death. To do this, I evaluated the requirement for BH3-only protein PUMA in the propagation of DNA damage and ER stress induced injury and went on to identify a different protein than p53 as a PUMA regulator in ER stress. In the second part of the project, the role of ATF4 and its downstream target, CHOP, was studied in ischemic neuronal injury. The overall problem, rationale and objective of each part of this study are presented separately.

1.9.A. ATF4 in DNA damage and ER stress. It is believed that DNA damage and ER stress are two central initiators of neuronal damage in neurodegenerative diseases. Studies have demonstrated the involvement of p53 in neuronal injury in these disorders. Previous to this work, it had been reported that PUMA was one of the main pro-apoptotic p53 target genes, and its function was regulated by the p53 protein and required for the induction of neuronal death after DNA damage. Additionally, a study on MEFs showed the p53 as a component of the ER stress induced apoptosis. However, the role of p53 in neuronal death-mediated by ER stress and regulation of PUMA in this stress paradigm were not completely identified.

On the other hand, one of the most studied genes activated following ER stress is ATF4. Several studies have shown that this gene could promote some anti, and pro-apoptotic genes in response to cellular stresses. However, its exact role in neurons in response to ER stress was not clear. Additionally, recent data obtained from a Gene Array screening (unpublished data) revealed significant upregulation of ATF4 mRNA, 8 hours after DNA damage insult. This test was performed in cortical neurons looking for the genes with roles in the DNA damage-induced neuronal death pathways. Therefore, the

project was started driven by the hypothesis that the upregulation of ATF4 was important in cortical neurons exposed to DNA damage. Based on these two cellular stresses, several objectives were established in this part of project: (1) To confirm ATF4 up-regulation after DNA damage; (2) To determine whether ATF4 was up-regulated in neurons in response to ER stress; (3) To test the relevance of the ATF4 signal to DNA damage and ER stress-induced neuronal death; (4) To evaluate the involvement of p53 in neuronal death-mediated by ER stress and specifically to test the effects of p53 in PUMA regulation in this stress paradigm; (4) To examine the effect of ATF4 in PUMA regulation following ER stress; and (5) To identify possible(s) ATF4 target gene(s) mediating its neuronal pro-apoptotic effects in ER stress. The second chapter of this dissertation included the results associated to this project.

1.9.B. ATF4 in Ischemic Neuronal Death. In this part of project, the role of ATF4 was examined in ischemic injury in vitro and in vivo. The problem, rational and objective of this study are presented.

In the third chapter, we investigated the role of ATF4 and its downstream target protein, CHOP, in ischemic neuronal death. Several studies have been demonstrated the endoplasmic reticulum dysfunction following brain ischemia/reperfusion. For example, the enhanced expression of the ER stress marker, BiP, in the affected area of the brain after focal ischemic injury suggested that ER stress might play an important role in mediating neuronal ischemic death. Given an obvious link between ATF4 and neuronal death (based on the first part of our project), the role of this protein was examined in ischemic neuronal injury. As it is described in chapter two, the major part of my research has primarily focused on addressing the downstream target of ATF4 in regulation of neuronal death

following ER stress. Using an in vitro hypoxia/reoxygenation model as a delayed and excitotoxic ischemic death as well as transient focal ischemia (MCAO) in vivo, we studied the role of ATF4-CHOP axis in ischemic injury. In this study, experiments are performed in ATF4 and CHOP transgenic mice.

Chapter 2.

Neuronal apoptosis induced by endoplasmic reticulum stress is regulated by ATF4-CHOP-mediated induction of the Bcl-2 homology 3-only member PUMA.

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Zohreh Galehdar, Patrick Swan, Benjamin Fuerth, Steven M. Callaghan, Tim M. Townes, David S. Park and Sean P. Cregan

Statement of Author Contribution

This manuscript represents the results of my studies of the role of ATF4-CHOP in regulation of Puma in ER stress-induced apoptosis. We define the role of ATF4 as a pro-death factor induced by ER stress in neurons. It is reported here that following ER stress, ATF4 regulates CHOP induction as a part of a pro-apoptotic pathway. Subsequently, CHOP activates Puma leading to cytochrome c release and the activation of intrinsic programmed cell death pathway. Moreover, for the first time, it is being presented that regulation of Puma in neuronal death-mediated by ER stress is independent of p53 tumor suppressor function.

The experiments in this manuscript were carried out by Z. Galehdar and P. Swan. All ATF4 experiments, ATF4 figures and data presented predominantly performed and prepared by Z. Galehdar. RNA samples for running the Q-PCR were also provided by her. The text for the manuscript was written and edited by Z. Galehdar with guidance and assistance from Dr. Sean P. Cregan and Dr. David S. Park.

ATF4-deficient mice were generated by Dr. Tim M. Townes and Joe Sun from University of Alabama at Birmingham, as previously reported. Original Gene Array analysis data used in DNA damage experiments was performed previously by Dr. Sean P. Cregan in Dr. Ruth S. Slack's laboratory.

Section: Cellular and Molecular

Neuronal Apoptosis Induced by Endoplasmic Reticulum is Regulated by ATF4-CHOP-Mediated Induction of the Bcl-2 Homology 3-Only Member PUMA

Zohreh Galehdar[*], Patrick Swan[*], Benjamin Fuerth, Steven M. Callaghan, Tim M. Townes, David S. Park[#] and Sean P. Cregan[#]

[*] Co-first authors – these authors contributed equally to this work
[#] Co-corresponding authors

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2.1 Abstract

An increasing body of evidence points to a key role of endoplasmic reticulum (ER) stress in acute and chronic neurodegenerative conditions. Extensive ER stress can trigger neuronal apoptosis, but the signaling pathways that regulate this cell death remain unclear. In the present study, we demonstrate that PUMA, a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family, is transcriptionally activated in cortical neurons by ER stress and is essential for ER-stress-induced cell death. PUMA is known to be a key transcriptional target of p53, but we have found that ER stress triggers PUMA induction and cell death through a p53-independent mechanism mediated by the ER-stress-inducible transcription factor ATF4 (activating transcription factor 4). Specifically, we demonstrate that ectopic expression of ATF4 sensitizes mouse cortical neurons to ER stress-induced apoptosis and that ATF4-deficient neurons exhibit markedly reduced levels of PUMA expression and cell death. However, chromatin immunoprecipitation experiments suggest that ATF4 does not directly regulate the PUMA promoter. Rather, we found that ATF4 induces expression of the transcription factor CHOP (C/EBP homologous protein) and that CHOP in turn activates PUMA induction. Specifically, we demonstrate that CHOP binds to the PUMA promoter during ER stress and that CHOP knockdown attenuates PUMA induction and neuronal apoptosis. In summary, we have identified a key signaling pathway in ER-stress-induced neuronal death involving ATF4–CHOP-mediated transactivation of the proapoptotic Bcl-2 family member PUMA. We propose that this pathway may be an important therapeutic target relevant to a number of neurodegenerative conditions.

2.2 Introduction

DNA damage and endoplasmic reticulum (ER) stress are believed to be central initiators of neuronal death in acute and chronic neurodegenerative conditions. Indeed, DNA strand breaks and markers of ER stress are common features of neurons destined to die in models of cerebral ischemia and neurodegenerative disease (Chen et al., 1997a, DeGracia and Montie, 2004, Paschen and Mengesdorf, 2005, Martin, 2008). Although the signaling pathways mediating DNA damage and ER stress-induced neuronal death are starting to be appreciated, the complexities of these signaling networks have yet to be fully defined.

One of the central players critical for the DNA damage response in neurons is the tumor suppressor protein p53 because it regulates several pathways involved in DNA repair and cell death (Vousden and Prives, 2009). Importantly, we and others have shown that p53 is essential for neuronal death induced by DNA damage (Xiang et al., 1996, D'Sa-Eipper et al., 2001, Morris et al., 2001). PUMA, a Bcl-2 homology 3 (BH3)-only family member, has been implicated as a proapoptotic p53-target gene (Han et al., 2001, Nakano and Vousden, 2001, Yu et al., 2001). We have demonstrated previously that PUMA is a critical downstream effecter of p53 in DNA damage-induced apoptosis in neurons (Cregan et al., 2004). Although the p53–PUMA pathway is clearly critical for DNA damage-induced neuronal death, its relevance to ER stress-induced apoptosis is unclear.

ER stress is known to activate a series of signals that comprise the unfolded protein response (UPR). The UPR includes at least three signaling pathways initiated by the kinases IRE1 and PERK and the transcription factor ATF6 (activating transcription factor 6) (Ron and Walter, 2007). These signals coordinate the cellular response to unfolded

proteins, which includes (1) downregulation of protein translation, (2) enhanced expression of ER chaperone proteins that promote protein refolding, and (3) activation of proteases involved in the degradation of misfolded proteins. Conversely, prolonged or severe ER stress can lead to the activation of apoptotic cell death. However, the mechanisms regulating ER stress-induced cell death have not been clearly defined.

Of relevance to the present study, the transcription factors ATF4 and CHOP (C/EBP homologous protein) have been implicated in the ER stress response. ATF4 is a member of the basic leucine zipper family of transcription factors (Hai and Hartman, 2001). During ER stress, ATF4 is believed to be induced in a translationally dependent manner by the PERK-eIF2α pathway (Harding et al., 2000b, Scheuner et al., 2001). ATF4, in turn, can regulate the expression of a number of factors, including the transcription factor CHOP (Fawcett et al., 1999, Harding et al., 2003). Although it has been established that ATF4 and CHOP are induced by ER stress, the functional relevance of this induction and the mechanism by which this pathway regulate cell death is less clear.

In the present study, we define a critical signaling pathway involved in the regulation of ER stress-induced apoptosis. Specifically, we demonstrate that Puma induction is critical for ER stress-mediated neuronal death and establish that Puma expression is regulated by an ATF4–CHOP signaling axis.

2.3 Materials and Methods

2.3.1. Animals

p53 mice and Bax mice were obtained from The Jackson Laboratory, mice harboring null mutations of Puma and Bim genes were generated in the laboratory of Dr. Andreas Strasser

(Walter and Eliza Hall Institute of Medical Research, Bundoora, Victoria, Australia), and mice carrying an ATF4 null mutation were obtained from Dr. Tim Townes and Joe Sun (University of Alabama at Birmingham, Birmingham, AL). All transgenic strains were maintained on a C57BL/6 background and genotyped as described previously (Bouillet et al., 1999, Cregan et al., 1999, Fortin et al., 2001, Masuoka and Townes, 2002, Villunger et al., 2003). Wild type and knockout littermates (experimental mice) were generated by breeding heterozygous mice.

2.3.2. Primary cortical neuron cultures

Cortical neurons were dissociated from embryonic days 14.5–15.5 male and female mouse embryos and cultured in Neurobasal media containing N2 and B27 supplements (Invitrogen) as described previously (Cregan et al., 2002). Drug treatments were initiated 2–3 d after plating. Stock solutions of tunicamycin, thapsigargin, camptothecin, and etoposide were prepared in DMSO (all from Sigma-Aldrich) and diluted in culture media immediately before adding to cultures.

2.3.3. Calcium phosphate transfection

The pCMV– ATF4 plasmid was a kind gift from Dr. Amy S. Lee (University of Southern California, Los Angeles, CA). A CHOP cDNA cloned into pCMV–Sport6 was purchased from Invitrogen and subcloned into pcDNA3. Cortical neurons were transiently transfected using calcium phosphate method as described previously (Aleyasin et al., 2004). Cortical neurons were plated in 24-well plates ($4x10^5$ cells per well) and after 2–3 d were cotransfected with 0.25μg of pGFP (as a reporter) and 0.75μg of either pCMV–ATF4, pCMV–CHOP, or pcDNA3 as an empty vector control.

2.3.4. Recombinant adenoviral infection

Human ATF4 cDNA was a kind gift from Dr. Amy S. Lee. Recombinant adenovirus expressing ATF4 was generated by subcloning ATF4 cDNA (HindIII– EcoRV fragment) into the pAdTrack vector under a cytomegalovirus (CMV) promoter. The pAdTrack vector also contains a second CMV promoter, which controls expression of green fluorescent protein (GFP). This construct was used to generate recombinant adenovirus, as described previously (He et al., 1998). Adenovirus expressing only GFP was used as control. Cortical neurons were plated at $4x10^5$ cells per well and transduced with recombinant adenoviruses at a multiplicity of infection (MOI) of 50 at the time of plating.

2.3.5. Nucleofection

Cortical neurons were transiently transfected with small interfering RNA (siRNAs) using the Nucleofection system according to the instructions of the manufacturers (Amaxa Biosystems). Briefly, 50 pmol of siRNA were added to freshly dissociated cortical neurons suspended in Amaxa Mouse Neuron Nucleofector solution ($5x10^7$ cells/ml). In rescue experiments, 2.5 μg of the expression plasmids pCMV– GFP or pCMV–GFP–CHOP were also added to the cell suspension. The cell suspension was then electroporated with the Nucleofector device using program setting O-05, and then cells were immediately transferred to tubes containing DMEM supplemented with 10% FBS. Neurons were plated at a cell density of $1x10^6$ cells/ml in DMEM/FBS, and the following day the media was changed to standard Neurobasal media containing N2/B27 supplements. Drug treatments were initiated after an additional 24 h incubation. HPLC purified siRNAs were obtained from Ambion (Applied Biosystems) and sequences are available on request.

2.3.6. Cell death and survival assays

Neuronal apoptosis was assessed by examining nuclear morphology in Hoechst 33258 stained cells as described previously (Cregan et al., 2002). Briefly, neurons were fixed in 4% paraformaldehyde (containing 0.2% picric acid in 0.1 M phosphate buffer, pH 7.1) for 30 min, washed in PBS, and stained with Hoechst 33258 (0.25 µg /ml) dye. Neurons were visualized by fluorescence microscopy, and images were captured using a CCD camera (Q-imaging) and Northern Eclipse software (Empix Imaging). The fraction of cells exhibiting an apoptotic nuclear morphology characterized by pyknotic and/or fragmented nuclei containing condensed chromatin was scored by an individual blinded to the treatments. In certain experiments, the fraction of apoptotic cells was also determined by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay as described previously (Cregan et al., 1999). Neuronal survival was assessed as described previously (Farinelli et al., 1998). Briefly, cells were lysed in 200 µl of lysis solution (10xPBS, 10% Triton X-100, 1M MgCl2, and 5% cetyldimethylethylammonium bromide) and loaded on a hemocytometer, and the number of intact nuclei was evaluated using phase-contrast microscopy. Percentage survival was calculated as the ratio of live (intact) cells in drug-treated versus nontreated wells from the same embryo and multiplied by 100%. When indicated, neuronal survival was also assessed by Live-Dead Cytotoxicity assay according to the instructions of the manufacturer (Invitrogen) and described previously (Fortin et al., 2001). Survival is reported as the percentage of cells exhibiting calcein-AM fluorescence. In all survival and apoptotic assays, a minimum of 500 cells were scored for each well.

2.3.7. Caspase activity assay

Neurons were harvested in caspase lysis buffer (10 mM HEPES, pH7.4, 1mM KCl, 1.5mM MgCl2, 1mM DTT, 1mM PMSF, 10% glycerol, 5 μg/ml leupeptin, and 2 μg/ml aprotinin), and 10 μg of protein was used in caspase-3-like activity assay as described previously (Cregan et al., 1999). Briefly, protein samples were added to caspase reaction buffer (25 mM HEPES, pH 7.4, 10mM DTT, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylamm onio]-1-propanesulfonate, and 10 μM caspase-3 substrate Ac-DEVD-AFC), and fluorescence produced by cleavage of Ac-DEVD-AFC (acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumaryl) substrate was measured on a SpectraMax M5 spectrofluorimeter (400 nm excitation, 505 nm emission) over a 1 h interval. Caspase activity is reported as ratio of fluorescence output in treated samples relative to corresponding nontreated controls.

2.3.8. Quantitative reverse transcription-PCR

RNA was isolated using Trizol reagent as per the instructions of the manufacturers (Invitrogen), and RNA concentration was measured on a spectrophotometer. Quantitative reverse transcription (RT)-PCR was performed using the QuantiFast SYBR Green RT-PCR kit (Qiagen) and 20 ng of RNA as described previously (Steckley et al., 2007). RT-PCR was performed on a Chromo4 detection system (MJ Research/Bio-Rad), and changes in gene expression were determined using the $\Delta(\Delta C_t)$ method using the ribosomal S12 transcript for normalization. Data are reported as fold increase in mRNA levels in treated samples relative to corresponding untreated control samples for each transcript. All PCRs exhibited high amplification efficiencies (>90%), and the specificity of the PCR products

were confirmed by sequencing. Primer sequences used for gene specific amplification has been shown **(Table 2.1)**.

Semi-quantitative RT-PCR was also performed using the AccesQuick RT-PCR kit (Promega) and 50ng of RNA. Cycling conditions were: 45^{o}C-45 min, 94^{o}C-2 min, [(94^{o}C-30 sec, 62^{o}C-30 sec, 72^{o}C-60 sec) x 30 cycles]. The cDNA product was purified from 2% agarose gels using a GFX purification kit (Amersham Biosciences, UK) as per the manufacturers' protocol.

2.3.9. Western blotting

Whole-cell extracts (50 μg of protein) were loaded and separated on 10–12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were probed with primary antibodies to ATF4/cAMP response element-binding protein-2 (CREB-2) (1:500; Santa Cruz Biotechnology), Gadd153/CHOP (1:500; Santa Cruz Biotechnology), PUMA (1:500; Prosci), or BIM (1:500; Stressgen) in blocking buffer overnight. Membranes were washed in TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20) and probed with HRP secondary (1:10,000), and immunoreactive bands proteins were visualized using SuperSignal ECL (Pierce). Blots were stripped and reprobed for β-actin (1:10,000; Santa Cruz Biotechnology) as a loading control.

2.3.10. Luciferase reporter assay

A DNA fragment encompassing the PUMA promoter region -828 bp to +89 bp relative to the transcription start site was amplified from mouse genomic DNA using the primers 5'-GATCGGTACCCAAGCCATTTAGCAGGAACC and 5'-CTAGGCTAGCTGCTGCT TCTGGAGTCTTCG. The Puma (0.9 kb)–LUC reporter construct was generated by

inserting the 0.9 kb Puma promoter fragment into the KpnI/NheI restriction sites of the pGL3basic vector (Promega). The 0.3 and 0.18 kb Puma promoter fragments were generated by PCR amplification of the Puma (0.9 kb)–LUC using the primers 5'-GATCGGTACCTGCTCCTCCTGCCTGGACCAG and 5' GATCGGTACCCGTGGGAGCCAGCGAGAG, respectively, and the common reverse primer 5'-CTAGGCTAGCTGCTGCTTCTGGAGTCTTCG. The corresponding PCR products were digested and inserted into the KpnI/NheI restriction sites of pGL3b vector to generate the Puma (0.3 kb)–LUC and Puma (0.18 kb)–LUC reporter constructs. Neurons were cotransfected with 0.5 μg of the Puma–LUC constructs or pGL3b and 0.25 μg of either pcDNA3 or pCMV–ATF4, and, after 4 h, neurons were treated with 200 nM thapsigargin. Luciferase activity was measured after 24 h using the Dual-Luciferase Reporter Assay System (Promega) according to the instructions of the manufacturers. The Renilla Luciferase vector phRL– simian virus 40 was included in all transfections and used to normalize the reporter luciferase activity. Reporter-based luciferase activity is reported as the ratio of luciferase activity produced in cells transfected with pCMV–ATF4 or pCMV–CHOP relative to pcDNA3 for each reporter construct.

2.3.11. Chromatin immunoprecipitation (ChIP) assay

Neuronal cultures ($20x10^6$ cells) were crosslinked with 0.5% formaldehyde and then harvested in SDS lysis buffer and sonicated to fragment DNA. Extracts were incubated with antibodies to p53 (CM5; Novocastra), ATF4/CREB-2 (C-20X; Santa Cruz Biotechnology), Gadd153/CHOP (sc-575; Santa Cruz Biotechnology), or rabbit IgG control and immunoprecipitated with protein-G Sepharose beads. Immunocomplexes were eluted and decrosslinked at 65°C, and DNA was recovered by phenol-chloroform

extraction. Quantitative PCR was performed using the Quantitect SYBER Green PCR kit according to the instructions of the manufacturer (Qiagen). Relative DNA binding of p53, ATF4, and CHOP was determined as the ΔC_t values between treated and untreated samples immunoprecipitated with the same antibody. ΔC_t values were corrected for any changes in nonspecific interactions as determined by corresponding IgG pull-down samples, and data are reported as fold enrichment ($2^{\Delta Ct}$) in DNA binding. Primer sequences used for amplification of the PUMA promoter were 5'-CTGTCCCCACGCTGTC (forward) and 5'-GCTTGCTTGCTGGTGTCG (reverse) and for the CHOP promoter were 5'-GACAAGTTCAGGAAGGACAGC (forward) and 5'-CGGAGGAGGTGAGTGAGTCA (reverse).

2.3.12. Data Analysis

Data are reported as mean and SEM. The value n represents the number of independent neuron cultures or number of embryos of indicated genotype from which independent neuron cultures were prepared involving at least three independent experiments. Differences between groups were determined by ANOVA and post hoc Tukey's test and were considered statistically significant when $p < 0.05$.

Table 2.1. Primers' sequences for performing semi-quantitative RT-PCR

Gene	Sequence	
	5'-Sense-3'	5'-Anti-Sense-3'
NOXA	CAACGCGGGCAGAGCTACCTGA	TGGGCTTGGGCTCCTCATCCTGCTC
PUMA	AGCACTTAGAGTCGCCCGT	GAGGAGTCCCATGAAGAGATTG
CHOP	ACAGAGGTCACACGCACATC	GGGCACTGACCACTCTGTTT
ATF4	AGTCGGGTTTGGGGGCTGAA	GGGGCAACCTGGTCGACTTTTATT
Bim	TAAGTTCTGAGTGTGACAGAGAAGG	CAGTTGTAAGATAACCATTTGAGGGTGG

2.4. Results

2.4.1. ER stress triggers neuronal apoptosis through a PUMA-mediated pathway that is activated independently of p53

Several studies have indicated that ER stress induces cell death through an intrinsic/ mitochondrial pathway regulated by Bax/Bak (Wei et al., 2001, Smith and Deshmukh, 2007). BH3-only Bcl-2 family proteins are known to be critical regulators of Bax/Bak activation during apoptosis (Bouillet and Strasser, 2002). Previous studies have suggested that the BH3-only family members Bim, Noxa and Puma can be induced by agents that induce ER stress (Reimertz et al., 2003, Li et al., 2006, Puthalakath et al., 2007). Therefore, we examined the expression of these family members in cortical neurons treated with the ER stressors tunicamycin (N-glycosylation inhibitor) and thapsigargin (SERCA, Ca^{2+}-ATPase inhibitor). We found that both of the ER stressors triggered a marked induction in Puma expression and a modest increase in Bim mRNA levels **(Fig. 2.1A)**. However, Noxa mRNA levels were not induced in response to either of these agents. PUMA and to a lesser extent BIM protein levels were also found to be induced by ER stress, although this was only evident in Bax-deficient neurons, presumably as a result of the rapid cell death that occurs in wild-type neurons **(Fig. 2.1B)**. We next examined whether these BH3-only members were required for ER stress-induced apoptosis in neurons. To address this, we treated $Puma^{+/+}$ and $Puma^{-/-}$ cortical neurons with the ER stressors tunicamycin and thapsigargin and assessed apoptosis by examining nuclear morphology. For comparison, sister cultures were treated with the DNA damaging agents camptothecin and etoposide. As shown in Figure 2.2, treatment of wild-type neurons with ER stressors **(Fig. 2.2A)** or DNA damaging agents **(Fig. 2.2B)** induced a substantial increase in the fraction of cells

(>80%) exhibiting an apoptotic morphology characterized by chromatin condensation and/or nuclear fragmentation. In contrast, in Puma-deficient neurons the ER stressors and DNA damaging agents induced only a modest increase in apoptosis (<20%). Consistent with the observed protection afforded by Puma deficiency, caspase-3 activity induced by both ER stressors and DNA damaging agents in wild type neurons was dramatically attenuated in Puma-deficient neurons **(Figure 2.2C)**. These results indicate that in addition to its key role in DNA damage induced cell death, Puma is also a critical regulator of neuronal apoptosis induced by ER stress. Because BIM expression was also modestly upregulated in response to ER stress, we examined tunicamycin- and thapsigargin- induced apoptosis in wild-type and Bim-deficient neurons. However, unlike Puma-deficient neurons, Bim-deficient neurons were not protected against thapsigargin- or tunicamycin-induced apoptosis, suggesting that BIM is not required for ER-stress-induced apoptosis in neurons **(supplemental Fig. S 2.1)**.

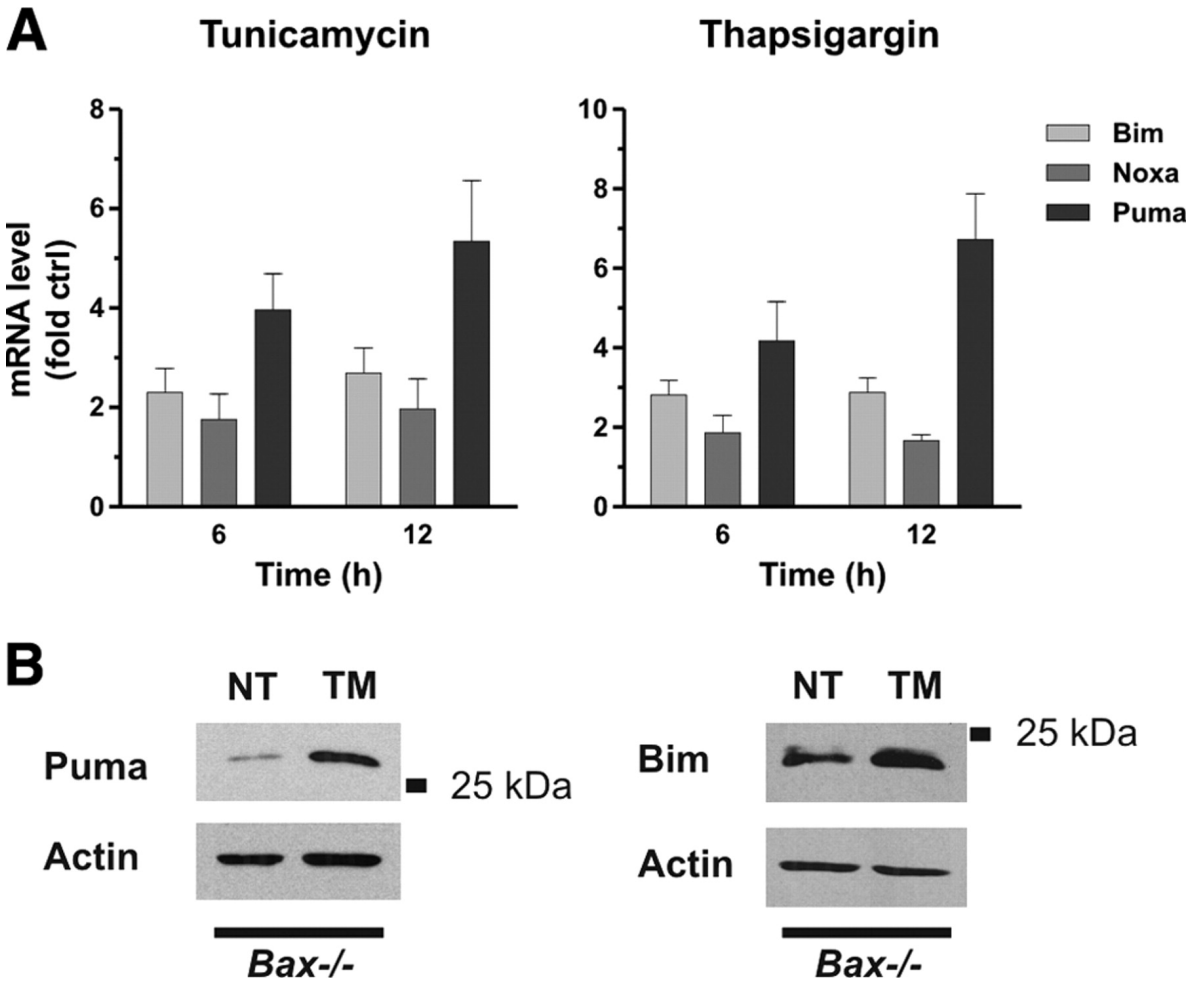
A
Tunicamycin
Thapsigargin
mRNA level (fold ctrl)
Time (h)
Time (h)
Bim
Noxa
Puma
B
NT
TM
Puma
25 kDa
Actin
Bax-/-
NT
TM
Bim
25 kDa
Actin
Bax-/-

Figure 2.1.

Figure 2.1. Puma expression is induced in cortical neurons during ER stress. ***A***, Cortical neurons were treated with tunicamycin (3 μg/ml) or thapsigargin (1μM), and Bim, Noxa, and Puma mRNA levels were assessed by real-time RT-PCR. Expression was normalized to ribosomal S12 levels and is reported as fold increase over untreated controls (n = 4). ***B***, Bax-deficient cortical neurons were treated with tunicamycin (TM), and Puma and Bim protein levels were assessed by Western blot at 24 h. NT, Nontreated.

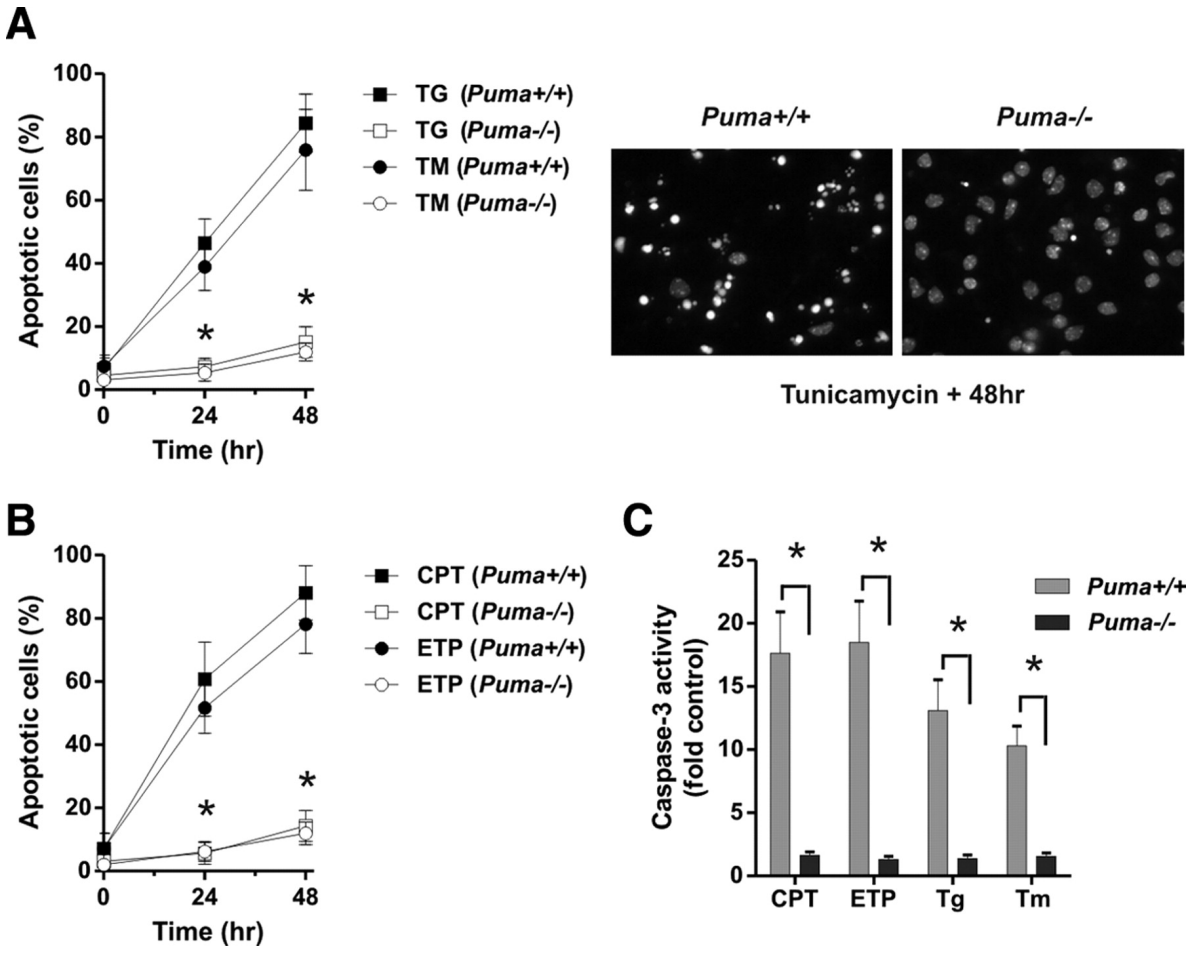
A
Apoptotic cells (%)
Time (hr)
TG (Puma+/+)
TG (Puma-/-)
TM (Puma+/+)
TM (Puma-/-)
Puma+/+
Puma-/-
Tunicamycin + 48hr
B
Apoptotic cells (%)
Time (hr)
CPT (Puma+/+)
CPT (Puma-/-)
ETP (Puma+/+)
ETP (Puma-/-)
C
Caspase-3 activity (fold control)
Puma+/+
Puma-/-
CPT
ETP
Tg
Tm

Figure 2.2.

Figure 2.2. PUMA is required for DNA damage-induced and ER stress-induced apoptosis in cortical neurons. ***A***, ***B***, Cortical neurons derived from Puma wild type and knockout littermates were treated with 1 μM thapsigargin (TG, Tg), 3 μg/ml tunicamycin (TM, Tm), 10 μM camptothecin (CPT), or 10 μM etoposide (ETP), and the fraction of apoptotic cells was determined at 24 and 48 h by assessing nuclear morphology after Hoechst staining. The fraction of apoptotic neurons was significantly decreased in $Puma^{+/+}$ versus $Puma^{-/-}$ cultures ($n \geq 7$; $*p < 0.001$). ***C***, Puma wild type and knockout cortical neurons were treated with DNA-damaging agents or ER stressors as above. Cell lysates obtained 20 h after treatment were assayed for caspase-3 activity ($n = 5$; $*p < 0.001$).

We and others have previously demonstrated that the transcriptional activator p53 is essential for PUMA induction and neuronal apoptosis induced by DNA damage (Cregan et al., 2004, Wyttenbach and Tolkovsky, 2006, Uo et al., 2007). Accordingly, we next investigated whether a p53–PUMA pathway is also involved in ER-stress-mediated neuronal death. To address this, we examined Puma expression in $p53^{+/+}$ and $p53^{-/-}$ cortical neurons after treatment with the ER stressors tunicamycin and thapsigargin. As shown in **Figure 2.3A**, Puma mRNA levels were increased by the ER stressors to a similar extent in $p53^{+/+}$ and $p53^{-/-}$ neurons, whereas, as expected, DNA-damage-induced Puma expression was abolished in the absence of p53. Furthermore, we found that p53 deficiency did not affect the level of ER-stress-induced neuronal apoptosis but, consistent with previous studies, dramatically reduced DNA damage-induced cell death **(Fig. 2.3B)**. Together, our data indicate that ER stress induces neuronal apoptosis through a PUMA-mediated pathway that is activated independently of p53.

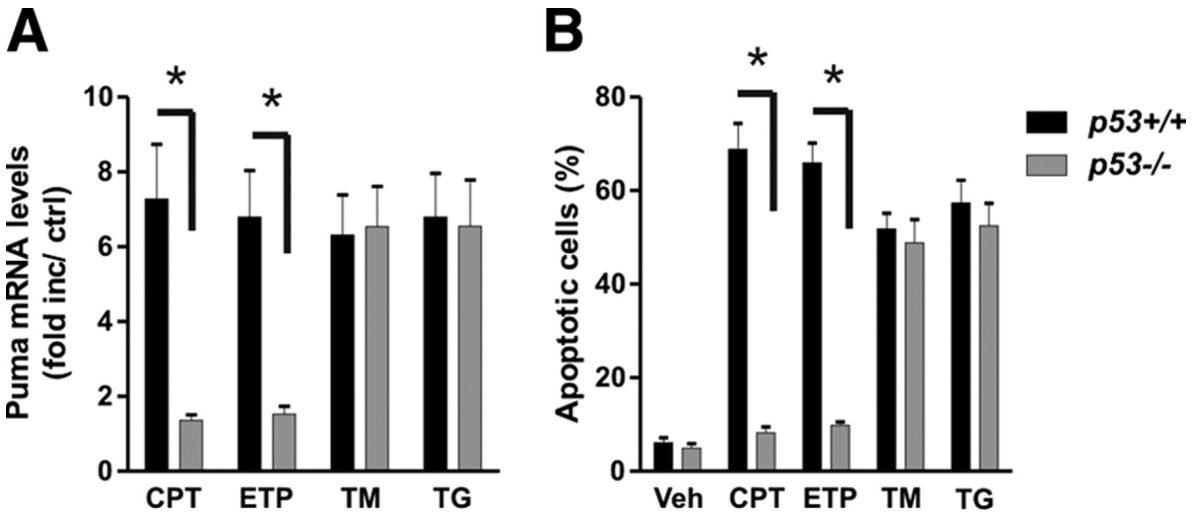
A
Puma mRNA levels
(fold inc/ ctrl)
10
8
6
4
2
0
*
*
CPT
ETP
TM
TG
B
Apoptotic cells (%)
80
60
40
20
0
*
*
Veh
CPT
ETP
TM
TG
p53+/+
p53-/-

Figure 2.3.

Figure 2. 3. ER stress triggers Puma induction and neuronal apoptosis through a p53-independent mechanism. Cortical neurons derived from p53 wild type and p53 knockout littermates were treated with thapsigargin (1 μm; TG), tunicamycin (3 μg/ml; TM), camptothecin (10 μm; CPT), or etoposide (10 μm; ETP). ***A***, RNA was extracted 12 h after treatment, and Puma mRNA levels were quantified by real-time RT-PCR. Puma expression was normalized to S12 levels and is reported as fold increase over untreated controls ($n \geq 6$; $^*p < 0.01$). ***B***, Neurons were stained with Hoechst dye 30 h after treatment, and the fraction of apoptotic cells was determined by assessing nuclear morphology ($n = 5$; $^*p < 0.01$). Veh, Vehicle.

2.4.2. ATF4 promotes ER stress-induced neuronal apoptosis

The above results clearly indicate that a transcriptional activator other than p53 is required for Puma induction during ER stress. The transcription factor ATF4 has been implicated in ER stress, but its functional role in the ER stress response and relevance to cell death remain unclear. Accordingly, we examined the potential role of ATF4 in ER-stress- and DNA-damage-mediated death pathways. Indeed, we observed a robust induction in ATF4 protein levels after treatment with the ER stressor tunicamycin but not in response to the DNA damaging agent camptothecin **(Fig. 2.4A)**. ATF4 was also rapidly induced in response to thapsigargin treatment, and increased protein levels were evident within 2 h of treatment **(Fig. 2.4B).**

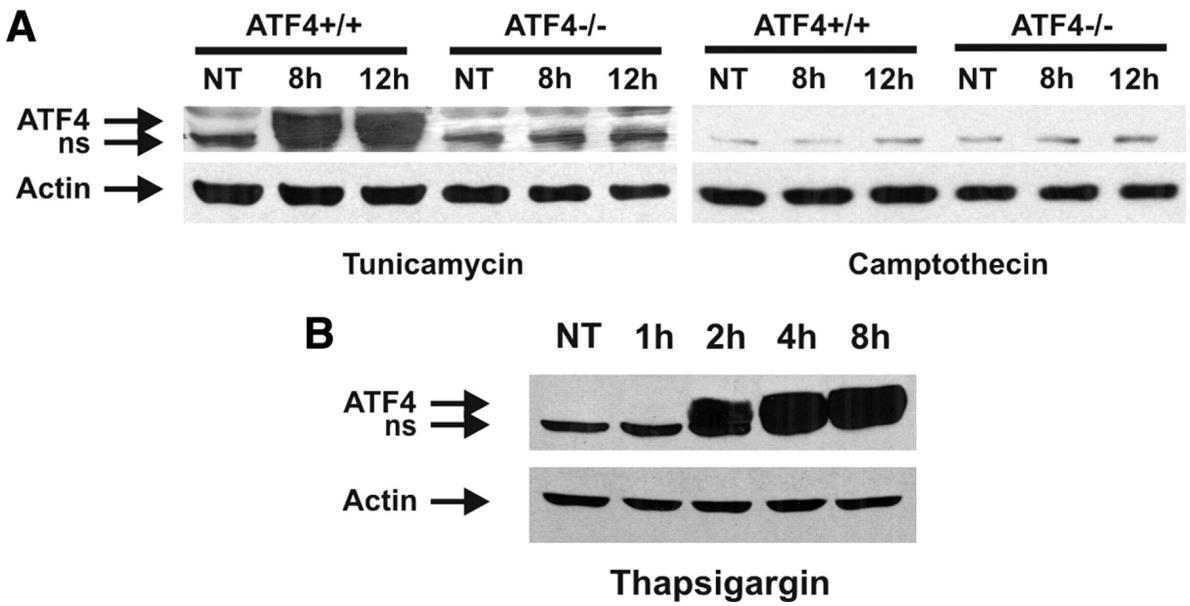
A
ATF4+/+
ATF4-/-
ATF4+/+
ATF4-/-
NT 8h 12h NT 8h 12h NT 8h 12h NT 8h 12h
ATF4
ns
Actin
Tunicamycin
Camptothecin
B
NT 1h 2h 4h 8h
ATF4
ns
Actin
Thapsigargin

Figure 2.4.

Figure 2.4. ATF4 expression is induced by ER stress but not DNA damage. ***A*****,** Cortical neurons derived from *ATF4*$^{-/-}$ mice and wild-type littermates were treated with tunicamycin (3 μg/ml) or camptothecin (10 μm), and ATF4 protein levels were assessed by Western blot. A nonspecific (ns) band was detected in extracts from both wild type and ATF4-deficient neurons. NT, Nontreated. ***B*****,** Cortical neurons were untreated or treated with thapsigargin (1 μm) for the indicated times, and ATF4 protein levels were assessed by Western blot.

We next examined whether gain or loss of function of ATF4 affects neuronal death induced by DNA damage or ER stress. In the first set of experiments, cortical neurons were cotransfected with plasmids expressing GFP and either ATF4 or an empty vector as control. After 24 h, transfected neurons were challenged with either tunicamycin or camptothecin, and the percentage of GFP-positive neurons exhibiting an apoptotic nuclear morphology was determined. As shown in Figure 2.5A, enforced expression of ATF4 sensitized neurons to tunicamycin-induced death. In contrast, ectopic expression of ATF4 protected neurons against camptothecin-induced apoptosis evident by an increase in neuronal survival from ~ 40 to 60% **(Fig. 2.5A)**. To ensure that these results were not an artifact of the transfection protocol, we repeated these experiments using recombinant-adenovirus-mediated ATF4 expression **(Fig. 2.5B)**. Consistent with the transfection results, we found that ectopic expression of ATF4 markedly enhanced cell death induction by the ER stressors tunicamycin (84.7 vs. 40% survival) and thapsigargin (71.7 vs. 21.8% survival) at 24 h after treatment **(Fig. 2.5B)**.

To address the role of endogenous ATF4, we assessed neuronal survival in wild type and ATF4-deficient neuronal cultures. As demonstrated in Figure 6A, ATF4-deficient neurons were found to be significantly more resistant than wild-type neurons to ER stress-induced cell death. For example, whereas only ~20% of $ATF4^{+/+}$ and $ATF4^{+/-}$ neurons remain alive 36 h after treatment with tunicamycin or thapsigargin, ~ 60–70% of ATF4-deficient neurons survive under the same conditions **(Fig. 2.6A)**. Similar results were found when neuronal survival and apoptosis was measured by live – dead and TUNEL assays, respectively **(Fig. 2.6C,D)**. In contrast, ATF4-deficient neurons were not resistant to DNA damage-induced cell death **(Fig. 2.6B)**. In fact, ATF4-deficient neurons exhibited

a modest increase in cell death compared with wild type neurons. Together, these results suggest that ATF4 promotes neuronal apoptosis during ER stress but can antagonize DNA damage- induced cell death.

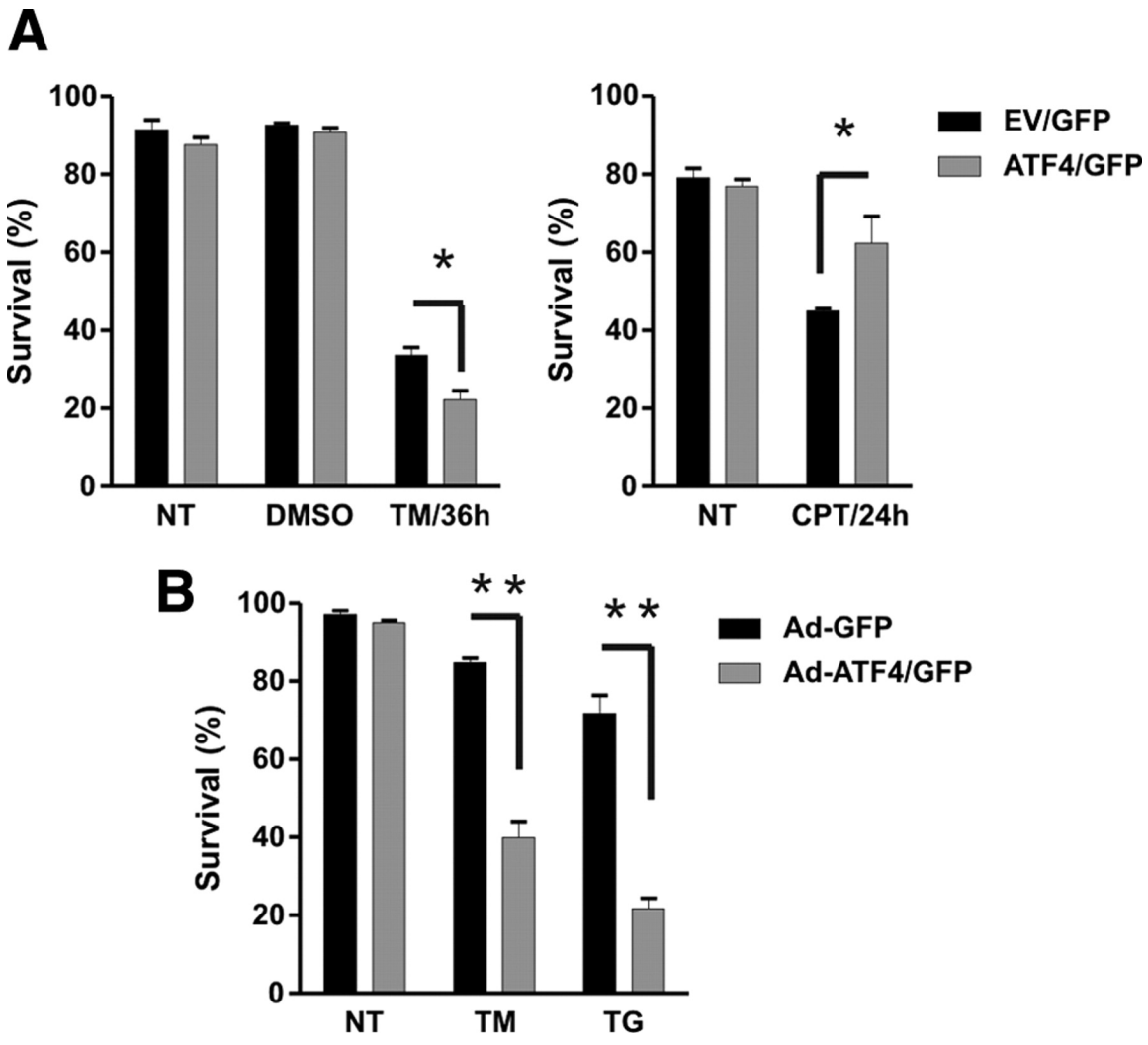
A
100
80
60
40
20
0
Survival (%)
NT
DMSO
TM/36h
*
EV/GFP
ATF4/GFP
CPT/24h
B
**
Ad-GFP
Ad-ATF4/GFP
TM
TG

Figure 2.5.

Figure 2.5. Ectopic expression of ATF4 sensitizes neurons to ER-stress-induced but not DNA-damage-induced apoptosis. ***A*****,** Cortical neurons were cotransfected with pGFP and either pcDNA3 [empty vector (EV)] or pcDNA3–ATF4, and, after 24 h, neurons were treated with camptothecin (10 μm), tunicamycin (2 μg/ml), or DMSO (0.1%) as a vehicle control. At the indicated times, neurons were fixed and stained with Hoechst 33258, and the fraction of apoptotic and nonapoptotic GFP-positive cells was assessed by fluorescence microscopy (n = 3; *p < 0.05). ***B***, Cortical neurons were infected with recombinant adenovirus expressing GFP (Ad–GFP) or coexpressing ATF4 and GFP (Ad–ATF4/GFP) at 50 MOI and, after 48 h, treated with tunicamycin (2 μg/ml) or thapsigargin (1 μm). After 24 h, cells were fixed and stained with Hoechst 33258, and the fraction of apoptotic and nonapoptotic GFP-positive neurons was determined (n = 3; **p < 0.01). (NT: Nontreated; TM: tunicamycin; TG: Thapsigargin; CPT: camptothecin).

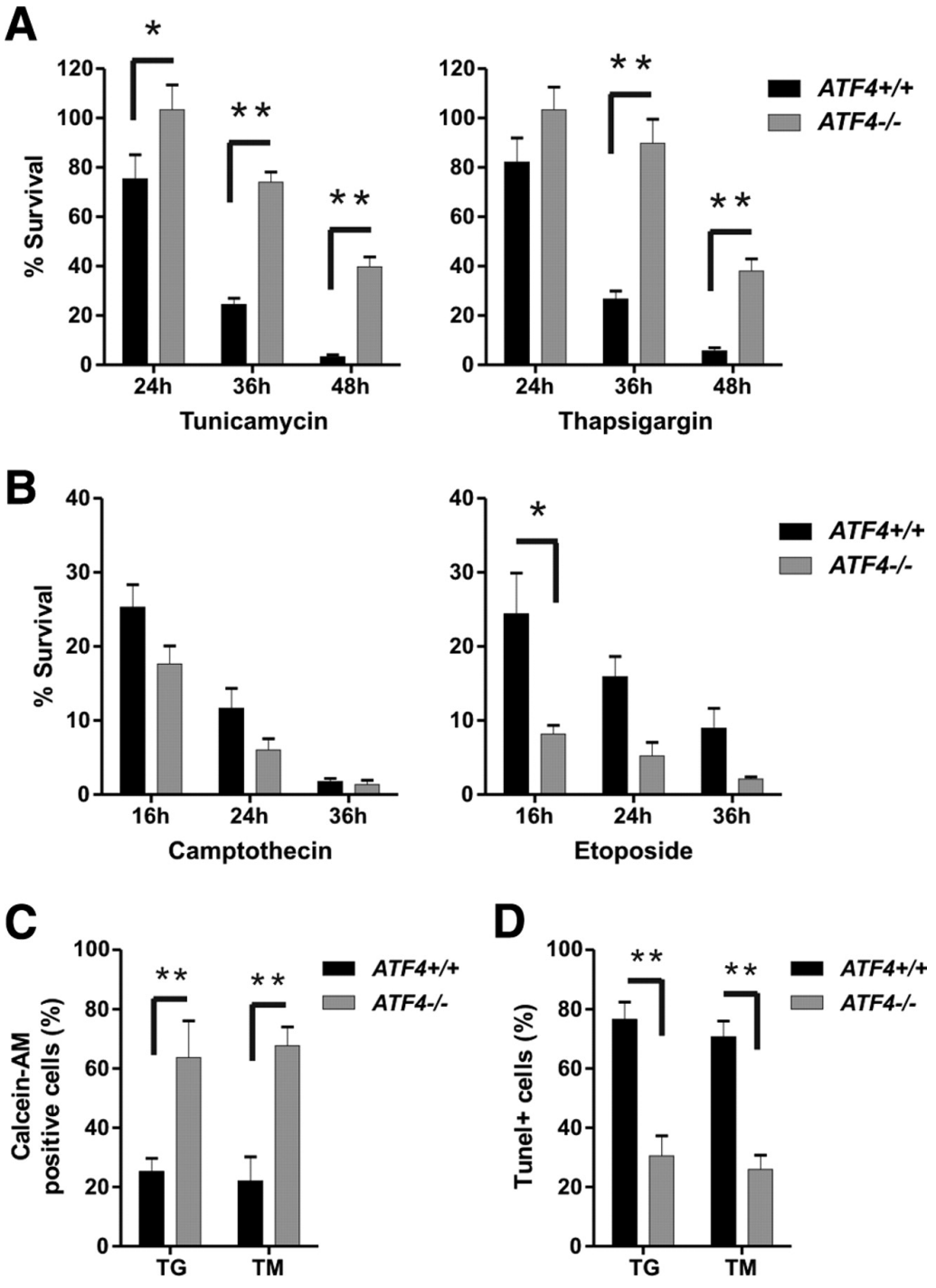
A
% Survival
120
100
80
60
40
20
0
*
**
**
24h
36h
48h
Tunicamycin
**
**
24h
36h
48h
Thapsigargin
ATF4+/+
ATF4-/-
B
% Survival
40
30
20
10
0
16h
24h
36h
Camptothecin
*
16h
24h
36h
Etoposide
ATF4+/+
ATF4-/-
C
Calcein-AM positive cells (%)
100
80
60
40
20
0
**
**
TG
TM
ATF4+/+
ATF4-/-
D
Tunel+ cells (%)
100
80
60
40
20
0
**
**
TG
TM
ATF4+/+
ATF4-/-

Figure 2.6.

Figure 2. 6. ATF4-deficient neurons are protected against ER-stress-induced but not DNA-damage-induced apoptosis. ***A***, ***B***, Cortical neurons derived from *ATF4*$^{-/-}$ embryos and wild type littermates were treated with the ER stressors tunicamycin (3 μg/ml) and thapsigargin (1 μm) or the DNA-damaging agents camptothecin (10 μm) and etoposide (10 μm). At the indicated times, cells were lysed, and the number of intact nuclei (viable cells) was counted. Survival was calculated as the ratio of viable drug-treated neurons to viable untreated neurons for each embryo ($n \geq 5$; $*p < 0.05$, $**p < 0.01$). ***C***, ***D***, Wild type and ATF4-deficient neurons were treated with thapsigargin (1 μm; TG) or tunicamycin (3 μg/ml; TM), and the percentage of calcein-AM-positive (live) cells and TUNEL-positive cells was measured after 36 h ($n = 3$; $**p < 0.01$).

2.4.3. ATF4 regulates Puma induction during ER stress

Given our observation that ATF4 promotes ER-stress-induced neuronal apoptosis, we investigated whether ATF4 is required for Puma induction by assessing Puma mRNA levels in wild type and ATF4-deficient neurons. As shown in **Figure 2.7**, Puma induction by both tunicamycin and thapsigargin was significantly reduced in ATF4-deficient neurons compared with wild-type neurons at both 8 and 12 h after treatment. In contrast, Puma expression induced by DNA damage was not affected by ATF4 deletion. Thus, ATF4 promotes Puma induction during ER stress but not in response to DNA damage. We next examined whether ATF4 can activate the Puma promoter. To address this, we generated a reporter construct, Puma (0.9 kb)–LUC, in which we inserted a ~ 0.9 kb mouse DNA fragment homologous to the previously reported human Puma promoter region upstream of luciferase in the pGL3b vector (Han et al., 2001, Yu et al., 2001).

To identify the ATF4-responsive region, we also generated the truncated Puma reporter constructs Puma (0.3 kb)–LUC and Puma (0.18 kb)–LUC that contained only the proximal ~300 and ~180 bp regions of the 0.9 kb Puma promoter fragment. As shown in **Figure 2.8A**, cotransfection of the Puma (0.9 kb)–LUC reporter construct with an expression plasmid for ATF4 (pCMV–ATF4) resulted in an approximately three fold increase in luciferase activity relative to empty vector control (pcDNA3). Ectopic expression of ATF4 caused a similar level of luciferase induction when cotransfected with the Puma (0.3 kb)– LUC but did not significantly activate luciferase expression from the Puma (0.18 kb)– LUC or pGL3b vectors. These results suggest that an ATF4-responsive element is present in the Puma promoter and is located in the nonoverlapping region of the 0.3 and 0.18 kb Puma LUC constructs (corresponding to a region ~90–210 bp upstream of

the transcription start site of Puma). Therefore, we examined this region for potential ATF4 binding sites using the Genomatix promoter analysis software and identified a conserved element exhibiting significant homology to the consensus ATF/CRE binding sequence and located 25 bp downstream of the previously identified p53 binding site **(Fig. 2.8B)**.

To determine whether ATF4 binds to the Puma promoter during ER stress in situ, we performed quantitative chromatin immunoprecipitation (ChIP) assays using PCR primers flanking the ATF4-responsive region and encompassing the putative ATF4 and p53 binding sites **(Fig. 2.8B)**. As expected, p53 binding to this region was enriched 4.5 fold after camptothecin treatment **(Fig. 2.8C)**. In contrast, p53 binding was not increased in neurons treated with ER stressors, consistent with the lack of p53 involvement in Puma expression in this death paradigm **(Fig. 2.8C)**. Curiously, we did not detect an increase in ATF4 binding in this region after either DNA damage or ER stress treatments **(Fig. 2.8D)**. However, ATF4 binding was significantly enriched at a previously described ATF4 response element located in the CHOP promoter (Ma et al., 2002), confirming that the ATF4 ChIP assay worked **(Fig. 2.8D)**. These results suggest that ATF4 likely regulates Puma expression during ER stress via an indirect mechanism.

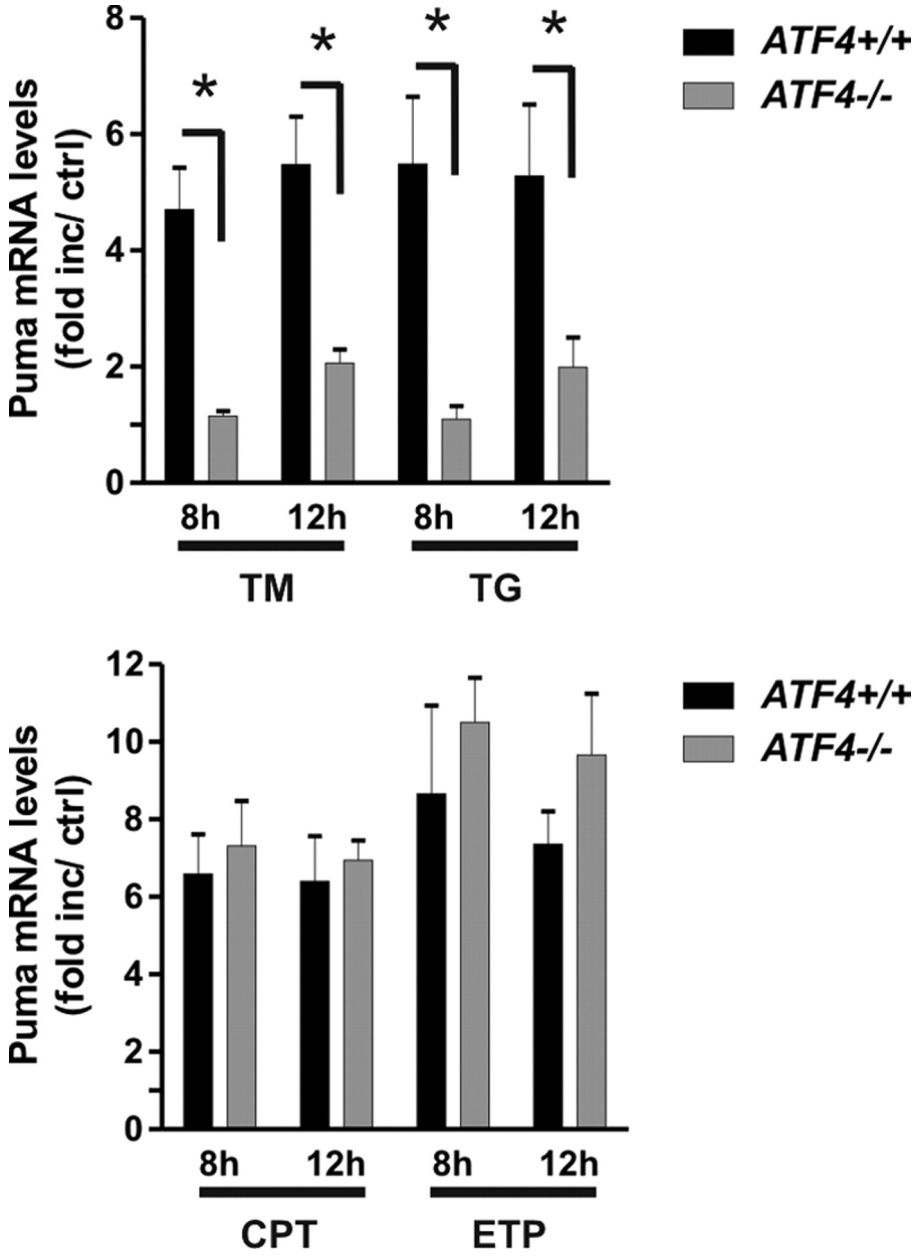
Puma mRNA levels
(fold inc/ ctrl)
8
6
4
2
0
*
*
*
*
8h
12h
8h
12h
TM
TG
ATF4+/+
ATF4-/-
Puma mRNA levels
(fold inc/ ctrl)
12
10
8
6
4
2
0
8h
12h
8h
12h
CPT
ETP
ATF4+/+
ATF4-/-

Figure 2.7.

Figure 2. 7. ATF4 regulates Puma induction during ER stress but not DNA damage. Cortical neurons derived from *ATF4*$^{-/-}$ and *ATF4*$^{+/+}$ littermates were treated with the ER stressors tunicamycin (3 μg/ml; TM) and thapsigargin (1 μm; TG) or the DNA-damaging agents camptothecin (10 μm; CPT) and etoposide (10 μm; ETP), and RNA was extracted after 8 and 12 h. Puma mRNA levels were quantified by real-time RT-PCR and are reported as fold increase over corresponding untreated controls ($n \geq 5$; $*p < 0.01$).

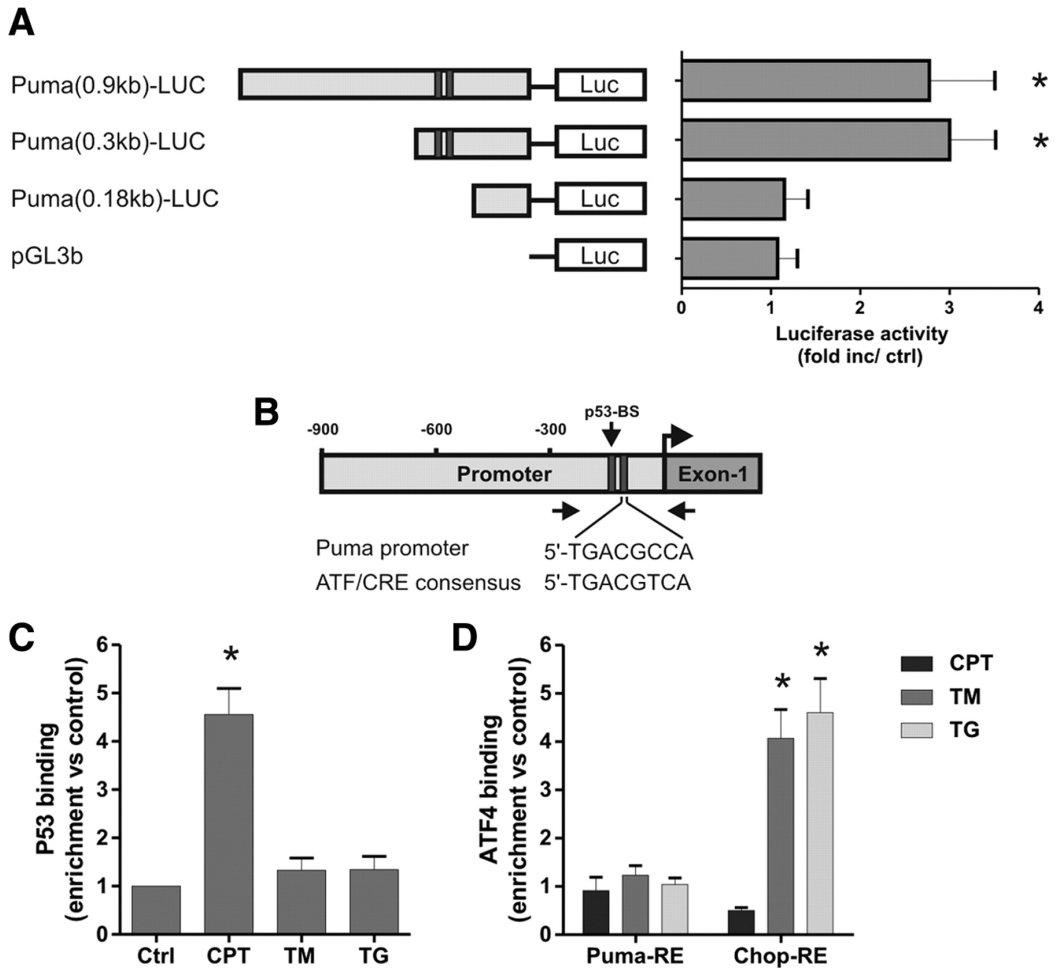
A
Puma(0.9kb)-LUC
Puma(0.3kb)-LUC
Puma(0.18kb)-LUC
pGL3b
Luc
*
*
0
1
2
3
4
Luciferase activity
(fold inc/ ctrl)
B
-900
-600
-300
p53-BS
Promoter
Exon-1
Puma promoter
5'-TGACGCCA
ATF/CRE consensus
5'-TGACGTCA
C
P53 binding
(enrichment vs control)
Ctrl
CPT
TM
TG
D
ATF4 binding
(enrichment vs control)
Puma-RE
Chop-RE
CPT
TM
TG

Figure 2.8.

Figure 2.8. ATF4 does not directly activate the Puma promoter during ER stress. ***A***, Neurons were cotransfected with the pGL3b or Puma–LUC reporter constructs and either pcDNA3 or pcDNA3–ATF4 and, after 4 h, treated with 200 nm thapsigargin. Luciferase activity was measured after 24 h and is reported as the ratio of luciferase activity produced in cells transfected with pcDNA3–ATF4 relative to empty pcDNA3 vector for each reporter construct ($n = 4$; $*p < 0.05$). ***B***, Schematic of the Puma promoter showing the location of the putative ATF/CRE and p53 binding sites. Arrows indicate the approximate positions of the PCR primers used in the ChIP assays. ***C***, Cortical neurons were treated with camptothecin (10 μm; CPT), tunicamycin (3 μg/ml; TM), or thapsigargin (1 μm; TG), and p53 binding to the Puma promoter was assessed after 8 h by ChIP assay. The level of p53 binding was quantified by real-time PCR and is reported as fold enrichment over untreated controls (Ctrl) ($n = 5$; $*p < 0.01$). ***D***, Cortical neurons were treated as above, and binding of ATF4 was assessed by ChIP assay and real-time PCR using primers specifically targeting the putative ATF4 response elements (RE) in the Puma and Chop promoters. Data are reported as fold increase over untreated control samples for each promoter region ($n = 5$; $*p < 0.01$).

2.4.4. ATF4 regulates Puma expression indirectly through CHOP induction during ER stress

The above results suggest that an intermediary factor is involved in ATF4-mediated Puma induction. Previous studies have suggested a relationship between ATF4 and the transcription factor CHOP, and indeed we observed enhanced binding of ATF4 to the CHOP promoter in neurons during ER stress **(Fig. 2.8D)**. Therefore, we hypothesized that ATF4 may regulate Puma induction through CHOP. Indeed, we found CHOP mRNA and protein levels to be markedly upregulated in neurons after treatment with tunicamycin or thapsigargin but not in response to DNA damage **(Fig. 2.9A,B)**. This increase in CHOP expression by ER stress was markedly reduced in ATF4-deficient neurons, consistent with its activation primarily by ATF4 in this paradigm **(Fig. 2.9C,D)**.

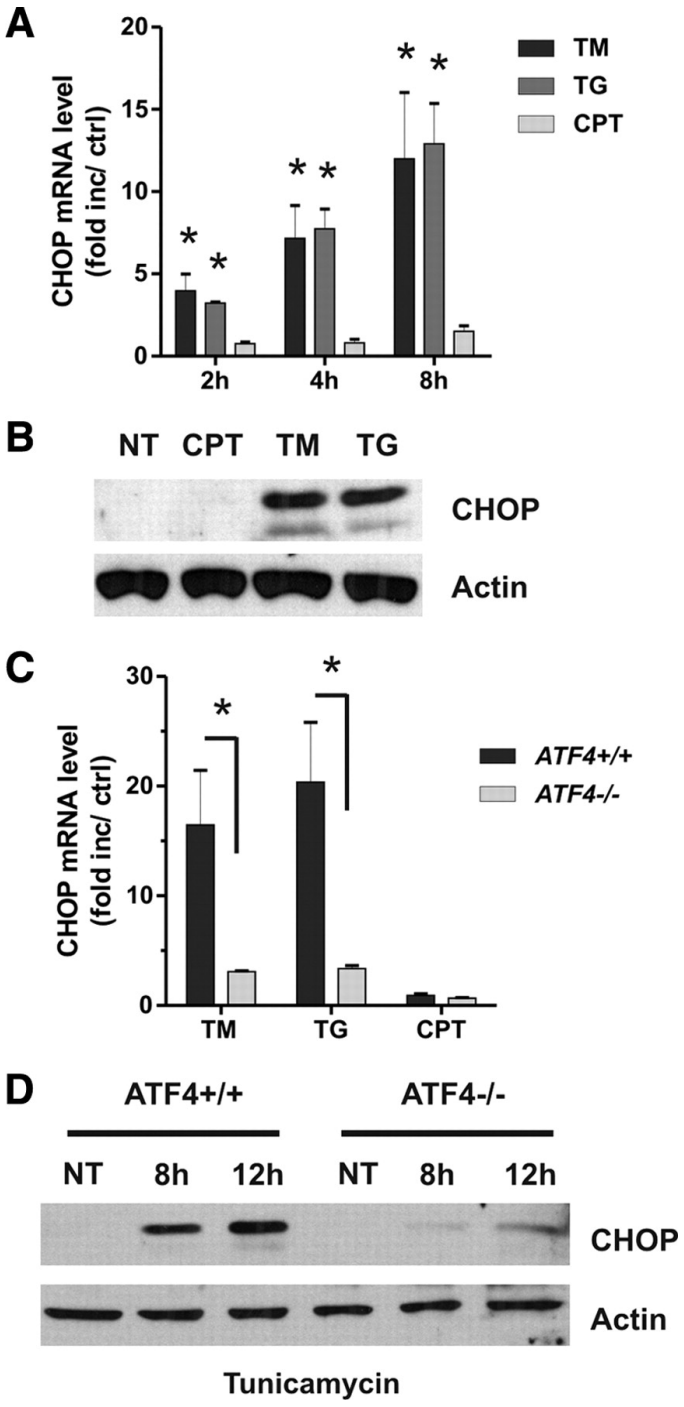
A
CHOP mRNA level
(fold inc/ ctrl)
20
15
10
5
0
2h
4h
8h
TM
TG
CPT
B
NT
CPT
TM
TG
CHOP
Actin
C
CHOP mRNA level
(fold inc/ ctrl)
30
20
10
0
TM
TG
CPT
ATF4+/+
ATF4-/-
D
ATF4+/+
ATF4-/-
NT
8h
12h
NT
8h
12h
CHOP
Actin
Tunicamycin

Figure 2.9.

Figure 2. 9. ATF4 regulates CHOP induction in response to ER stress but not DNA damage. ***A***, Cortical neurons were treated with camptothecin (10 μm; CPT), tunicamycin (3 μg/ml; TM), or thapsigargin (1 μm; TG), and CHOP mRNA levels were quantified by real-time RT-PCR after 2, 4, and 8 h ($n = 4$; $*p < 0.01$). Ctrl, Control. ***B***, CHOP protein levels were assessed by Western blot 10 h after treatment with camptothecin, tunicamycin, or thapsigargin. NT, Nontreated. ***C***, Cortical neurons derived from $ATF4^{+/+}$ and $ATF4^{-/-}$ littermates were treated with camptothecin, tunicamycin, or thapsigargin, and, after 8 h, CHOP mRNA levels were quantified by real-time RT-PCR ($n \geq 4$; $*p < 0.01$). ***D***, $ATF4^{+/+}$ and $ATF4^{-/-}$ cortical neurons were treated with tunicamycin and after 8 and 12 h CHOP protein levels were assessed by Western blot.

To determine whether CHOP contributes to Puma induction by ER stress we transfected cortical neurons with siRNAs directed against CHOP or non-targeting control siRNA using nucleofection technology. Neurons transfected with the CHOP targeting siRNAs exhibited a marked reduction in ER stress induced CHOP expression **(Fig. 2.10A)**. Importantly, we found that CHOP knockdown reduced Puma mRNA induction by tunicamycin and thapsigargin by approximately 30-40% **(Fig. 2.10B)**. In contrast, Puma induction by the DNA damaging agent camptothecin was not affected by transfection with the CHOP siRNAs **(Fig. 2.10B)**. Similarly, neurons transfected with siRNAs targeting CHOP exhibited a significant decrease in ER stress induced neuronal apoptosis, but not DNA damage induced neuronal cell death **(Fig. 2.10C)**. Similarly, we found that CHOP knockdown significantly increased neuronal survival after tunicamycin or thapsigargin treatment as measured by the live– dead cytotoxicity assay **(Fig. 2.10D)**.

To confirm the specificity of the CHOP siRNA knockdown effect, we nucleofected neurons with the Chop– siRNA-1 that targets the 5' untranslated region of the CHOP mRNA and a GFP fused CHOP expression plasmid lacking the 5' untranslated region (pCMV–GFP–CHOP) or the control vector pCMV–GFP. As shown in Figure 10E, in contrast to pCMV-GFP, ectopic expression of the siRNA-resistant GFP–CHOP protein partially restored tunicamycin-induced Puma expression and neuronal cell death in Chop– siRNA-transfected neurons **(Fig. 10E,F)**. It was noted that the rescue of Puma induction was not complete, which is likely attributable to the lower transfection efficiency (~60% GFP-positive cells) of the expression plasmids compared with siRNA (>85%).

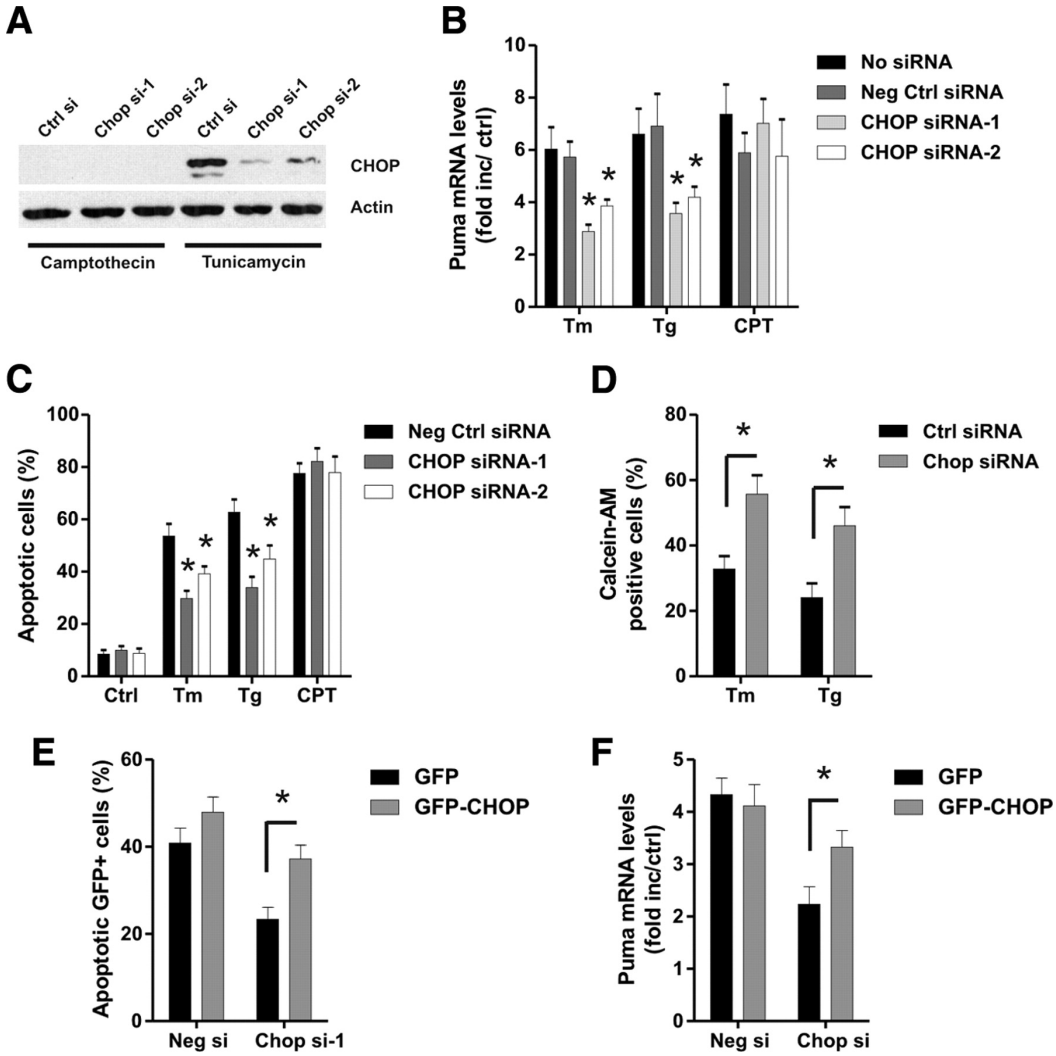
A
Ctrl si
Chop si-1
Chop si-2
Ctrl si
Chop si-1
Chop si-2
CHOP
Actin
Camptothecin
Tunicamycin
B
Puma mRNA levels (fold inc/ ctrl)
No siRNA
Neg Ctrl siRNA
CHOP siRNA-1
CHOP siRNA-2
Tm
Tg
CPT
C
Apoptotic cells (%)
Neg Ctrl siRNA
CHOP siRNA-1
CHOP siRNA-2
Ctrl
Tm
Tg
CPT
D
Calcein-AM positive cells (%)
Ctrl siRNA
Chop siRNA
Tm
Tg
E
Apoptotic GFP+ cells (%)
GFP
GFP-CHOP
Neg si
Chop si-1
F
Puma mRNA levels (fold inc/ctrl)
GFP
GFP-CHOP
Neg si
Chop si

Figure 2.10.

Figure 2. 10. CHOP knockdown diminishes Puma expression and neuronal apoptosis induced by ER stress but not DNA damage. Cortical neurons were nucleofected with two different siRNAs directed against CHOP or a nontargeting control siRNA (Ctrl si), and, 24 h later, the neurons were treated with camptothecin (10 μm; CPT), tunicamycin (3 μg/ml; Tm), or thapsigargin (1 μm; Tg). ***A***, Protein was extracted 12 h after treatment, and CHOP protein was assessed by Western blot. Ctrl, Control. ***B***, RNA was extracted 12 h after treatment, and Puma mRNA levels were quantified by real-time RT-PCR ($n = 4$; $*p < 0.05$). ***C***, Neurons were stained with Hoechst after 36 h, and the fraction of apoptotic cells was determined by assessing nuclear morphology ($n = 4$; $*p < 0.05$). ***D***, Cortical neurons nucleofected with Chop–siRNA-1 or negative control siRNA were treated with thapsigargin (1 μm) or tunicamycin (3 μg/ml), and the percentage of calcein-AM-positive (live) cells was determined at 36 h ($n = 3$; $*p < 0.05$). ***E***, ***F***, Cortical neurons were nucleofected with Chop–siRNA-1 or negative control siRNA and either pCMV–GFP or pCMV–GFP–CHOP, and, after 18 h, neurons were challenged with tunicamycin (3 μg/ml). ***E***, The fraction of apoptotic GFP-positive neurons was assessed by Hoechst staining 30 h after treatment with tunicamycin ($n = 3$). ***F***, Puma mRNA levels were quantified by real-time RT-PCR 10 h after tunicamycin treatment and are expressed as fold increase over corresponding untreated controls ($n = 3$).

We next performed reporter assays to determine whether CHOP can activate the Puma promoter from the ATF4- responsive region. As shown in Figure 2.11A, cotransfection of the Puma (0.3 kb)–LUC reporter construct with an expression plasmid for CHOP (pCMV–CHOP) resulted in a >2.5-fold increase in luciferase activity relative to empty vector control (pcDNA3). In contrast, pCMV–CHOP did not induce luciferase activity when cotransfected with the Puma (0.18 kb)–LUC or pGL3b reporter constructs **(Fig. 2.11A)**. Interestingly, we found that enforced expression of CHOP was sufficient to induce apoptosis in wild-type neurons but not *Puma*$^{-/-}$ neurons, suggesting that PUMA is required for CHOP-induced cell death **(Fig. 2.11B)**. We then performed ChIP assays to determine whether CHOP binds to the Puma promoter in situ during ER stress. As shown in **Figure 2.11C**, CHOP binding to the Puma promoter increased approximately threefold after treatment with tunicamycin or thapsigargin but was not increased by camptothecin treatment. These results suggest that CHOP can directly regulate the Puma promoter during ER stress but not in response to DNA damage. Together, our results indicate that ER stress induces neuronal apoptosis through a p53-independent pathway involving ATF4- and CHOP-mediated transcriptional induction of the proapoptotic Bcl-2 family member PUMA.

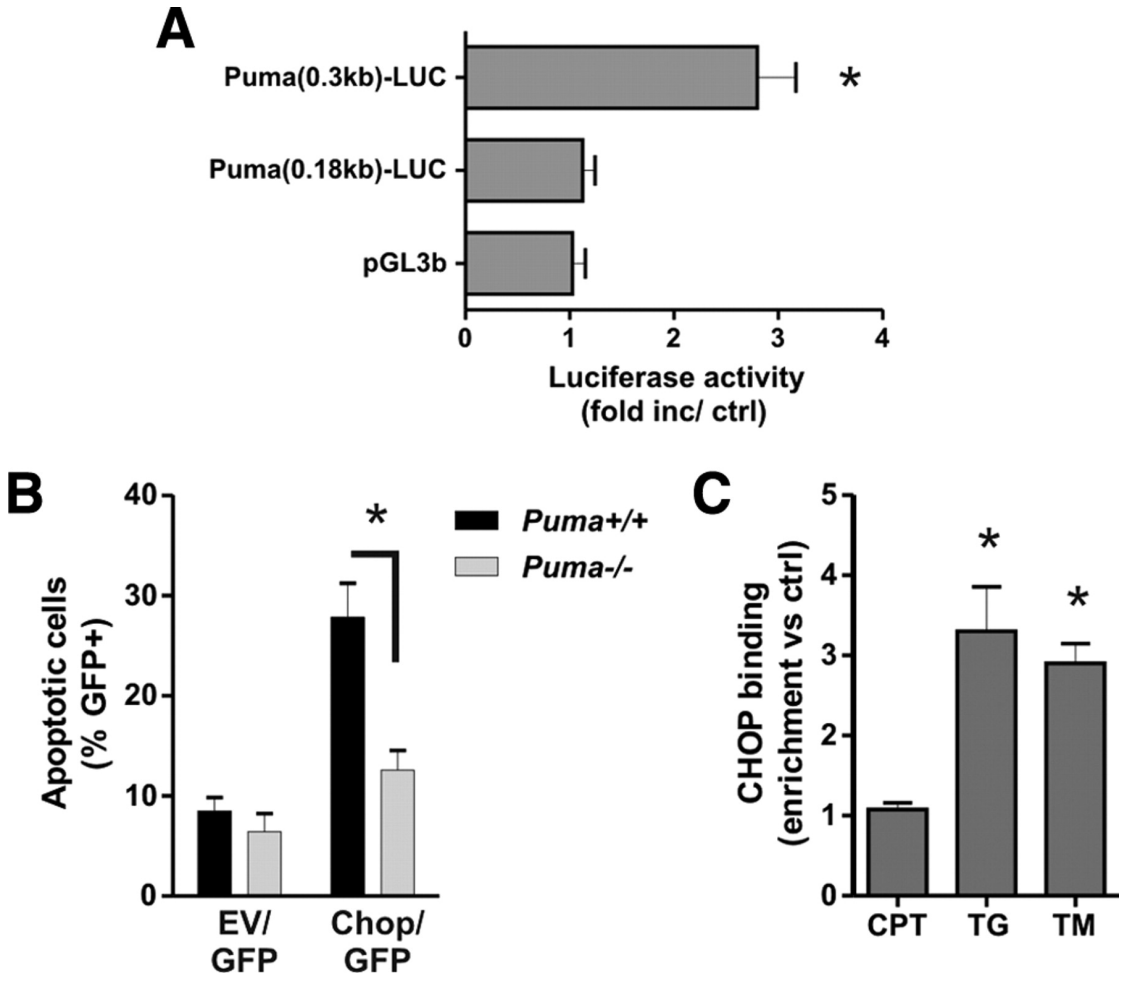
A
Puma(0.3kb)-LUC
Puma(0.18kb)-LUC
pGL3b
*
0
1
2
3
4
Luciferase activity
(fold inc/ ctrl)
B
Apoptotic cells
(% GFP+)
40
30
20
10
0
*
Puma+/+
Puma-/-
EV/
GFP
Chop/
GFP
C
CHOP binding
(enrichment vs ctrl)
5
4
3
2
1
0
*
*
CPT
TG
TM

Figure 2.11.

Figure 2.11. CHOP activates the Puma promoter during ER-stress-induced apoptosis. ***A***, Neurons were cotransfected with the pGL3b or Puma–LUC reporter constructs and either pcDNA3 or pcDNA3–CHOP, and luciferase activity was measured after 24 h. Luciferase activity is reported as the ratio of luciferase activity produced in cells transfected with pcDNA3–CHOP relative to empty pcDNA3 vector for each reporter construct ($n = 4$; $^*p < 0.05$). ***B***, Cortical neurons derived from $Puma^{+/+}$ and $Puma^{-/-}$ littermates were nucleofected with pGFP and either pcDNA3 [empty vector (EV)] or pcDNA3–CHOP. Neurons were Hoechst stained 48 h after transfection, and the fraction of GFP-positive neurons exhibiting an apoptotic nuclear morphology was determined ($n = 3$; $^*p < 0.05$). ***C***, Cortical neurons were treated with camptothecin (10 μm; CPT), tunicamycin (3 μg/ml; TM), or thapsigargin (1 μm; TG), and CHOP binding to the Puma promoter was assessed after 12 h by ChIP assay. The level of CHOP binding was quantified by real-time PCR and is reported as fold enrichment over untreated controls ($n = 4$; $^*p < 0.05$).

2.5 Discussion

An increasing body of evidence points to a key role of ER stress in a wide variety of acute and chronic neurodegenerative conditions. Indeed, protein aggregates and markers of the ER stress response have been observed in dying neurons in animal models of cerebral ischemia (Hu et al., 2001, Kumar et al., 2001) and Alzheimer's, Parkinson's, and Huntington's diseases (Ryu et al., 2002, Katayama et al., 2004, Reijonen et al., 2008). Therefore, it has become essential to identify the signaling pathways that regulate ER-stress-induced neuronal death.

2.5.1. ER stress induces neuronal apoptosis via a PUMA-dependent but p53-independent pathway

Recent studies suggest that ER stress induces apoptosis through an intrinsic (mitochondrial) pathway (Masud et al., 2007). The BCL-2 family proteins are key regulators of mitochondrial permeabilization, and the proapoptotic family members Bax and Bak have been implicated in ER-stress-induced cell death (Wei et al., 2001, Smith and Deshmukh, 2007). The BH3-only subfamily of Bcl-2 proteins are activated in a cell-type- and stimulus dependent manner and function by directly or indirectly promoting Bax/Bak activation (Bouillet and Strasser, 2002). In this study, we demonstrate that transcriptional activation of the BH3- only family member PUMA is required for both ER-stress- and DNA-damage-induced apoptosis in neurons but that the upstream regulators of Puma induction are distinct in the two paradigms. Others and we have shown previously that DNA damage- induced Puma expression in neurons is mediated by the tumor suppressor p53 (Cregan et al., 2004, Wyttenbach and Tolkovsky, 2006). However, this is clearly not

the case with ER stress because p53 deletion had no significant effect on Puma induction or cell death. Consistent with the lack of involvement of p53, we did not detect an increase in expression of the p53- responsive BH3-only member Noxa (Oda et al., 2000). Nonetheless, the involvement of p53 in ER stress may be impacted by the cellular context. For example, it has been reported previously that tunicamycin can induce Puma expression in Saos-2 cells that lack p53 function (Reimertz et al., 2003). Conversely, PUMA induction in response to ER stress in mouse embryonic fibroblasts has been reported to be dependent on p53 (Li et al., 2006).

2.5.2. ATF4 regulates ER stress induced Puma expression and neuronal apoptosis

We have determined that ATF4 is rapidly induced in response to ER stress but not DNA damage and that ATF4 is a key regulator of ER-stress-induced neuronal apoptosis. Interestingly, ATF4 has also been shown to regulate the expression of genes involved in amino acid metabolism and redox functions, suggestive of a cellular protective function (Harding et al., 2003). The reason for this apparent dichotomy is not clear, but ATF4 is known to dimerize with a number of transcription factors, including FIAT, Nrf2, NF-IL6, as well as AP-1 and Maf family members, and it is likely that this can alter its biological function (Tanaka et al., 1998, Hai and Hartman, 2001, He et al., 2001). The dual nature of ATF4 is reflected in several other proteins that can play both pro-survival and death roles, such as p53 a protein involved in both DNA repair and cell death (Offer et al., 2002) and nuclear factor-κB (NF-κB) (Aleyasin et al., 2004). Importantly, we have determined that ATF4 promotes ER-stress-induced neuronal apoptosis by regulating the transcriptional induction of Puma. Thus, ER stress and DNA damage induce Puma expression via distinct pathways involving ATF4 and p53, respectively. It has been reported recently that ATF4

also plays a proapoptotic role in oxidative-stress-induced apoptosis in neurons (Lange et al., 2008). Interestingly, we have demonstrated recently that PUMA plays a critical role in regulating oxidative-stress-induced neuronal apoptosis and that Puma induction in this context involves both p53 and p53-independent mechanisms (Steckley et al., 2007). Thus, it would be interesting to determine whether ATF4 also contributes to Puma induction during oxidative-stress-induced neuronal death.

2.5.3. CHOP regulates PUMA induction in neurons during ER stress

Despite our finding that ATF4 is an important regulator of PUMA induction, we were unable to detect a direct interaction of ATF4 with the PUMA promoter, suggesting that an ATF4- responsive intermediate may be involved. The stress-inducible transcription factor CHOP is known to be activated by a number of pathways, including ATF4, ATF2, ATF6, and Ire1/XBP-1 (Yoshida et al., 2000, Ma et al., 2002, Averous et al., 2004). We demonstrate that CHOP is induced in response to ER stress but not DNA damage and that at least in this context its induction occurs predominately through ATF- mediated transactivation. Previous studies have implicated CHOP as a proapoptotic factor. For example, enforced expression of CHOP has been shown to induce cell death (Maytin et al., 2001), and CHOP-deficient cells have been reported to be resistant to ER-stress-induced cell death (Zinszner et al., 1998, Oyadomari et al., 2002). However, the mechanism by which CHOP engages the apoptotic pathway has remained unclear. The involvement of the Bcl-2 family has been suggested by the findings that CHOP-induced cell death is associated with translocation of Bax to the mitochondria and that enforced expression of Bcl-2 can block CHOP-induced cell death (Matsumoto et al., 1996, Gotoh et al., 2004). Importantly, we have determined that CHOP knockdown decreases ER-stress induced

Puma expression and neuronal apoptosis. Consistent with this, we found that CHOP can activate the Puma promoter and that it binds to the Puma promoter in situ during ER stress. Furthermore, we show that enforced expression of CHOP can induce apoptosis in wild-type but not Puma-deficient neurons, indicating that PUMA is required for CHOP-induced neuronal death. Paradoxically, we found that ectopic expression of ATF4 was not sufficient to induce neuronal cell death but rather sensitized neurons to ER-stress-induced apoptosis. This likely reflects a requirement for ER stress signals in the activation of ATF4. Indeed, it has been shown previously that ectopic expression of ATF4 is not sufficient to induce CHOP expression in the absence of an ER stress signal (Harding et al., 2000a). The half-life of ATF4 under nonstressed conditions is very short because it is rapidly targeted for proteasomal degradation (Lassot et al., 2001). Thus, it is assumed that ER stress signals directly or indirectly affect the stability of the ATF4 protein. It is also possible that ER stress leads to the induction of an additional factor required for efficient ATF4-mediated transactivation. It should be noted, however, that even ectopic expression of CHOP only resulted in a relatively modest increase in neuronal apoptosis (~30% cell death after 48 h). Thus, it is likely that ER stress signaling can potentiate the proapoptotic functions of CHOP as well. Although our study clearly demonstrates the important involvement of ATF4–CHOP signaling in ER stress-induced neuronal apoptosis, it is evident that inhibition of this pathway does not completely abrogate Puma induction or cell death. This suggests that additional factors contribute to Puma induction during ER-stress-induced apoptosis. In addition to ATF4, ER stress activates the Ire1α and ATF6 arms of the unfolded protein response, and it is possible that these pathways may also contribute to Puma induction. Indeed, both proapoptotic and anti-apoptotic functions have been

attributed to these pathways (Lin et al., 2007, Yokouchi et al., 2007). Interestingly, during activation by ER stress, Ire1α exhibits dual functions in inducing the expression of the prosurvival transcription factor XBP1 and activating the proapoptotic JNK signaling pathway (Urano et al., 2000, Yoshida et al., 2001, Calfon et al., 2002). Thus, it is possible that Ire1–JNK signaling could contribute to Puma induction through activation of the transcription factor c-jun. Furthermore, several transcription factors, including E2F1, Foxo3a, p73, and NF-κB, have been implicated in the transcriptional regulation of Puma, but their relevance in ER stress has not yet been examined (Hershko and Ginsberg, 2004, Melino et al., 2004, You et al., 2006, Wang et al., 2009a).

2.5.4. ATF4-CHOP-PUMA pathway in neurodegenerative conditions

In the present study, we have identified a key signaling pathway in ER-stress-induced neuronal apoptosis involving the transcription factors ATF4 and CHOP and their downstream target PUMA. Several lines of evidence suggest that this pathway may be relevant and important in brain injury and neurodegenerative conditions. For example, induction of ATF4, CHOP, and Puma levels have been reported in affected brain regions in models of cerebral ischemia (Paschen et al., 1998, Jin et al., 2001, Morimoto et al., 2007, Niizuma et al., 2009). Furthermore, both ATF4- deficient and CHOP-deficient mice have been reported to exhibit smaller infarcts after ischemia–reperfusion injury (Tajiri et al., 2004, Lange et al., 2008). Similarly, both CHOP and PUMA have been implicated in dopaminergic neuronal death in Parkinson's disease models (Holtz and O'Malley, 2003, Biswas et al., 2005a, Silva et al., 2005). Finally, it has been reported recently that Puma expression is induced in degenerating motor neurons in a mouse model of amyotrophic lateral sclerosis and that Puma deletion reduces motor neuron apoptosis in this model

(Kieran et al., 2007).

In summary, our results demonstrate that an ATF4–CHOP– PUMA signaling pathway plays a key role in the regulation of ER-stress-induced neuronal apoptosis. We suggest that this pathway may be an important therapeutic target relevant to a variety of neurodegenerative conditions.

2.6. Supplemental Figure

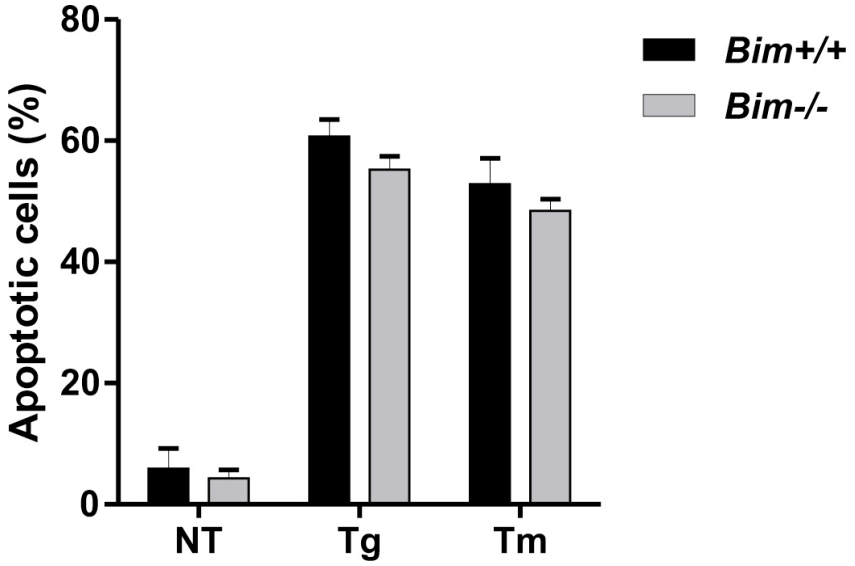

Figure S 2.1.

Figure S2.1. BIM is not required for ER stress induced apoptosis in cortical neurons. Cortical neurons derived from wildtype and Bim-null littermates were treated with Thapsigargin (1μM) or Tunicamycin (3μg/ml) and after 36 hours cells were stained with Hoechst 33342 and the fraction of apoptotic cells was determined by assessing nuclear morphology (n=8).

Chapter 3.

The role of ATF4-CHOP pathway in neuronal damage induced by cerebral ischemic injury

Zohreh Galehdar, Farzaneh Safarpour, Steve M. Callaghan, Sean P. Cregan, David S. Park.

Statement of Author Contribution

This manuscript represents the results of my studies of the role of ATF4 signaling pathway and its downstream target, CHOP, in ischemic neuronal death. The data obtained from *in vitro* and *in vivo* experiments are presented. Some of the data are preliminary. This manuscript is still under preparation.

All in vitro experiments in this manuscript were carried out by Z. Galehdar, including cell culture data, as well as animal breeding for the in vitro and in vivo experiments. The Middle Cerebral Artery Occlusion procedure (MCAO) was performed with help from F. Safarpour.

3.1. Abstract:

An increasing body of evidence points to a key role of ER stress in a wide variety of acute and chronic neurodegenerative conditions including stroke. Indeed, markers of the ER stress response have been observed in dying neurons in animal models of cerebral ischemia. To further decipher the significance of these signals, we investigate the role of ATF4-CHOP signaling pathway in ischemic neuronal injury. Ischemic stroke results from a transient or permanent reduction in cerebral blood flow in the brain. In spite of much research in trying to develop therapeutic strategies, most clinical trials have failed. These failures demonstrate that effective treatments require a more complete understanding of molecular signals that lead to neuronal death. However, stroke is complex and distinct mechanisms may regulate rapid and/or delayed neuronal death. The signaling pathways regulating these mechanisms however are not fully defined. Previous studies had suggested that ER stress playing a pivotal role in post-ischemic neuronal death. Yet, the relevance of ER stress signals was not fully known in ischemic neuronal injury. Accordingly, this thesis research attempts to explore the functional role of ER stress -inducible pathway, ATF4-CHOP axis, in different models of neuronal death evoked by ischemia. The data indicates that ATF4 is essential in delayed type of death in vitro. In focal ischemia model (tMCAO) ATF4 also plays a role as a mediator of death signal *in vivo*. However, CHOP function looks more complex, and our data did not support the role of CHOP in ischemic neuronal death.

3.2. Introduction:

Brain is a unique organ with high metabolic demand and low energy reserves. Although this organ represents only 2 percent of the body weight, it receives almost 18 percent of cardiac output, 20 percent of oxygen supply, and 25 percent of total body glucose consumption[11]. Due to these unique requirements, the brain is very susceptible to damage from transient or permanent deprivation of blood supplies including glucose and oxygen. When the low amount of oxygen and glucose do not meet the brain metabolic demands, resulting to cerebral ischemic stroke[12]. Eventually, long duration of ischemia and lack of blood supply will lead to the irreversible loss of neuronal function and neuronal death.

Stroke is a serious neurological problem worldwide. In Canada also, a stroke attack occurs approximately every ten minutes.[13] About 80% of all stroke cases are induced by ischemia (Thrift et al., 2001). Within the ischemic stroke, there are two major zones of injury: the center of ischemic region with acute and necrotic cell death (core), and the region surrounding the core area known as penumbra. The penumbra is the ischemic region that despite ischemic core zone, experiences mild to moderate damage with higher chance of cell survival. Penumbra displays a more delayed type of cell death with characteristics of apoptosis (Dirnagl et al., 1999). The signaling pathway (s) that regulate ischemic neuronal death in this region is not fully identified.

For decades, numerous studies have focused on the vascular aspects of stroke to limit ischemic damage. Currently, the most effective therapeutic strategy for stroke consists of

[11] http://www.acnp.org
[12] http://www.intechopen.com/books/acute-ischemic-stroke, Excitotoxiciy and oxidative stress in acute ischemic stroke, R.R. Bretón & J.C.G. Rodríguez, 2012
[13] http://www.canadianstrokenetwork.ca

re-establishing blood circulation in the affected area of the brain through lysing the blood clot. Currently, tissue plasminogen activator (tPA) is the only approved therapeutic drug administered in ischemic stroke (Kleindorfer et al., 2004, Adeoye et al., 2011). Although the effect of tPA on stroke treatment has been confirmed, a narrow time frame window for receiving this drug makes it useful only to few patients (Lansberg et al., 2009). Therefore, to overcome this problem, understanding the mechanism (s) and key molecular events by which neuronal death takes place is necessary. Many studies focus on elucidating the molecular pathway of delayed neuronal death in ischemic injury[14] (Won et al., 2002). These mechanism (s) may also have relevance to neuronal death in other neurodegenerative diseases.

The mechanisms of neuronal death in ischemia are complex. Based on the multiple parameters such as severity, duration and location of ischemia within the brain, various complex mechanisms may be involved in ischemic neuronal injury. Endoplasmic reticulum stress (ER stress) has been postulated to play a vital role in ischemic neuronal death (Nakka et al., 2010, Srinivasan and Sharma, 2011). Indeed, protein aggregates and markers of ER stress have been observed in animal models of cerebral ischemia (Hu et al., 2001, Kumar et al., 2001). We have also shown that ATF4 is translationally induced during ER stress in neurons (Galehdar et al., 2010). Activating transcription factor 4 (ATF4) is a stress-responsive transcription factor (Harding et al., 2000a) that has been considered to promote neuronal death following ER stress (Galehdar et al., 2010). ATF4, in turn, can

[14] http://www.intechopen.com/books/advances-in-the-preclinical-study-of-ischemic-stroke, Delayed neuronal death in ischemic stroke: Molecular pathways, V. Li, et al. 2012

regulate the expression of a transcription factor CHOP, a transcription factor implicated in apoptosis (Fawcett et al., 1999, Scheuner et al., 2001, Galehdar et al., 2010). However, the pro-death role of CHOP is controversial and it may depend on the nature of the toxic stimulus. Therefore, although it has been established that ATF4-CHOP pathway is induced by ER stress, the functional relevance of this induction in regulation of neuronal death following cerebral ischemia is less clear. Understanding the relationship between this pathway and ischemic neuronal injury may help to identify an important therapeutic target relevant to stroke and a number of other neurodegenerative conditions.

3.3. Material and Methods:

3.3.1. Animals

All procedures and experiments involving animal subjects, performed for the scientific purposes, were approved by the University of Ottawa Animal Care Committee and conformed to the guidelines set forth by the Animal Care Council of Canada and Canadian Institutes of Health Research.

3.3.2. ATF4 knockout Mice

ATF4 transgenic mice with C57BL/6J genetic background were generously provided by Dr. Tim Townes (Masuoka and Townes, 2002). Now, this transgenic mice is commercially available from The Jackson laboratory ($Atf4^{tm1Tow/J}$). The ATF4 knockout mice are infertile, therefore, all ATF4 Knockout mice were generated by breeding heterozygote ATF4 mutants and genotyped by PCR.

3.3.3. DDIT3 (CHOP) Null Mice

DDIT3 (CHOP) homozygous breeding pairs were obtained from The Jackson Laboratory ($Ddit3^{tm2.1Dron}$/J). These transgenic mice were originally on mixed C57BL/6J/129S background, but since they have been backcrossed to C57BL/6J more than 10 times, genetically they were considered as pure C57Bl/6J background mice. The phenotype of the DDIT3 (CHOP) deficient mice used in this study was similar to the C57Bl/6J wild-type mice. DDIT3 (CHOP) null mice are fertile. Therefore, breeding knockout DDIT3 (CHOP) transgenic mice was performed to produce knockout CHOP. Primarily, PCR was performed to confirm the genotype.

3.3.4. Primers' Sequences and PCR Conditions

The following primers were used for ATF4 genotyping: primers for the wild type ATF4 alleles,ATATTGCTGAAGAGCTTGGCGGC(forward),GTTTCTACAGCTTCCTCCACT CTT (common-reverse). To detect the targeted allele, the following antisense (reverse) primer, ATATTGCTGAAGAGCTTGGCGGC (Neo forward), was used. ATF4 null and heterozygote mice were genotyped by PCR according to the following conditions: 95^{o}C, 2min (1 cycle); 94^{o}C, 20 sec; 62^{o}C, 30 sec; 68^{o}C, 1:30 min (30 cycles); 70^{o}C, 5min. To genotype DDIT3 (CHOP) transgenic mice, the following primers' sequences were obtained from the Jackson laboratory: ATGCCCTTACCTATCGTG (common), AACGCCAGGGTTTTCCCAGTCA (mutant reverse), GCAGGGTCAAGAGT GTG (wild type reverse). Genotype conditions were followed according to the protocol provided by the Jackson Laboratory.

3.3.5. Primary Neuronal Cell Culture

Primary cerebellar granule neuronal culture (CGN) was prepared as previously described (Cregan et al., 1999, Rashidian et al., 2009). CGN is a reliable and reproducible model to study the excitotoxiciy component of stroke. Briefly, cerebella of 7-9 days postnatal mice were dissected individually to prepare primary cultures of cerebellar granule neurons. Based on the designed experiments, cells were plated at a density of either $5x10^5$ or $3x10^4$ on poly-D-lysine (Sigma) coated four-well dishes and 96-well plate, respectively. In order to obtain pure neuronal culture and preventive of grown of proliferative cells such as glia, Cytosine-β-arabinoside (10 μm; Sigma) was added to the cells 18-24 hours after plating.

3.3.6. Hypoxia/Reoxygenation

A Humidified environmental chamber was used to induce hypoxia in neurons, as described previously (Rashidian et al., 2005) with some modifications. The hypoxia chamber was set at 37^oC, 1% O_2, and 5% CO_2. Briefly, two different neuronal death paradigms were triggered. For more delayed model of neuronal death, cells were incubated in the chamber on days 7-8 after plating, for 24 hours, in the presence of the NMDA blocker, MK-801 (10 μM, Research Biochemicals). The MK-801 is added to the culture right before incubation in the hypoxic chamber. Finally, cultures were allowed to re-oxygenated by incubating under normoxic conditions, at 37°C incubator for different time points depends upon the experiments. Control plates were kept in the incubator without exposure to hypoxia. For an excitotoxic death paradigm, cells were incubated in the hypoxia chamber without adding NMDA blocker, for 4.5-5 hours followed by 2 hours re-oxygenation.

3.3.7. Glutamate Excitotoxiciy Model of Neuronal Death

Glutamate toxicity model of death was performed as described previously with minor modifications (O'Hare et al., 2005). Briefly, CGNs were plated in 96-wells plates. After seven days of plating, cells were treated by glutamate at a final concentration of 50 μM and incubated at 37^{o}C for 60 minutes. Then glutamate was washed off with conditioned medium for two times. After final wash, 500 μl of 1:1 conditioned media to fresh media was added to each well. Cultures were incubated again in 37^{o}C for 2 hours. This experiment was performed in the presence or absence of NMDA blocker, MK-801 (10 μM).

3.3.8. Assessment of gene induction, using Semi-quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacture's protocol. A total of 50ng of RNA was used performing RT-PCR using Access quick RT-PCR system (Promega) or Superscript One-Step RT-PCR with Platinium Taq kit (Invitrogen). The primers were used at a final concentration of 0.5 μM. S12 was used as a loading control. The primers used for the semi-quantitative RT-PCR included: CTGCCTTTCACCTTGGAGAC (sense), CTGCTCCTTCTCCTTCATGC (antisense) DDIT3; GCACTTAGAGTCGCCCG (Sense); CCTCGATGACATCCTTGG (sense), GGAAGGCATAGCTGCTGG (antisense) S12. The cDNA synthesis was performed at 42^{o} C for 45 min, followed by 94^{o} C for 2 min. This was followed by 30 cycles (DDIT3), or 25 cycles (S12) at 94^{o} C for 1 min, 55-64^{o}C for 30 sec, and 72^{o}C for 1 min. The final product was observed using 2% agarose-ethidium bromide gel. Correct band for ATF4, and CHOP were processed by densitometry using ImageJ software. Transcripts of genes

of interest were normalized against S12 as a loading control. Data were reported as fold increase in mRNA levels in treated samples comparing to corresponding untreated control samples. (n=3)

3.3.9. SDS-PAGE and Western Blot analysis

Total protein was extracted from cells or brain tissues, as described previously (Smith et al., 2003, Gonzalez et al., 2008). Approximately 30-50 µg protein was separated using 12% SDS-polyacrylamide gel and transferred to 0.45 µm nitrocellulose membranes. Blots were probed with primary antibodies to CHOP (Mouse monoclonal, abcam), (1:500) overnight at 4^{o}C. Membranes were probed with secondary antibodies at 1:10,000 after washing 3 times with 1X PBS-T. Immunoreactivity was detected using ECL (*Thermo Scientific* Pierce). ß-Actin primary antibody (1:3000) was used as a loading control for 15 min at room temperature, and protein levels were normalized against β-actin signals. Data are presented as mean ± SEM.

3.3.10. Transient Middle Cerebral Artery Occlusion (tMCAO)

As a focal ischemia model, the middle cerebral artery occlusion (MCAO) procedure was performed. The goal of this procedure was to produce a focal cerebral ischemia by blocking a middle cerebral artery using intraluminal thread. In this experiment, the 9-12 weeks old male mice, weighing 20-25 g, were selected from ATF4 and CHOP mice colonies. The knockout mice were compared with their wild type littermates. This technique was followed as described previously, with some modifications (Clark et al., 1997). Briefly, a midline neck incision was made along the neck in deeply anaesthetize mouse. The soft tissues were pulled apart gently in order to make the common carotid

artery visible. Then, the left common carotid artery was dissected free from the surrounding nerves carefully. As a next step, a ligature was tied around the common and left external carotid arteries. A small whole was made in the common carotid artery. Subsequently, arteries were clamped to prevent bleeding. As a final step, an occluding silicon-coated monofilament (around 11 mm length, 10 mm width silicon coated) was introduced via the common carotid artery into the internal carotid artery toward the circle of Willis (junction of the anterior and middle cerebral arteries), in order to block the origin of the middle cerebral artery. During surgery, the body temperature of the animals was maintained at 36°-37°C using a heating plate. To induce transient ischemia, the occlusion continued for 45 minutes, and finally the monofilament was removed to restore blood flow. In the control (Sham) mice, all procedures were performed except that the monofilament was not inserted into the MCA.

3.3.11. Histology, Perfusion and Fixation

At the designated times following MCAO (three days), mice were anesthetized and perfused with ice-cold 0.9% saline solution (~ 10 ml) to remove the blood from the animal, followed by fixation with cold 4% paraformaldehyde (PFA) (PH 7.4). The perfused brain were removed and postfixed with the same fixative (PFA) overnight at 4°C. The next day, PFA was removed and fixed brains were cryoprotected in sufficient volume of 10% buffered-sucrose/ 0.2% sodium azide. The sucrose solution was changed twice per day for five consecutive days. Coronal sections at the thickness of 14 μm were cut and placed on slides, at the level of cortex and striatum, with cryostat microtome. The section slides were stored in -80° C for further analysis. Brain sections were stained with 0.1% aqueous cresyl violet solution for more accurate analysis of infarct volume and apoptotic cells. The

penumbra region of the ipsilateral cortex and striatum was compared to the contralateral non-lesion area of each brain section.

3.3.12. Cell survival analysis

Cells were lysed using cell lysis buffer as described previously (Gonzalez et al., 2008). Percentage survival was calculated as the number of live nuclei in treated cells compared with untreated control. Data are presented as mean SEM of three independent experiments.

3.4. Results:

3.4.1. ATF4 is necessary for delayed neuronal death-induced by hypoxic insult, *in vitro*

Growing evidence suggests that ER stress may participate in post-ischemic neuronal death (Morimoto et al., 2007, Oida et al., 2008). In our previous study we have shown that following ER stress, a transcriptional response occurs through activation of transcription factor 4 (ATF4) in neurons (Galehdar et al., 2010). Selective translation of ATF4 resulted in neuronal death. To test the functional importance of ATF4, as an apoptotic member of the unfolded protein response pathway in delayed models of ischemic death, we used an *in vitro* model of ischemia where neuronal loss occurs in the presence of the NMDA blocker MK-801 (**Fig. 3.1)**. Glutamate is the chief excitatory neurotransmitter in the human and mammalian brain (Choi, 1988). Glutamate receptor blockers such as MK-801, an experimental NMDA antagonist, have demonstrated the ability to reduce or eliminate brain damage from acute conditions such as stroke, ischemia/hypoxia (Lipton and Rosenberg, 1994). Therefore, mouse Cerebral Granule Neuronal cultures (CGNs) were subjected to hypoxia, in presence of MK-801, seven days after plating. Following 24 hours hypoxia,

cultures were transferred to normal oxygen condition at 37°C for 24 hours reoxygenation. Afterward, cells were lysed and cell survival assessment was performed. Cells with condensed or fragmented nuclei were considered as dead cells and excluded from counting. Cells with intact nuclei were counted as healthy cells. As shown in **Figure 3.1**, ATF4-deficient neurons were found to be significantly more resistant than wild-type neurons to cell death induced by hypoxia-reoxygenation. For example, whereas only ~44% of $ATF4^{+/+}$ neurons remained alive 24 hours after exposure to hypoxia- reoxygenation, ~92% of ATF4-deficient neurons survived under the same conditions. Importantly, in this initial experiment, there was more death in wild type neurons exposed to hypoxic insults alone compared to non-treated littermates cells, without oxygenation (~63% survival). Hypoxia alone did not affect the survival levels in ATF4-deficient neurons. These data indicate that ATF4 contributes to death of neurons in delayed model of ischemia, and the absence of ATF4 in neurons confers significant resistance to this death paradigm.

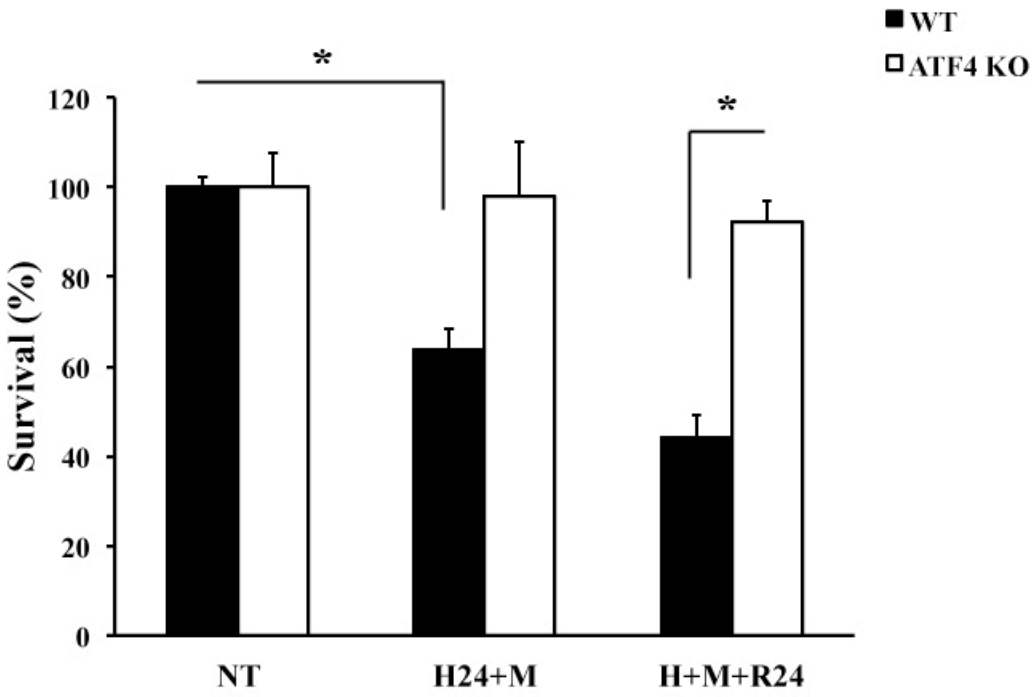

Figure 3.1.

Figure 3.1. ATF4-deficient neurons are resistance to delayed ischemic models of death induced by nonexcitotoxic hypoxic insults. CGNs obtained from ATF4 knockout pups and their wild type littermate controls were subjected to 24 hours hypoxia followed by 24 hours reoxygenation. Survival was examined by nuclei integrity. Quantitation of survival was shown in the presence of NMDA receptor antagonist, MK-801. Results are expressed as percentage (%) of control (n = 3). The data are mean ± SEM. * denotes significance ($p< 0.05$, t test). (NT: non-treated cells; H+M: Hypoxia+MK-801; H+M+R24: Hypoxia+MK-801+24 hours Reoxygenation).

3.4.2. Rapid excitotoxic neuronal death does not depend on ATF4 pro-death function

It has been suggested that ER stress may be involved in more rapid excitotoxic death (Sokka et al., 2007). Therefore, we next determined whether ATF4 participated in this type of cell death paradigm. To test this, we examined whether ATF4 deficiency was protective in models of excitotoxic hypoxia where death is induced in the absence of NMDA blocker, MK-801 **(Fig. 3.2A)**. As shown in **Figure 3.2A**, in contrast to the delayed model of ischemic death, ATF4 appeared not to play a functional role in the rapid hypoxic cell death model. The lack of functional effects of ATF4 was also confirmed using direct glutamate exposure as an alternative model of rapid cell death **(Fig. 3.2B)**. As shown in **Figure 3.3B**, similar results to excitotoxic hypoxia were obtained by glutamate toxicity. Taken together, these data suggest that ATF4 plays a significant role only in delayed modes of death.

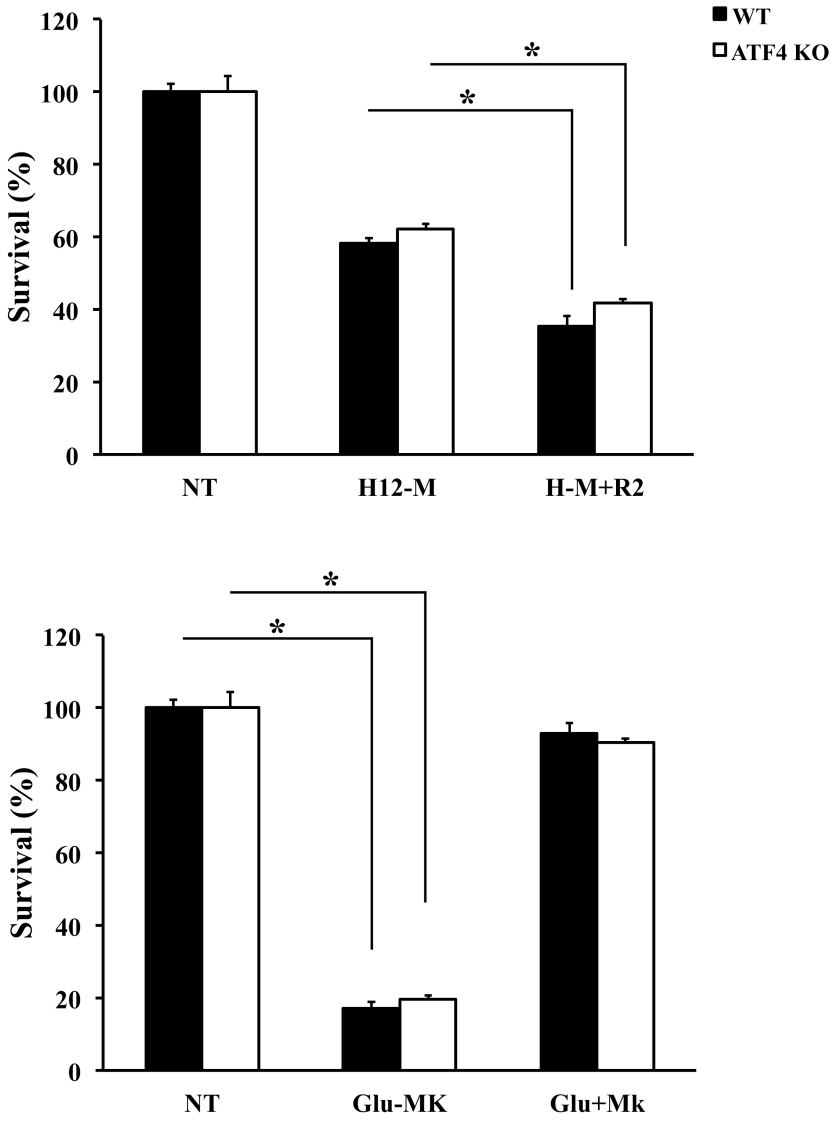
WT
ATF4 KO
Survival (%)
120
100
80
60
40
20
0
*
*
NT
H12-M
H-M+R2
Survival (%)
120
100
80
60
40
20
0
*
*
NT
Glu-MK
Glu+Mk

Figure 3.2.

Figure 3.2. ATF4-deficient cerebral granule neurons (CGNs) are not resistant to excitotoxiciy. CGNs were cultured from ATF4-deficient mice and wild type littermates. **(A)** Cells were subjected to 5 hours hypoxia followed by 2 hours reoxygenation, in the absence of MK-801. **(B)** Cells were subjected to glutamate (50 μM, for 60 minutes. After removing glutamate by final wash, cultures were incubated for 2 hours with 1:1 conditioned media to fresh media. Cells were lysed and intact healthy nuclei were counted. Survival was expressed as the percentage relative to untreated cells for each genotype. Excitotoxic insults have the same effect on both genotypes neurons. ATF4 KOs neurons did not display any difference in survival compared with wild type littermates. Each error bar represents the mean ± SEM. (n=3). *denotes significance ($p< 0.05$, t test) (NT: Nontreated; H: Hypoxia; M: MK-801; R: Reoxygenation; H-M: Hypoxia without MK-801; H-M+R2: Hypoxia without MK-801 followed by 2 hours Reoxygenation; Glu: Glutamate).

3.4.3. CHOP is induced in response to hypoxic insults

Our previous studies indicate that CHOP is upregulated after ER stress treatment in neurons. Indeed we showed that ATF4 specifically mediated the induction of CHOP under ER stress conditions. Given the importance of CHOP in ER-mediated responses in neuronal death, we hypothesized that CHOP may be induced following a delayed model of ischemic neuronal death, and its expression may depend upon the ATF4. To test this, we determined whether the CHOP message was upregulated following non-excitotoxic hypoxic insults in wild type neurons. In addition, we examined whether CHOP upregulation was attenuated in the neurons of ATF4-deficient littermates. CGNs were exposed to 24 hours hypoxia in the presence of MK-801, followed by 3, 6 or 12 hours reoxygenation. As shown in **Figure 3.3**, induction of CHOP message was detectable as early as 3 hours after reoxygenation and reached a maximum (~ 4.5 fold) induction at 6 hours. Although the CHOP mRNA level decreased at 12 hours reoxygenation, it remained high in comparison to non-treated cells. ATF4-deficient neurons did not show any increase in CHOP mRNA, indicating that induction of CHOP message depends upon the presence of ATF4. Consistent with an increase in message, the CHOP protein was also induced in ATF4 wild type with an increase detectable at 6 hours (p value, 0.036). Protein levels were still induced 12 hours after ischemia (**Fig. 3.4**).

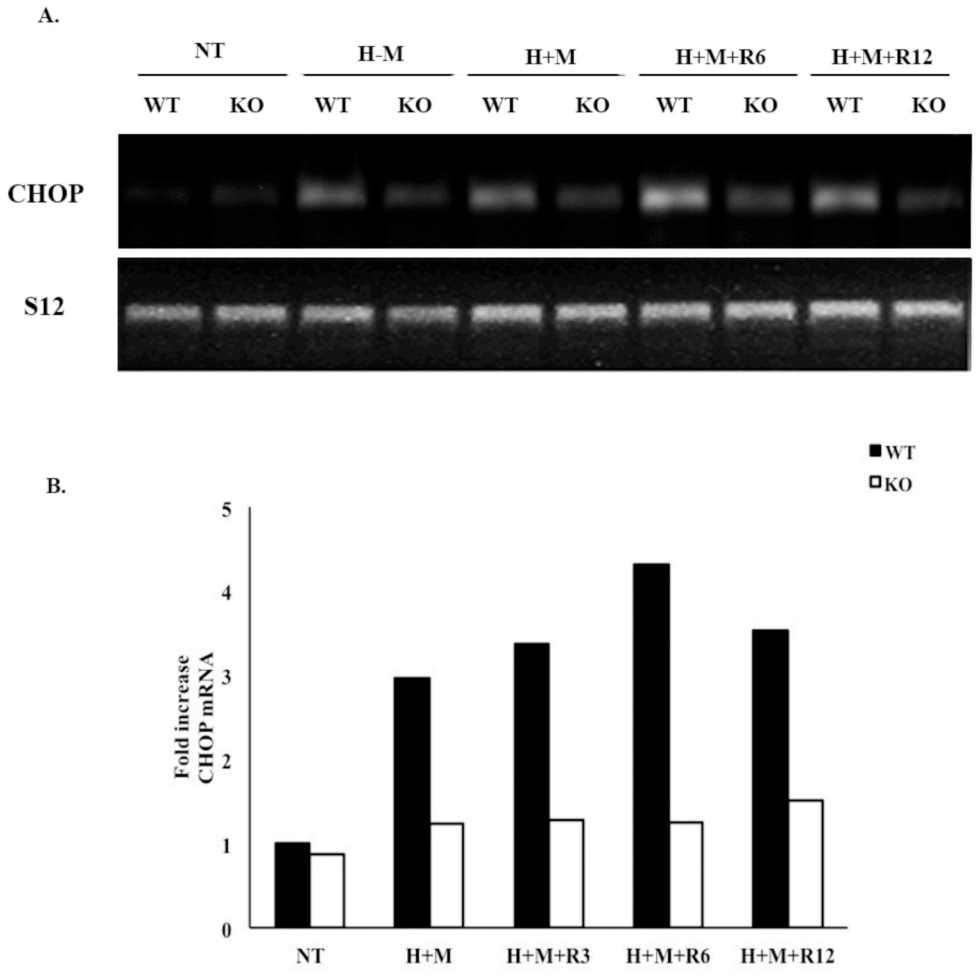
A.
NT
H-M
H+M
H+M+R6
H+M+R12
WT
KO
WT
KO
WT
KO
WT
KO
WT
KO
CHOP
S12
B.
WT
KO
Fold increase CHOP mRNA
0
1
2
3
4
5
NT
H+M
H+M+R3
H+M+R6
H+M+R12

Figure 3.3.

Figure 3.3. CHOP mRNA levels are upregulated after hypoxia-reoxygenation in the presence of ATF4. Total mRNA was extracted from CGNs exposed to hypoxia for 24 hours followed by exposure to periods of reoxygenation (3,6,12 hours). **(A)** Semi-quantitative RT-PCR was performed to examine the level of CHOP message. **(B)** Densitometric analysis of all signals was performed, and CHOP signals were normalized to S12 as a loading control. Data are presented as fold increase relative to nontreated sample for wild type genotype of each separate experiment. This figure represents the average of two independent experiments.

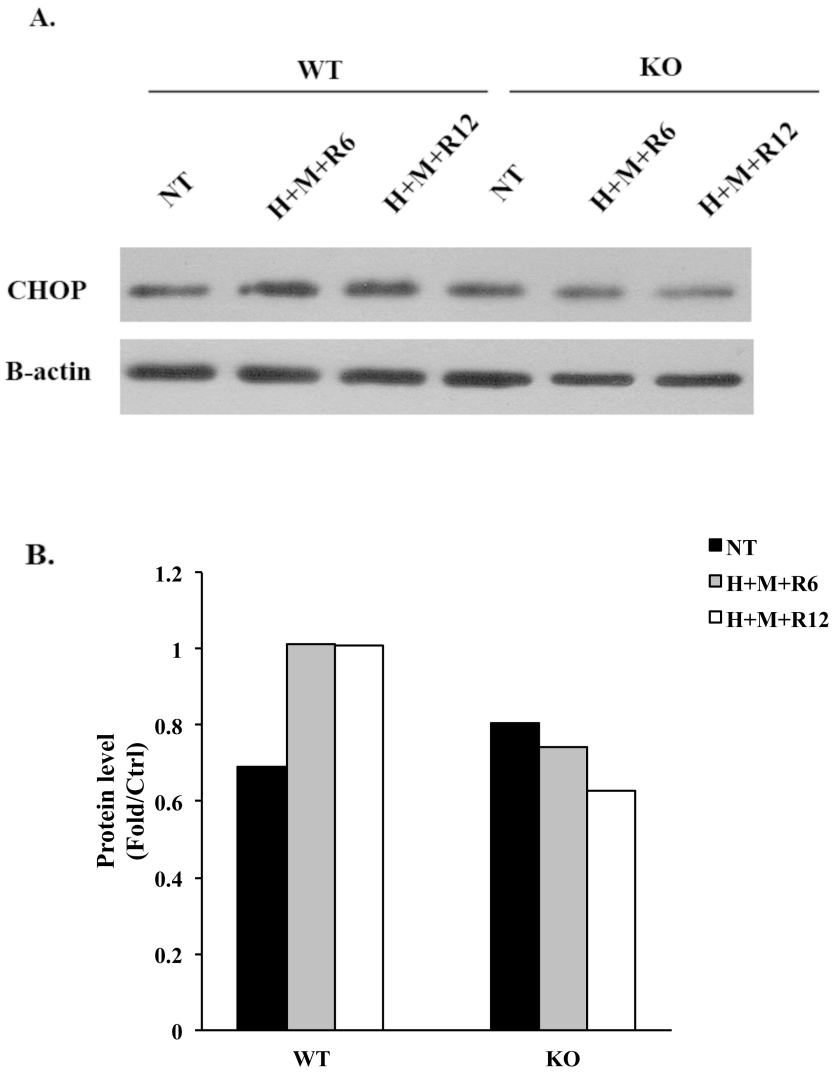
A.
WT
KO
NT
H+M+R6
H+M+R12
NT
H+M+R6
H+M+R12
CHOP
B-actin
B.
NT
H+M+R6
H+M+R12
Protein level
(Fold/Ctrl)
1.2
1
0.8
0.6
0.4
0.2
0
WT
KO

Figure 3.4.

Figure 3.4. ATF4 regulates CHOP protein induction following hypoxia-reoxygenation. CGNs were exposed to 24 hours hypoxia followed by 6 and 12 hours reoxygenation. Total protein was extracted and purified from cells and protein levels were analyzed by Western Blotting **(A)**. Densitometry of all signals was performed and CHOP signal was normalized to β-actin as the loading control **(B)**. Data are presented as fold increase relative to nontreated control cells. This figure represents the result obtained from two independent experiments. (NT: nontreated; H+M+R6: Hypoxia+MK-801+6hours reoxygenation; H+M+R12: Hypoxia+MK-801+12 hours reoxygenation; Ctrl: Control)

3.4.4. CHOP deficiency does not affect cell death in delayed model of ischemic neuronal death

To address the role of endogenous CHOP in ischemic damage, as a next step, we assessed the level of neuronal survival in CHOP deficient CGNs and their wild type littermates. Percentage survival was calculated as the number of remaining live nuclei in treated wells compared with untreated cultures. Surprisingly, CHOP deficiency was found not to affect the level of death in delayed model of ischemia. As shown in **Figure 3.5**, CHOP-deficient neurons exhibited almost the same amount of death compared to wild type littermates' neurons (36% vs. 39% survival). Interestingly, hypoxia without reoxygenation also induced neuronal death in this experiment. However, consistent with hypoxia-reoxygenation, there was no difference between KO neurons and wild type. This result suggests that CHOP does not promote neuronal apoptosis at least during this delayed model of ischemic death.

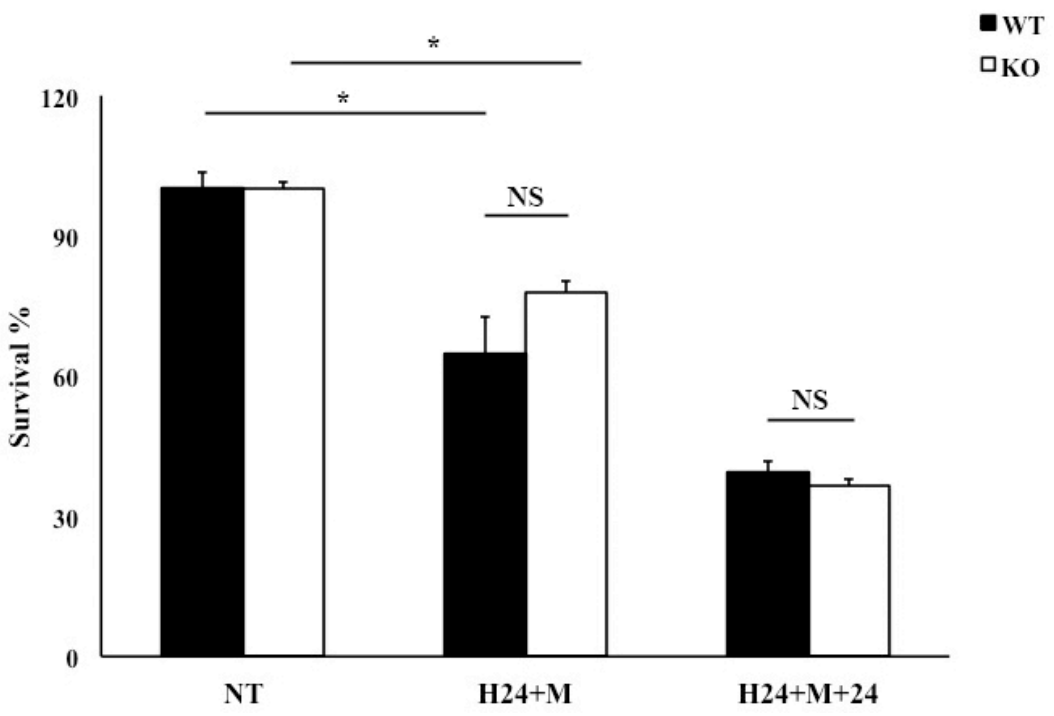

Figure 3.5.

Figure 3.5. CHOP deficiency dose not protect neurons against death-induced by hypoxia-reoxygenation. CGNs obtained from CHOP null pups and their wild type littermates were exposed to hypoxia or 24 hours, in the presence of MK-801. Reoxygenation was performed after hypoxia for 24 hours. Cells were lysed and intact nuclei were counted as healthy cells. Percentage survival was calculated in comparison to non-treated cultures. Error bars represent the mean ± SEM. (n=3). * denotes significance ($p< 0.05$, t test). (NT: Untreated; H: Hypoxia; M: MK-801; NS: Not significant).

3.4.5. ATF4 as mediator of delayed death in MCAO, a focal model of ischemic injury

We next asked whether ATF4 played a role in ischemic delayed death in adult models of injury. This question is significant, because it is possible that ATF4 signaling in glia or other non-neuronal constituents may have affect neuronal survival, which would not be seen in the dissociated cultures. To examine this question, we used a 45-min transient Middle Cerebral Artery Occlusion (tMCAO) model of delayed ischemia in which neuronal death of the striatum and cortex is observed. Male ATF4 null mice and their wild type littermates were subjected to surgery. Depending on the duration of blood flow restriction, different motor and behavioral deficits result. To assess the success of operation, we measured the level of blood flow in the middle cerebral artery region after occlusion and reperfusion compared to blood flow before the surgery (data not shown). In addition, for further confirmation, we checked the animals for neurological deficits after blood restoration. In most cases animals showed decreased resistance to lateral push and circling movement.

Several previous studies have revealed that apoptosis induced by transient MCAO model reached its peak at 24–48 h of stroke. However, an increase in infarct volumes can continue even as late as 3 days after reperfusion [reviewed in (Liu and McCullough, 2011)]. Therefore, brains were collected 3 days after injection, and coronal sections of the striatum and cortex were collected as described previously (Smith et al., 2003) and stained with cresyl violet for more acute infarct volume estimation. Regions with infarct were distinguished from non-damaged normal regions. Damaged regions displayed detectable shrunken with pyknotic nuclei in dying neurons, while healthy neurons showed round soma and clear intact nuclei (**Figure 3.6**). As is shown in **Fig 3.6**, a large area of injury is

appeared in the ipsilateral region (affected region by MCAO) of the wild type ATF4 brains. The brain of ATF4-deficient mice did not show any infarcts. Control mice of both genotypes (sham) did not show any significant damage. These results are consistent with our *in vitro* data. Our preliminary evidence suggests that ATF4 plays a critical role in delayed death *in vivo*. We will need more data to confirm these findings. Nevertheless, our results are consistent with previous studies (Lange et al., 2008). These studies indicate that ATF4 knockout brains were more resistant to ischemic injury (Lange et al., 2008).

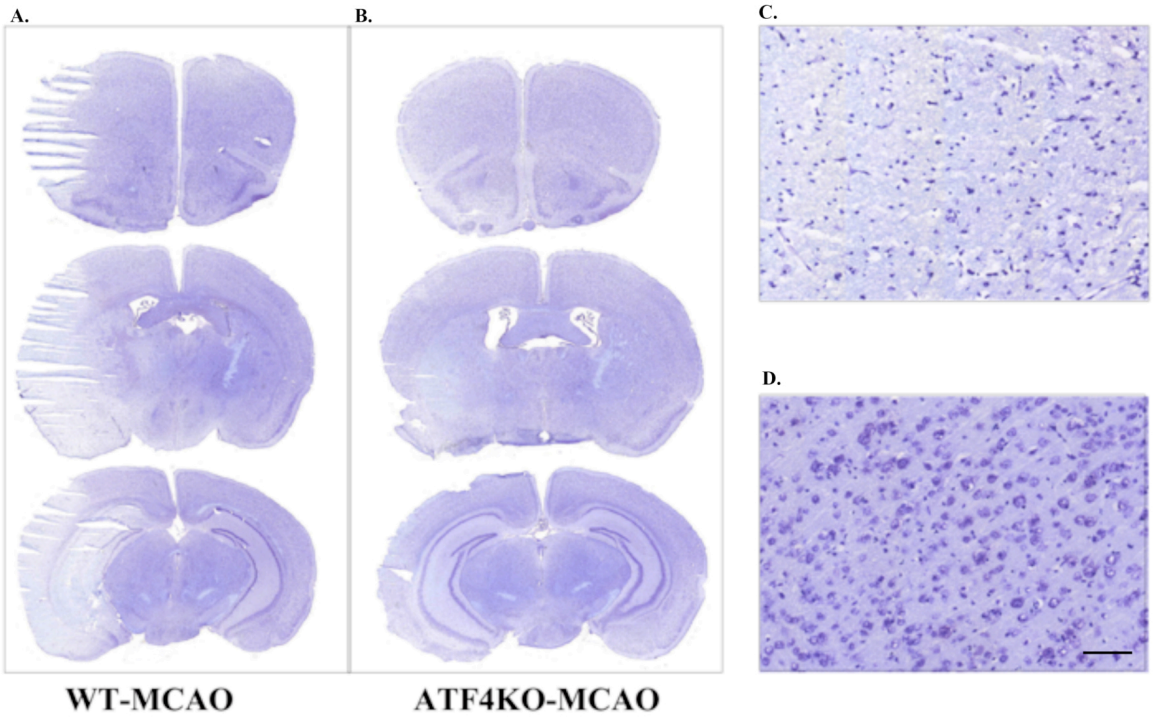

Figure 3.6.

Figure 3.6. ATF4 deficiency provides significant protection from 45 minutes tMCAO-induced delayed neuronal death in vivo. (A) Representative sections from the cortex region of wild type animals 3 days after surgery. **(B)** Representative sections from the cortex region of ATF4-deficient animals after surgery. Sections are stained by cresyl violet. **(C)** Shrunken compacted dead neurons. **(D)** Healthy neurons with round soma and intact nuclei. (n=9). Scale bar: 20 μm

3.5. Discussion

Although the pivotal role of ER stress in animal models of ischemia has been hypothesized, the mechanism of ER stress-induced apoptosis in ischemic injury is not fully defined. Therefore, it has become essential to identify the signaling pathways that regulate ER-stress-induced neuronal death following ischemia. In the present investigation, we used both *in vitro* and *in vivo* paradigms of ischemia/hypoxia. The goal of this study was to explore the role of the ER stress-related transcription factor ATF4 in ischemic neuronal death. Our results are significant because: (i) They provide clear evidence of the importance of pro-apoptotic role of ATF4 in ischemic neuronal death in vitro and in vivo (ii) They define the conditions under which ATF4 participate in stroke-induced death signaling. Because both rapid (excitotoxic) and delayed death appears in ischemic damage in brain, our results serve to resolve an outstanding question of whether ATF4 acts differentially in excitotoxiciy vs. more delayed death. Our results point to a model by which ATF4 regulates only a delayed model of ischemic death. The evidence for this finding can be summarized as follows: (1) in ischemic models of delayed death, using NMDA receptor antagonist in vitro, ATF4 deficiency significantly blocks death, whereas it is not protective on *in vitro* models of excitotoxic injury. These results are important because the main attempt to develop effective therapeutic strategies focuses on salvaging the ischemic penumbra, where the neuronal death happens in a more delayed form. (2) Our *in vitro* results are also consistent with our data *in vivo*. For example, in a focal model of stroke, ATF4 appears to play a significant apoptotic role. The large size of the infarct in the wild type mice brains vs. ATF4-deficient, following MCAO, confirmed this role.

How might ATF4 mediate a death signal in ischemic injury? We have shown that

transcription factor CHOP is regulated directly by ATF4 following ER stress to induce cell death. Our *in vitro* data show that ischemic insults elevate the CHOP message and protein in CGNs in the presence of ATF4. However, our preliminary results do not support the apoptotic role of CHOP, since there is no protection in the CHOP null neurons vs. wild type in response to delayed ischemic death. It is likely ATF4 does not act through CHOP pathway to induce cell death in these cellular paradigms. However, we cannot exclude the role of CHOP in this study since we need to perform further experiments including: 1) Increase the number of "n" for each *in vitro* experiments to confirm the results. 2) Performing tMCAO in CHOP KO mice and compare the infarct region with wild type brains. 3) Examining the protein expression of CHOP in ATF4 KO brains following tMCAO compare to wild type. 4) Rescue experiments with ATF4 and CHOP *in vitro* and *in vivo*.

Chapter 4.

General Discussion

Summary

Neurodegenerative diseases are one of the major health problems, striking primarily in mid to late life. As the population is aging, in particular in developed countries, the incidence of these diseases is expected to rapidly increase. Unfortunately, the pathogenesis of many of these diseases remains unknown. Presently, there has been much effort dedicated to develop clinical strategies to treat and prevent neurodegenerative conditions. One of the key achievements of these approaches is a profound understanding of pro-apoptotic signals in the nervous system.

Numerous studies have suggested that ER stress is involved in several human diseases, from cancer to chronic and acute neurodegenerative disorders (Moenner et al., 2007) [reviewed in (Kim et al., 2008)]. However, the exact causal effects and contributions of ER stress in neuronal disease processes are not clearly known. The endoplasmic reticulum (ER) is an important cellular organelle responsible in quality control of proteins for normal cell functions (Brodsky and Skach, 2011). Accumulation of misfolded proteins has been known as a common sign of many neurodegenerative diseases (Soto, 2003, Winklhofer et al., 2008). Accumulation of misfolded proteins disturbs function of the ER leading to ER stress.

ER stress activates adaptive signaling pathways known as the unfolded protein response (UPR) that primarily counteracts the effects of the cellular stress. This adaptive response acts through activation of the ER chaperones, enhancement of degradation of misfolded/unfolded proteins, and inhibition of global protein synthesis, in order to decrease the load of misfolded proteins within the ER (Ron and Walter, 2007). However, if the stress is severe or continues for a long time, function of the ER is severely impaired

resulting in cell death [reviewed in (Xu et al., 2005)]. The cellular signals involved in ER stress-mediated neuronal apoptosis are not yet fully understood.

Neuronal DNA damage also has been closely linked with neurodegenerative disorders such as Parkinson's diseases, and Alzheimer's diseases (Zhang et al., 1999, Coppede and Migliore, 2009). Although the signaling pathways mediating DNA damage-induced neuronal death are starting to be elucidated, the complexities of these signaling networks have yet to be fully defined.

The main goal of this dissertation was to identify molecular mechanisms that may mediate neuronal degeneration, triggered by DNA damage and ER stress. Specifically this work was directed to understanding the role of ATF4, a leucine zipper transcription factor in neuronal death. The results presented in this thesis support a number of conclusions: 1) ATF4 is required for neuronal cell death-induced by ER stress but not DNA damage. 2) The pro-apoptotic BH3-only protein PUMA is an ATF4 target in ER stress-induced neuronal death. 3) Neuronal death is regulated through p53-dependent pathway following DNA damage, and p53-independent pathway in response to ER stress. 4) ATF4 requires CHOP transcription factor to regulate pro-apoptotic function of PUMA in ER stress. The results associated to this part of our research project were published on the year 2010 (Galehdar et al., 2010, J. Neuroscience).

For the second part of project, an in vitro and in vivo model of ischemic cell death was used to explore the effects of ATF4 deficiency in ischemic neuronal death. The role of ATF4-CHOP pathway was examined in this model. This work has been reflected as part of the body of the second manuscript, which is under preparation. Therefore, this general discussion will be divided into two sections relevant to the results presented in chapters

two and three of this thesis. In this chapter (chapter 4) the brief description of major findings as well as the possible future directions is discussed. The latter will be based both on our published results presented here as well as on our preliminary results and observations.

The ER stress related ATF4-CHOP-PUMA pro-apoptotic pathway

Overview of major findings

In chapter two, critical insight was provided into signals that participate in DNA damage and ER stress-induced neuronal death. P53 involvement in the regulation of neuronal death-induced by DNA damage has been well studied (Morris et al., 2001, Sedarous et al., 2003, Martin et al., 2009). In addition the important role for the up-regulation of PUMA, a BH3-only Bcl-2 family protein, has been shown for p53-mediated cell death (reviewed in chapter 1:General introduction). On the other hand, it has been shown that ER stress is one of the central initiators of neuronal death. Despite the importance of p53 in DNA damage-neuronal death paradigms, question remains as to the role of this protein in ER stress-mediated death. Our results indicate that ATF4 but not p53 promotes neuronal apoptosis induced by ER stress. This indicates that the function of ATF4 depends upon the stress signal, as it does not have any role in regulation of death caused by DNA damage. Relevant to the apoptotic nature of the ATF4 signal, it was shown that exogenous expression of ATF4 promotes neuronal death, and that ATF4 deficiency significantly delays death after ER stress (Galehdar et al., 2010). Based on previous studies, expression of several BH3-only family members was examined following ER stress including Bim, Noxa, and PUMA. Our data indicate that PUMA has pro-apoptotic role in ER stress. Importantly, we found that ATF4 but not p53 promotes death-

induced by ER stress through regulation of PUMA. However, this regulation is not through direct activation of PUMA. Our study suggests that CHOP, a stress-inducible transcription factor functions as an ATF4-responsive intermediate target gene to regulate pro-apoptotic activity of PUMA. CHOP knockdown by siRNA technique diminishes Puma expression and neuronal apoptosis-induced by ER stress, suggesting that PUMA is a downstream target of CHOP. Specifically, we show that CHOP binds to the PUMA promoter directly during ER stress to regulate PUMA apoptotic function. In conclusion, our results define, for the first time ATF4-CHOP-PUMA, as a key-signaling pathway in ER stress-induced neuronal apoptosis.

Future directions

Although our results clearly demonstrate the important involvement of ATF4–CHOP-PUMA signaling in ER-stress-induced neuronal apoptosis, it is evident that inhibition of this pathway does not completely abrogate cell death. This suggests that additional factors contribute to Puma induction during ER-stress-induced apoptosis. Therefore, further studies are required to elucidate other potential apoptotic factors. For example, in addition to ATF4, ER stress activates other UPR signaling pathways such as Ire1α and ATF6 arms. It is possible that these pathways may also contribute to Puma induction and neuronal apoptosis. One of the approaches could be to explore the consequences of inhibition of these two genes, either by deficiency (KO neurons) or downregulation (RNAi approach), over CHOP inhibition. In addition, several transcription factors such as Foxa3a (forkhead box, class O, 3a) have been implicated in PUMA regulation (You et al., 2006). However, the relevance of them in ER stress has not yet been examined. Therefore, strategies to identify the possible link between this gene and ER stress in neurons may help to identify another upstream regulatory gene of PUMA.

The ATF4-CHOP apoptotic pathway in ischemic models of neuronal death

Overview of major findings

Chapter three of this thesis describes the effects of ATF4-CHOP pathway in ischemic neuronal damage. In first part of our project, we identified the ATF4-CHOP as a key regulatory pathway in neuronal death in an ER stress model. Several lines of evidence suggest that this pathway may play a role in brain injury. For instance, ATF4 and CHOP mRNA and protein expressions have been identified in ischemic region of mice brain following global ischemia, (Tajiri et al., 2004, Morimoto et al., 2007). However, the relevance of ATF4-CHOP pathway in neuronal death-induced by ischemic stroke is not fully defined. Currently, we investigate the possible contribution of ATF4-CHOP axis in neuronal ischemic injury following hypoxia-reoxygenation *in vitro* and middle cerebral artery occlusion (MCAO) *in vivo*. In this part, we show that ATF4-deficient neurons are resistant to more delayed ischemic model of death *in vitro*, whereas it does not play a significant role in rapid ischemic death including excitotoxic model of hypoxia and glutamate toxicity. Our results also indicate that ATF4 deficiency reduces expression of CHOP message and protein following nonexcitotoxic hypoxic insults. Surprisingly, our results show that CHOP deficiency does not affect neuronal death in delayed model of ischemic death in vitro. We also examine the effect of ATF4 deficiency *in vivo*, following 45 minutes transient MCAO (tMCAO). The brains of ATF4-deficient mice showed much smaller infarct compare to wild type littermates following MCAO suggesting that ATF4 is a mediator of neuronal death in stroke. Collectively, our preliminary results in this part of our study reinforce the concept that CHOP may serve a more complex role in neuronal death, depends upon the model of cellular stress. In addition, ATF4 may not function

through CHOP to regulate cell death following ischemic insults.

Future directions

In this part of our study, we attempt to understand the molecular events that control neuronal death following ischemia. Our data suggests a complex scenario. Although the ER stress-induced expression of ATF4-CHOP correlated with neuronal apoptosis, we were unable to confirm linking CHOP function with ischemic neuronal injury. However, more experiments need to confirm this study. How ATF4 regulates ischemic death is an important question for future studies. In this regard, recent studies showed that TRB3 (tribbles homolog 3) is an ER stress-inducible gene involved in cell death. Expression of this gene in hypoxic neuronal cultures has also been shown (Ohoka et al., 2005, Halterman et al., 2010). Whether ATF4 may act through TRB3 to regulate ischemic injury is an interesting hypothesis for future studies.

The work presented in this dissertation represent accurate and novel contributions to the current knowledge about the roles of ER stress in neuronal death. My main goal has been to be objective when interpreting data and I remained passionate about the potential outcomes of my projects. It is my intention to see this goal reflected in this thesis.

Appendix 1: REFRENCES

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