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Éric Chi-Ching Cheung
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Cellular and Molecular Medicine)
GRADE / DEGREE

Department of Cellular and Molecular Medicine
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The dual roles of apoptosis-inducing factor (AIF) in neuronal life and death

TITRE DE LA THÈSE / TITLE OF THESIS

Ruth Slack
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Éric Shoubridge

Stephen Lee

John Bell

Rashmi Kothary

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The dual roles of apoptosis-inducing factor (AIF) in neuronal life and death

Eric Chi Ching Cheung

This thesis is submitted as partial fulfillment of the requirements for the Degree of

Doctorate of Philosophy

Department of Cellular and Molecular Medicine

University of Ottawa

Ottawa, Ontario, Canada

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ISBN: 978-0-494-48393-0
Our file Notre référence
ISBN: 978-0-494-48393-0

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Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Ruth Slack. She helps me going through this invaluable PhD experience by her scientific guidance and advice, as well as her encouragement and her advice on how to become a successful independent researcher. Without her support, I would not be able to achieve all the accomplishments during my PhD study.

I would also like to sincerely thank Dr. Sean Cregan, who taught me how to design and execute elegant experiments. I have learned a lot from his fastidious attention to experimental procedures and his thoroughness in the analysis of experimental results. These have been invaluable to me during my PhD research, and will continue to be so in the future.

I would also like to thank the members of my thesis advisory committee: Drs. Martin Holcik, Stephen Lee, and Heidi McBride, for their comments and input for my project, and for providing numerous letters of recommendation on my behalf.

I am privileged to have the opportunity to work with some exceptional local and international scientists. I would like to thank the lab of Dr. Stephanie Bennett for their help in the *in vivo* excitotoxicity model, the lab of Dr. Heidi McBride for the mitochondrial studies, Drs. Gordon Shore and Nancy Steenaart at McGill University for the help in the design of the mitochondrially anchored AIF, Drs. Josef Penninger and Nicholas Joza in Vienna, Austria, for providing the AIF-flox mice before it was published, and for taking the time and effort in critically reading my second paper. Last but not least, to the lab of Dr. Luca Scorrano in Padua, Italy, for their help in the Opa1 project. Not only have I obtained from them the technical and conceptual assistance to my study, but more importantly I am

able to truly experience the power of collaborations across different scientific fields which make the research move forward faster.

I would like to thank the "life-side" of the lab: Dr. Jackie Vanderluit, Dr. Kerry Ferguson, Kelly McClellan, and Stephen McNamara for showing me the exciting research in developmental neurobiology, and for helping me in some of the *in vivo* studies in my thesis. Many thanks to Jason MacLaurin, Steve Callaghan, Carl McIntosh, Linda Jui and Eric Ouellet for their technical assistance. And also thanks to the "death-side" of the lab: Andre Fortin, Nicole Arbour, Nicole Legrand, Karine Pilon-Larose, and Dr. Melissa Kelly. Apart from the science, I have also shared many good times with all of you during conferences and numerous activities outside the lab. For all of these and more, I thank all of you. You guys have made this lab the most lively I have ever seen, and made the long hours and many late nights in the lab a bit less unbearable.

On a more personal note, I would like to thank my family for always believing in me. Without my family's support and guidance when I was younger, I would not be able to accomplish all of these.

Finally, I would like to acknowledge the funding agencies. The research presented in this thesis was supported by Canadian Institute of Health Research, the Canadian Stroke Network, Heart and Stroke Foundation of Canada, and the University of Ottawa.

Abstract

Development of drugs to rescue neuronal cell death during acute neuronal injury such as stroke is challenging because of the multiple neuronal death pathways. Apart from the caspase pathway, neurons can also die in the absence of caspases. One of the key factors in the caspase independent pathway is the mitochondrial protein Apoptosis-inducing factor (AIF) which is released and translocated to the nucleus during cell death. In this thesis, I have first demonstrated that AIF is a critical factor in both DNA damage induced Bax dependent neuronal cell death and Bax independent excitotoxic cell death using the *Hq* mice that have reduced AIF expression. These results are the first to directly show the role of AIF in acute neuronal injury using both *in vivo* and *in vitro* models.

Recent studies have also revealed an unexpected role of AIF in the mitochondria to maintain survival. It is therefore possible that after cell death induction, the release and subsequent loss of AIF from the mitochondria alone, but not its pro-apoptotic activity, is enough to kill the cell. To resolve this controversy, I have designed an AIF mutant construct that is mitochondrially anchored and cannot be released during cell death. The expression of this construct cannot protect cells from dying in the presence of the wildtype AIF that is able to translocate to the nucleus. This indicates that even when AIF is preserved in the mitochondria, the apoptotic activity of AIF can still kill the cell. This is the first definitive evidence that AIF is indeed pro-apoptotic during cell death, in addition to its survival role in healthy cells.

AIF has suggested to be a reactive oxygen species (ROS) scavenger or to stabilize mitochondrial complex I, however, the exact role is still unclear. Using the forebrain specific

AIF knockout animals, I have showed that rather than scavenging ROS, AIF may instead regulate mitochondrial structure and function through the mitochondrial fusion protein Opa1. Taken together, this thesis illustrates the mechanisms of AIF in neuronal cell life and death, which are essential for the design of AIF as a drug target for acute neuronal injury such as stroke.

List of abbreviations

aa: amino acid

Ab: Antibody

AC3: active caspase 3

Ad: adenovirus

Afg3L2: ATPase family gene 3-like 2

AIF: Apoptosis-Inducing Factor

AIF-exB: AIF exon B

AIFL: AIF long

AIFsh: AIF short

AIFsh2: AIF short 2

Akt: Ak transforming

ALS: Amyotrophic lateral sclerosis

AMID: AIF homologous mitochondrion-associated inducer of death

AMPA: alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid

ANOVA: analysis of variance

ANT: adenosine nucleotide translocator

Apaf-1: Apoptotic activating factor-1

Ara-C: cytosine arabinoside

ATP: adenosine tri-phosphate

ATPase: adenosine tri-phosphatase

a.u.: arbitrary unit

Bad: Bcl2 antagonist of cell death

BAF: Boc-aspartyl (Ome)-fluoromethylketone

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2 associated X

Bcl: B-cell lymphoma

Bcl-w: Bcl weak

Bcl-xL: Bcl-x long

BH1-4: Bcl-2 homology domain 1-4

Bid: BH3 interacting domain death agonist

Bim: Bcl-2-interacting mediator

Bmf: Bcl-2-modifying factor

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

calcein-AM: Calcein Acetoxymethyl Ester

CI/II/III/IV: complex I/II/III/IV

C-terminal: Carboxyl terminal

CA1/3: Cornu Ammonis 1/3

CAD/ICAD: caspase-activated DNase/inhibitor of caspase-activated DNase

Caf4: CCR4 (chemokine (C-C motif) receptor 4) Associated Factor 4

Campto: camptothecin

CCD: charge-coupled device

Ccp: cytochrome c peroxidase

C. elegans: *Caenorhabditis elegans*

CGNs: cerebellar granule neurons

CK: creatine kinase

CMT2a: Charcot-Marie-Tooth type 2A

CMX: chloromethyl-x-rosamine

CNS: central nervous system

Co-IP: coimmunoprecipitation

Con-A: concavalin A

Conc.: concentration

Cox-IV: cytochrome C oxidase subunit IV

Cpt: camptothecin

CP: cortical plate

Cps-6: CED-3 Protease Suppressor-6

Cre: cyclization recombination

CsA: cyclosporine A

CV: cresyl violet

Cyclothiazide: 6-chloro-3,4-dihydro-3-(5-norbornen-2-yl)-2H-1,2,4-benzothiazidiazine-7-sulfonamide-1,1-dioxide

Cyp-D: cyclophilin D

cyt c: cytochrome c

d: deoxy

Da: Dalton

DAP3: death associated protein 3

dATP: deoxy-adenosine triphosphate

DEVD: Aspartate-Glutamate-Valine-Aspartate

DFHR: dihydrofolate reductase

DKO: double knockout

DIV: days in vitro

dnDrp1: dominant negative Drp1

Δm_{Ψ}: mitochondrial membrane potential

dn: dominant negative

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

Dnm1: dynamin 1

Drp1: dynamin related protein 1

E: embryonic day

EDC: 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride

EDTA: ethylenediamine tetra-acetic acid

EGL-1: egg laying defective 1

EGTA: ethylene glycol-O-O'-bis-{2-amino-ethyl}-N,N,N',N',-tetraacetic acid

EM: electromicroscopy

Endo G: endonuclease G

ER: endoplasmic reticulum

ES: embryonic stem

ERK: extracellular regulated MAP kinase

ETC: electron transport chain

FAD: flavin adenine dinucleotide

FCCP: p-trifluoromethoxy carbonyl cyanide phenyl hydrazone

FEN-1: flap endonuclease-1

Flox: flanked locus of X-over P1

Foxg1: forkhead box G1

Fzo: fuzzy onion

g: gram

GDAP1: ganglioside-induced differentiation-associated protein-1

GFP: green fluorescence protein

Glut: glutamate

GTPase: guanosine triphosphatase

GYKI 52466: 4-(8-methyl-9H-1,3-dioxolo [4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride

h: hour

H_2O_2: hydrogen peroxide

HEK293: human embryonic kidney 293

hFis: human Fis (fission)

HIV Rev: human immunodeficiency Regulator of Virion

HK: hexokinase

Hq: Harlequin

Hrk/DP5: Harakiri/Death Protein 5

HtrA2: high temperature requirement protein A 2

Hsp: hereditary spastic paraplegia

Hrs: hours

i-AAA protease: inner membrane AAA protease

IAP: inhibitor of apoptosis protein

IM: inner membrane

IMS: intermembrane space

InsP3R: inositol (1,4,5) triphosphate receptor

IR: gamma irradiation

IZ: intermediate zone

K: kilo

KA: kainic acid, (2S,3S,4R)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid

L: litre

M: molar

m*: milli

*m: meter

μ: micro

m-AAA protease: matrix AAA protease

MCAO: middle cerebral artery occlusion

Mcl-1: myeloid cell leukemia sequence 1

Mdv1: mitochondrial division 1

MEF: mouse embryonic fibroblast

Mfn: mitofusin

Mgm1: mitochondrial genome maintenance 1

MGPY: methyl green pyronine Y

min: minute

MitoSox: mitochondrial superoxide indicator

MK-801: (5S,10R)-(1-5-methyl-[3,7]-10,11,-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine

MLS: mitochondrial localization signal MPT: mitochondrial permeability transition

MMP: mitochondrial

MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

MnSOD: manganese superoxide dismutase

MOI: multiplicity of infection

MOMP: mitochondrial outer membrane permeabilization

MPP^+: 1-methyl-4-phenylpyridinium

MPT: mitochondrial permeability transition

mRNA: messenger RNA

mt: mitochondrial

mtHsp70: mitochondrial heat shock protein 70

MTP18: Mitochondrial protein 18 kDa

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Mule: Mcl-1 ubiquitin ligase E3

mut: mutated

n: nano

N-terminal: amino terminal

NAC: N acetyl cysteine

NAD(P): Nicotinamide adenine dinucleotide (phosphate)

NDUFA8: NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8

NES: nuclear export signal

NLS: nuclear localization signal

NMDA: N-methyl-D-aspartic acid.

nNOS: nitric oxide synthase

NO: nitric oxide

OGD: oxygen glucose deprivation

OM: outer membrane

Opa1: optical atrophy 1

Oxphos: oxidative phosphorylation

P: postnatal

p53: protein 53

p66(shc): 66 kilodalton isoform of the growth factor adapter Shc

PAR: poly (ADP)-ribose

PARG: poly(ADP)-ribose glycohydroase

PARL: Presenilin-associated rhomboid-like

PARP: poly(ADP)-ribose polymerase

PBR: benzodiazepine receptor

PBS: phosphate buffered saline

Pcp1p: pyrrolidone-carboxylate peptidase

PEST: proline-glutamic acid-serine-threonine

Pfu: plaque forming units

PH3: phospho-histone H3

PI: propidium iodide

PCR: polymerase chain reaction

PNS: peripheral nervous system

PPII: poly-proline-2

Prg3: p53 regulated gene 3

PU: pyruvate uridine

Puma: p53-upregulated modulator of apoptosis

Q/R: glutamine/arginine

Ras: rats sarcoma

Rbd1p: rhomboid 1 protein

RNA: ribonucleic acid

RNAi: RNA interference

RNS: reactive nitrogen species

ROS: reactive oxygen species

S: second

S.D.: standard deviation

SH3: Src homology 3

Shc: Src homology 2 domain containing

siRNA: small interference ribonucleic acid

Smac/DIABLO: second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point

SVZ: subventricular zone

TBI: traumatic brain injury

tBid: truncated Bid

TEM: transmission electron microscopy

TIM: Translocases in the inner membrane

TMRE: tetramethylrhodamine ethyl

tPA: tissue plasminogen activator

TPR: tetraticopeptide repeat

ts: temperature sensitive

TUNEL: terminal deoxynucleotide transferase biotin-dUTP nick end labeling

UV: ultra violet

V: volt

VDAC: voltage dependent anion channel

WAH: worm AIF homolog

WW: tryptophan-tryptophan

Yme1L: Yeast mitochondrial escape 1 like

zVAD-fmk: zVal-Ala-Asp(OMe)-fluoromethylketone

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ο θάνατος είναι κάτι που δεν πρέπει να φοβόμαστε, γιατί όταν ζούμε δεν υπάρχει και όταν υπάρχει δε ζούμε.
Επίκουρος, ἐπιστολὴ πρὸς Μενοικέα

Death is nothing to us, since when we are, death has not come; and when death has come, we are not.
Epicurus, Letter to Menoeceus

曰：敢問死。
曰：未知生，焉知死。
論語 先進第十一

"May I ask about death?"
"You do not yet understand life, how can you understand death?"
Confucius, The Analects Chapter 11

Chapter I: Introduction

Stroke is the fourth leading cause of death in Canada, causing around 16,000 deaths each year. A stroke results from a sudden loss of blood supply to the brain, either due to an interruption of blood flow from a blot clot (ischemic stroke) or the rupture of the blood vessel (hemorrhagic stroke). During stroke, blood supply to the brain is severely limited and the availability of glucose and O_2 drops dramatically which leads to a reduction of energy production (ATP) in neurons. This causes the activation of various pathways that lead to cell death (Figure 1-1) (Lipton, 1999). Since neurons require ATP to maintain the membrane potential, a decrease in ATP level causes neuronal depolarization and synaptic release of glutamate. The resulting build-up of glutamate leads to overstimulation of ionotrophic glutamate receptors and the influx of Ca^{2+} into the neurons. Mitochondria accumulate much of this excessive Ca^{2+}, and pathologically high concentration of Ca^{2+} induces mitochondrial dysfunction and mitochondrial permeability transition (MPT). Ca^{2+} also activates neuronal nitric oxide synthase (nNOS), which produces nitric oxide (NO) that undergoes further reactions to produce reactive oxygen and nitrogen species (ROS/RNS) (Arundine & Tymianski, 2003; Arundine & Tymianski, 2004; Michaelis, 1998; Sattler & Tymianski, 2001). Loss of mitochondrial activity and the presence of ROS/RNS lead to DNA damage and subsequent activation of the pro-apoptotic Bcl-2 proteins at the mitochondria. Pro-apoptotic Bcl-2 proteins and high concentration of Ca^{2+} induce the release of pro-apoptotic proteins in the mitochondrial intermembrane space that activate different cell death pathways (Green & Kroemer, 2004; Kroemer & Martin, 2005; Kroemer & Reed, 2000). Even if the blood flow is restored after the ischemic insult (reperfusion), the surviving neurons under hypoxic and excitotoxic stress can still go on to die because of the sudden influx of O_2 and

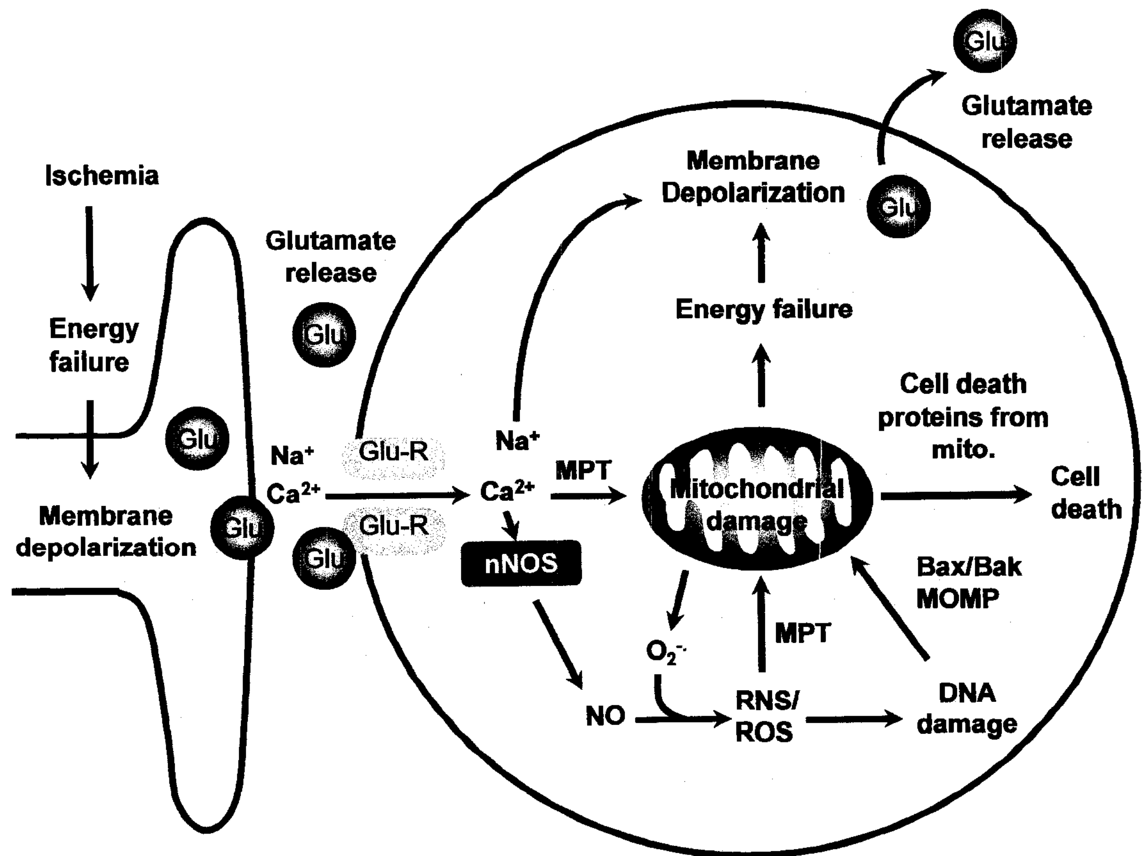
glucose, which leads to a burst of secondary ROS production from the mitochondria and subsequent DNA damage and induction of cell death (Crack & Taylor, 2005; Lipton, 1999). Mitochondria, therefore, are the key cell death players in ischemic injury, not only for ATP production, but also as one of the major producers of ROS. They also initiate the cell death machinery by releasing its pro-apoptotic proteins into the cytosol.

In this thesis, I will provide definitive proof that the mitochondrial intermembrane protein, apoptosis-inducing factor (AIF), is a proapototic factor for neuronal cell death. Apart from the pro-apoptotic role, this thesis is the first to describe a novel role of AIF in maintaining mitochondrial structure which is critical for proper mitochondrial function and cell survival. The next sections will serve as a background to understand the type of neuronal injuries after stroke, the consequences of these injuries to mitochondria, and the activation of AIF and other pathways that lead to cell death. A more detailed introduction of AIF will then be given. To understand the novel role of AIF in maintaining mitochondrial structure, the mitochondrial fission and fusion pathways will be also discussed.

1: Excitotoxicity

Excitotoxicity refers to the over-stimulation of the ionotropic glutamate receptors that occurs in acute neuronal injury such as ischemia and traumatic brain injury (Arundine & Tymianski, 2004; Nicholls & Budd, 1998). During ischemia, neurons fail to maintain the ionic gradient across the plasma membrane (i.e. depolarization) due to the energy depletion caused by the reduction of blood supply. Pathological increase of glutamate in the synaptic cleft is then induced by two mechanisms. Depolarization activates the presynaptic voltage dependent Ca^{2+} channels that release glutamate into the synaptic cleft. Due to the energy

Figure 1-1: Overview of the pathways involved in neuronal cell death during ischemia. After ischemia, the pathological activation of glutamate receptors (section 1) induces cell death through the activation of different cell death pathways including Ca^{2+} induced ROS/RNS production through the activation of nitric oxide synthase (nNOS) (section 1.3), mitochondrial permeability transition (MPT) (section 2), and the Bax/Bak mediated mitochondrial outer membrane permeabilization (MOMP) (section 3). Pro-apoptotic proteins such as cyt c and AIF are then released from the mitochondria and subsequently lead to cell death (section 4 and 5).



Ischemia
Energy failure
Membrane depolarization
Glutamate release
Glu
Na+
Ca2+
Glu-R
Glu-R
Na+
Ca2+
MPT
nNOS
Membrane Depolarization
Glutamate release
Energy failure
Cell death proteins from mito.
Mitochondrial damage
Cell death
Bax/Bak MOMP
MPT
O2-·
NO
RNS/ ROS
DNA damage

depletion and ionic gradient imbalance across the plasma membrane, the presynaptic reuptake of glutamate from the synaptic cleft is also impaired (Dirnagl et al, 1999). The combined effect of these two mechanisms causes a pathological increase of glutamate in the synaptic cleft, stimulating the ionotrophic glutamate receptors on the post-synaptic membrane. A high amount of Ca^{2+} thus enters the cytoplasm through these receptors and these ions engage different pathways that lead to cell death. The following section will focus on the roles of different types of ionotropic glutamate receptors during excitotoxicity, and the consequences of high amount of Ca^{2+} ions in the cells.

1.1: NMDA receptors

Compared to other types of glutamate receptors, NMDA receptors are more important than the non-NMDA receptors in excitotoxicity because of their higher affinity to glutamate, higher permeability to Ca^{2+}, and slower desensitization after binding to glutamate. In resting membrane potential, Ca^{2+} influx through NMDA receptors are blocked by Mg^{2+} within the channel. When the plasma membrane becomes depolarized, this Mg^{2+} blockade is relieved. This permits the influx of Ca^{2+} and to a lesser extent Na^{+} ions into the cytoplasm when glutamate binds to the receptors. The co-agonist glycine is also required for full activation (Dirnagl et al, 1999; Michaelis, 1998). Since NMDA receptors have a central role in excitotoxicity and other neurological disorders, multiple types of pharmacological antagonists have been found. Unfortunately, none of these inhibitors have suceeded in clincial trials, because the pro-survival role of the receptors are often overlooked (Ikonomidou & Turski, 2002; Kemp & McKernan, 2002). In addition, the differences

between synapatic versus extrasynaptic NMDA receptors are not taken into account (Hardingham & Bading, 2003). Indeed, a complete blockade of NMDA receptors irrespective to their subtypes and function cannot rescue neurons, and may even exacerbate cell death during acute injury (Dirnagl et al, 1999, Michaelis 1998). To solve this problem, the pro-survival role of the NMDA receptors must be separated from its cell death activity, which is highly dependent on Ca^{2+} entry through this receptor. Once the Ca^{2+} ions enter the cell, they trigger a cell death factor in close proximity to the NMDA receptors. Neuronal nitric oxide synthase (nNOS) is one of the factors, and it can be induced by Ca^{2+} influx following NMDA receptor activation (Arundine & Tymianski, 2003; Dawson et al, 1991; Zhang et al, 1994). nNOS is tethered to NMDA receptors through PSD95 which brings it in close proximity to the Ca^{2+} influx (Aarts et al, 2002; Cao et al, 2005; Sattler et al, 1999). Once nNOS is activated, NO is produced, which can cause an increase in ROS/RNS (see section 1.3). The excessive activation of NMDA receptors following pathological glutamate release after acute injury activates nNOS, which is dependent on the influx of Ca^{2+} specifically through these receptors.

1.2: Non NMDA receptors: KA and AMPA receptors

While the roles of NMDA receptors are critical in the pathology of stroke, the non NMDA receptors such as KA (kainic acid) and AMPA receptors may also have a role in excitotoxicity. Compared to NMDA receptors, these receptors have a lower permeability to Ca^{2+} and become desensitized to glutamate in a shorter period of time. In the presence of drugs to suppress this rapid desensitization, the activation of either AMPA or KA receptors

can induce neuronal cell death *in vitro* and *in vivo,* due to membrane depolarization which removes the Mg^{2+} blockade of NMDA receptors (Jayakar & Dikshit, 2004; Liu & Zukin, 2007). AMPA receptors are unique because they can undergo RNA editing at the amino acid Q/R site of the GluR2 subunit that can dictate the permeability of the receptor to Ca^{2+} (Peng et al, 2006). How activation of AMPA receptors induce cell death is not yet clear. While NMDA receptors are linked to nNOS, the interacting partners of AMPA receptors are poorly understood. It is possible that AMPA receptors induce cell death in a different mechanism compared to NMDA and KA receptors, since the latter two receptors eventually lead to PARP and AIF activation for cell death (Chapter 3, (Cheung et al, 2005; Mandir et al, 2000; Yu et al, 2002)). Similar to NMDA receptors, AMPA receptors can also induce excitotoxicity but the mechanism of action may be quite different. The KA receptor subunits GluR5-6, similar to AMPA receptor subunit GluR2, also undergo RNA editing in which the Q form is permeable to Ca^{2+} ions but the R form is not. Unlike AMPA receptor GluR2, most of the GluR5 and 6 are not edited to the impermeable R form, which causes neurons that have a higher amount of KA receptors vulnerable to excitotoxicity than those that exclusively have AMPA receptors (Chittajallu et al, 1999; Michaelis, 1998). Although this increase of Ca^{2+} through KA receptors may not activate nNOS, an increase of Ca^{2+} ions can damage the mitochondria and activate other apoptotic proteases (see section 1.3). While the activation of all three types of glutamate receptors lead to cell death, the pathways invoked may be very different among these receptors. Chapter 2 will show the participation of AIF specifically in NMDA and KA induced excitotoxicity, while not involving AMPA.

1.3: Ca^{2+} and neuronal cell death

The key event during excitotoxicity is the influx of Ca^{2+}, which in turn recruits several pathways that cause neuronal cell death. In addition to the activation of nNOS (section 1.2), Ca^{2+} influx can recruit other pathways to induce cell death. In this section we will discuss the activation of different cell death pathways after Ca^{2+} influx into the cytoplasm that may also be responsible for neuronal cell death.

As the Ca^{2+} ion is an important second messenger in the cell, the Ca^{2+} ion concentration is tightly controlled through different mechanisms. ER and mitochondria are the two major sinks for excess Ca^{2+} ions. Ca^{2+} can also be extruded from the cell through Ca^{2+}-ATPase or Na^{+}-Ca^{2+} exchanger at the plasma membrane. Since most of these mechanisms rely on ATP, during ischemia when the energy supply is disrupted, the affected neurons lose the ability to control the level of Ca^{2+} ions (Lipton, 1999). Loss of Ca^{2+} control and the influx of Ca^{2+} ions through the ionotropic glutamate receptors create a pathologically high amount of Ca^{2+} ions in the cytoplasm which is taken up by the mitocondria or the ER. Excessive Ca^{2+} ions in the mitochondria interrupts the mitochondrial membrane potential and leads to the disruption of the flow of electrons in the electron transport chain. These leaked electrons react with oxygen to produce superoxide ($O_2^{\cdot-}$), which is one of the key components in the production of various reactive oxygen and nitrogen species (ROS/RNS) (Dirnagl et al, 1999; Sattler & Tymianski, 2000). For example, $O_2^{\cdot-}$ can react with NO (produced by nNOS) to produce a highly reactive peroxynitrate ($ONOO^{-}$) and other ROS/RNS such as $NO_2^{\cdot}$ and $OH^{\cdot}$. These ROS/RNS can also be produced by mitochondrial independent pathways such as the activation of xanthine synthase via calpain, and the metabolism of arachidonic acid (Mikkelsen & Wardman, 2003), both processes are Ca^{2+} dependent. The production of these ROS/RNS induces a vicious cycle of oxidative stress,

since these toxic compounds can induce mitochondrial damage (Giulivi et al, 1995), inactivation of ATP synthesis (Brookes et al, 1999; Heales et al, 1999) and inactivation of ROS scavengers such as MnSOD (Radi et al, 2002). These ROS and RNS componds induce widespread protein and DNA damage via nitration and oxidation (Abramov et al, 2007; Lau et al, 2006), which in turn trigger the activation of the cell death pathways via the Bcl-2 proteins and the release of pro-apoptotic proteins in mitochondria (see section 3.2).

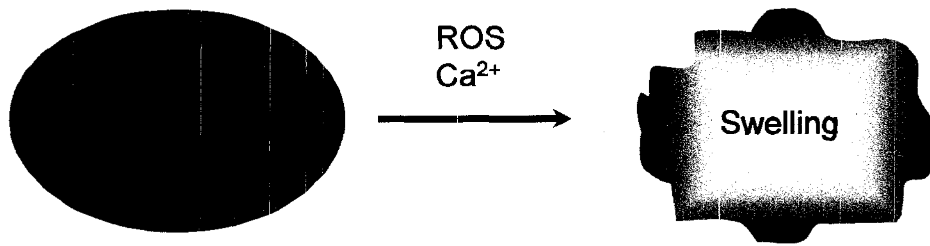
In addition to the production of ROS/RNS, Ca^{2+} can also directly activate several cell death proteins in neurons following excitotoxic insult. For example, calpains can be activated by elevated levels of Ca^{2+}, which in turn cleaves and inactivates a variety of vital proteins (reviewed in (Chan & Mattson, 1999; Hara & Snyder, 2007)). Recently, it has also been suggested that calpain can assist the release of AIF from the mitochondria (see section 5.3). Endonucleases such as the Ca^{2+}-Mg^{2+} dependent DNAse I-like endonucleases can also be activated by Ca^{2+} during excitotoxicity. These endonucleases can induce intranucleosomal DNA fragments in the absence of caspase activity (Gwag et al, 1997; Ko et al, 2000; Won et al, 2002). Ca^{2+} activates calcineurin which in turn dephosphorylates and relieves the inhibition of Bad, a pro-apoptotic BH3 only protein, via 14-3-3 protein (Wang et al, 1999). A high concentration of Ca^{2+} in the mitochondria can also induce the opening of the mitochonrdial permeability transition (MPT) pore which also allows the release of the proapoptotic factors in the mitochondria (see section 2) (Bernardi & Forte, 2007; Green & Kroemer, 2004; Zamzami et al, 2005). An increase in Ca^{2+} in the cytoplasm, therefore, can lead to ROS/RNS induced damage in protein, DNA and mitochondria, as well as the activation of pro-apoptotic proteins and enzymes. Most of these pathways converge to the

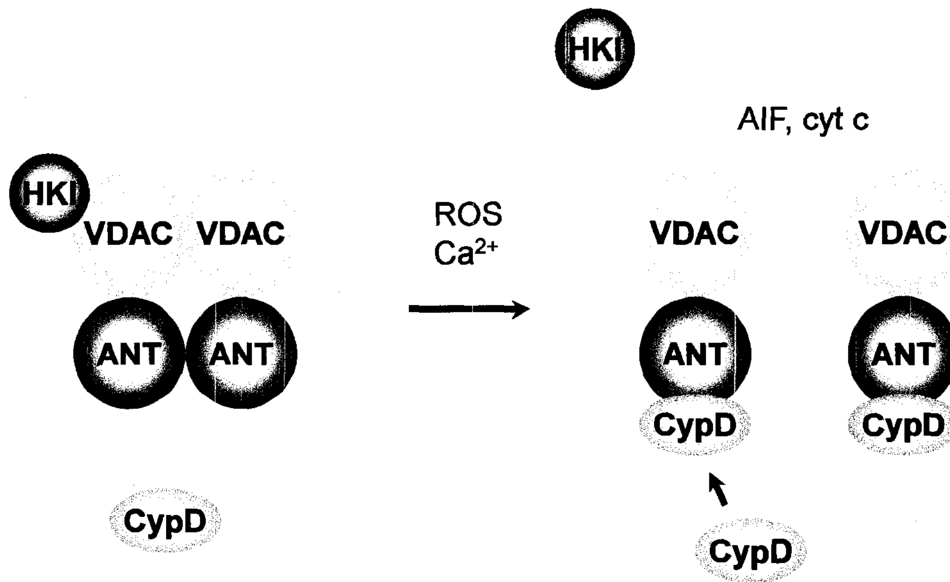
permeabilization of mitochondria and the release of proapototic proteins, which will be discussed in sections 2 and 3.

2: Mitochondrial permeability transition pore (MPT pore) (Figure 1-2)

In order to maintain the electrochemical potential to generate ATP, the mitochondrial inner membrane is not permeable to ions and solutes. Mitochondrial permeability transition (MPT) occurs when the inner membrane becomes permeable to solutes with a molecular weight up to 1500 Da, which allows water and solutes to pass through the inner membrane (Bernardi et al, 2006; Green & Kroemer, 2004; Kroemer et al, 2007; Rasola & Bernardi, 2007; Zamzami et al, 2005). This can be inhibited by cyclosporine A (CsA) and bongkrekic acid (BA) and requires Ca^{2+} and voltage across the membrane (Bernardi et al, 2006; Bernardi et al, 1999; Crompton, 1999). MPT is usually triggered by an influx of Ca^{2+} into the mitochondria during certain types of cell death such as excitotoxicity, during which the Ca^{2+} threshold can also be lowered by various factors such as an increase in ROS/RNS (Bernardi, 1999; Zoratti & Szabo, 1995). Transient MPT pore opening may occur in physiological situations to maintain Ca^{2+} homeostasis; however, persistent MPT pore opening can lead to collapse of the membrane potential, ATP depletion, massive release of Ca^{2+}, cristae unravelling and swelling of the matrix. Changes in mitochondrial matrix volume and structure disrupt the integrity of the outer mitochondrial membrane, and consequently the mitochondrial apoptotic factors such as cyt c and AIF are released (Kroemer & Reed, 2000; Susin et al, 1998). During neuronal cell death, a combination of

Figure 1-2: The mitochondrial permeability transition (MPT). In the presence of high level of Ca^{2+} and/or ROS, the inner membrane of the mitochondria becomes permeable to solutes with molecular weight up to 1500 Da, which allows water and solutes to pass through the inner membrane. This leads to the collapse of the membrane potential and swelling of the matrix. As a result the pro-apoptotic proteins in the intermembrane space such as cyt c and AIF are released. The MPT pore may consist of VDAC, ANT, and Cyp-D. See text for details.


ROS
Ca2+
Swelling


HKI
AIF, cyt c
HKI
VDAC
VDAC
ROS
Ca2+
VDAC
VDAC
ANT
ANT
ANT
ANT
CypD
CypD
CypD
CypD

increased intracellular Ca^{2+}, ROS, and perturbation in metabolism can trigger MPT. Various MPT inhibitors such as CsA and BA can protect neurons after various neuronal injuries (Cao et al, 2001; Ferrand-Drake et al, 1999; Folbergrova et al, 1997; Friberg et al, 1998; Matsumoto et al, 1999; Muranyi & Li, 2005; Okonkwo et al, 1999; Okonkwo & Povlishock, 1999; Russell et al, 2002; Vanderluit et al, 2003). These indicate that MPT is a crucial element in neuronal cell death, particularly in excitotoxicity and ischemia that involves Ca^{2+}overload and ROS induced cell death.

MPT is thought to be originated by a supramolecular complex in the mitochondria called the MPT pore (Figure 1-2) (Kroemer et al, 2007; Rasola & Bernardi, 2007; Zamzami et al, 2005). As discussed below the exact composition of MPT pore remains controversial and may vary under different conditions; but the consensus is that it consists of three proteins: the inner membrane adenine nucleotide translocator (ANT), the outer membrane voltage dependent anion channel (VDAC) and the matrix cyclophilin D (Cyp-D) which has protein folding activity (Crompton, 1999; Galat & Metcalfe, 1995; Halestrap & Brennerb, 2003; Kroemer & Reed, 2000; Tsujimoto et al, 2006; Tsujimoto & Shimizu, 2006). Other factors such as the Bcl-2 family members, the mitochondrial creatine kinase (CK), hexokinases (HK), and the outer membrane benzodiazepine receptor (PBR) can act as regulators of the MPT pore but are not considered to be a part of the pore (Henry-Mowatt et al, 2004; Mathupala et al, 2006; McEnery et al, 1992; Robey & Hay, 2006; Schlattner et al, 2006; Sharpe et al, 2004). HK and CK, in particular, have been suggested to act as a link between energy metabolism and control of cell death. Reduction of energy metabolism induces HK dissociation from VDAC which sensitizes mitochondria to undergo MPT (Robey & Hay, 2006). Most of the core components are originally identified using specific drugs that can abolish MPT, such as CsA that inhibits Cyp-D and BA that inhibits ANT.

(Cesura et al, 2003; Halestrap & Brennerb, 2003; Halestrap et al, 1997; Marzo et al, 1998; Verrier et al, 2003; Waldmeier et al, 2003; Zoratti & Szabo, 1995). When these proteins are reconstituted in liposomes or lipid layers, they exhibit similar channel properties as the MPT pore in isolated mitochondria (Brustovetsky & Klingenberg, 1996; Debatin et al, 2002; Dierks et al, 1990; Szabo et al, 1993; Szabo & Zoratti, 1993). Also, fractionation of mitochondrial membranes lead to the identification of VDAC-ANT-Cyp-D complex which has properties similar to endogenous MPT pore when it is reconstituted in membranes (Beutner et al, 1998). Most importantly, as discussed above, MPT drugs such as CsA and BA act on specific proteins which inhibit different forms of cell death during ischemic or traumatic brain damage. These biochemical studies have identified the mitochondrial proteins ANT, VDAC and Cyp-D as the core components of the MPT pore.

Recent studies using genetically modified mice have questioned the true identity of the MPT pore. In contrast to the biochemical studies mentioned above, mice deficient in MPT pore components demonstrate different requirements of these proteins in cell death. Although ANT overexpression can induce cell death (Bauer et al, 1999), ANT deficiency does not abolish Ca^{2+}-induced CsA-sensitive MPT in mice. The deficiency only raised the amount of Ca^{2+} required to induce mitochondrial swelling. These results suggest that ANT is not required for the formation of the MPT pore. At most, it functions merely as a modulator (Kokoszka et al, 2004). Unlike $ANT^{-/-}$ mice, $Cyp\text{-}D^{-/-}$ mice are significantly resistant to Ca^{2+} and ROS triggered MPT, and as a result survival is increased after ROS treatment (Baines et al, 2005; Nakagawa et al, 2005). Importantly, $Cyp\text{-}D^{-/-}$ mice are more resistant to *in vivo* models of cerebral ischemia, suggesting that Cyp-D plays a critical role in neuronal cell death during ischemia (Schinzel et al, 2005). In contrast to ROS mediated cell death, Cyp-$D^{-/-}$ cells do not display resistance to DNA damaging agents or ER stress initiators, which

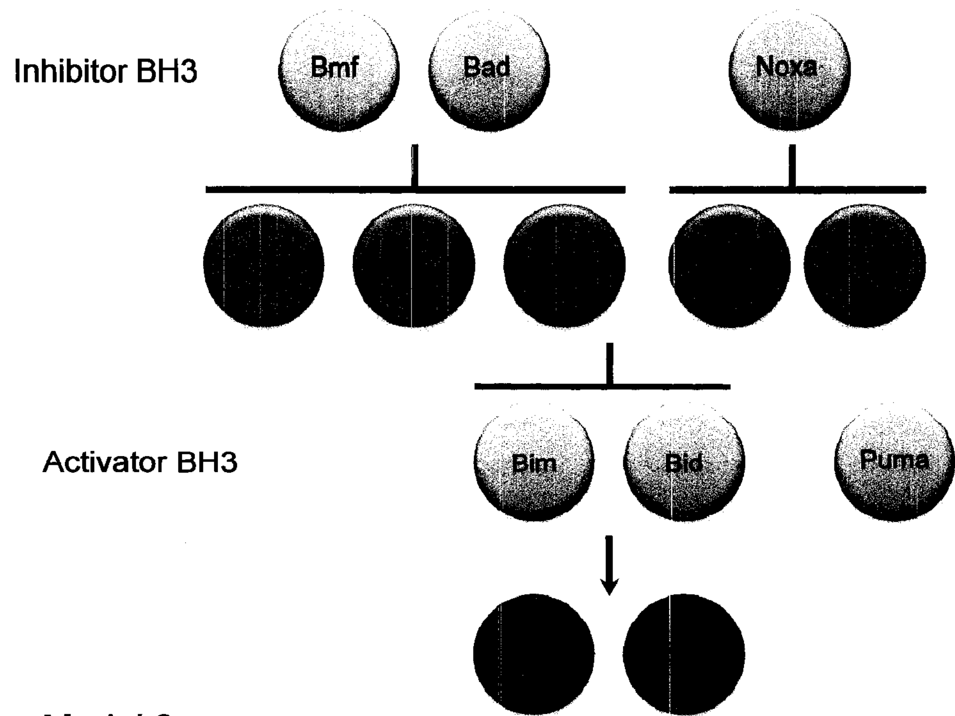
rely on the classical Bcl-2 apoptotic pathway. Similarly, $CypD^{-/-}$ cells are as sensitive to Bax and tBid as wildtype cells (Baines et al, 2005; Nakagawa et al, 2005; Schinzel et al, 2005). These results suggest that cell death induced by pro-apoptotic Bcl-2 family proteins does not depend on Cyp-D mediated MPT, contrary to previous reports that (1) Bax/Bak may cooperate with VDAC to induce MPT, and (2) over-expression of Bcl-2 can inhibit MPT pore to open (Marzo et al, 1998; Shimizu et al, 1998). It is still possible, however, that the Bcl-2 family of proteins may have an indirect effect on MPT by controlling the Ca^{2+} homeostasis from ER to mitochondria (Mathai et al, 2005; Pinton & Rizzuto, 2006; White et al, 2005). Since VDAC has at least three isoforms (VDAC1, 2, and 3) in mitochondria, it has been more difficult to assess the role of VDAC in MPT. $VDAC1^{-/-}$ and $VDAC3^{-/-}$ mitochondria have the same basic properties of MPT as the wildtype controls (Krauskopf et al, 2006). VDAC1/3 double knockout cells with siRNA of VDAC2 (essentially all three isoforms are absent) also do not behave differently than control cells in terms of MPT induced cell death (Baines et al, 2007). Although these results indicate that VDAC is dispensable for MPT induced cell death, it should be noted that this does not necessarily mean that VDAC does not play a role in cell death. A recent study using erastin to induce cell death (which is neither apoptotic, nor necrotic) demonstrates that VDAC2 and 3 are the direct targets, and deletion of these proteins can confer protection (Yagoda et al, 2007). Nevertheless, from these mutational studies, it seems that VDAC and ANT are not part of the MPT pore. It is possible that drugs such as BA which was previously thought to inhibit ANT could be inhibiting other components of MPT pore. The exact components of the MPT pore, therefore, remain to be determined. From the cell death studies of the $CypD^{-/-}$ mice, it is clear that CypD is involved in ROS and Ca^{2+} induced cell death, typically found following an ischemic injury.

3: Bcl-2 family proteins and mitochondrial outer membrane permeabilization (MOMP)

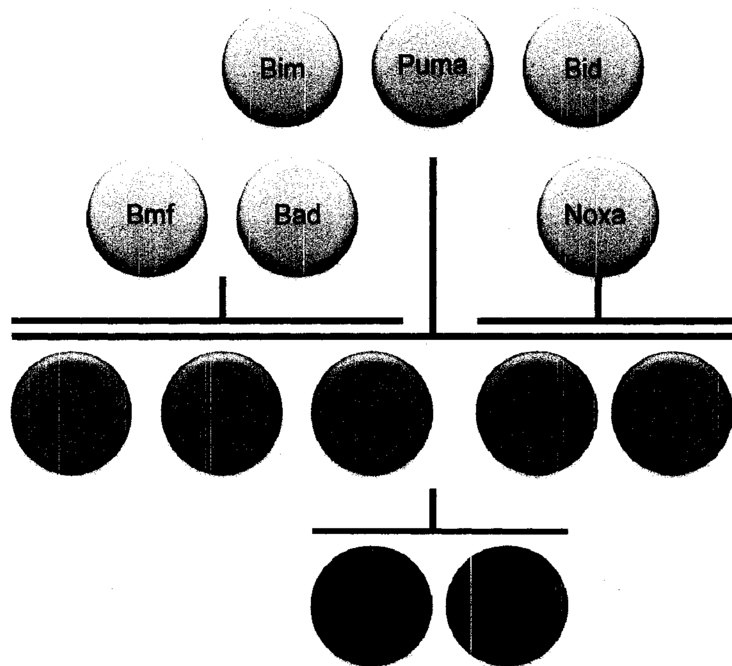
The Bcl-2 family members control the cell death pathway primarily at the mitochondria and to some extent at the ER. Activation of the pro-apoptotic Bcl-2 family members induces MOMP and the release of apoptotic factors from the intermembrane space (IMS) of the mitochondria (Adams & Cory, 2007; Newmeyer & Ferguson-Miller, 2003; Sharpe et al, 2004). This family of proteins consists of three major subpopulations that contain shared homology with conserved regions termed Bcl-2 homology domains 1 through 4 (BH1-4). Anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1, have all four BH1-4 domains (Motoyama et al, 1995; Nakayama et al, 1993; Opferman et al, 2003; Rinkenberger et al, 2000; Veis et al, 1993; Wilson-Annan et al, 2003), and are capable of inhibiting cell death. The multi-domain pro-apoptotic Bcl-2 family includes Bax and Bak (Knudson et al, 1995; Lindsten et al, 2000; Wei et al, 2001), and have apoptogenic activities mainly at the mitochondria (Cheng et al, 2001; Scorrano et al, 2003; Zong et al, 2003). The third and most structurally unique Bcl-2 family subgroup is the pro-apoptotic BH3-only proteins which include Bim, Bid, Bad, Hrk/DP5, Bmf, Noxa, Puma, and Mule (Bouillet et al, 1999; Datta et al, 2002; Imaizumi et al, 2004; Puthalakath et al, 2001; Ranger et al, 2003; Sax et al, 2002; Schuler et al, 2003; Villunger et al, 2003; Zhong et al, 2005). This subset of the Bcl-2 family shares homology with the other members through a single BH3 domain, which is essential for interactions between Bcl-2 family members. This population of proteins is differentially regulated in response to injury such that different stressors activate specific BH3-only proteins, which leads to the activation of the multi-domain proapoptotic Bax/Bak and induce MOMP (Huang & Strasser, 2000; Kuwana et al, 2005).

Figure 1-3: Activation of Bax/Bak by BH3 only proteins: the two models. Two models are proposed for the activation of Bax/Bak by BH3 only proteins. Model 1 is the hierarchy model involving two different class of BH3 only proteins, Model 2 is the indirect activation model in which there is only one class of BH3 only proteins. See text for details.

Model 1



Model 2



3.1: Activation of Bax/Bak by BH3-only proteins (Figure 1-3)

In healthy cells, the pro-apoptotic Bax/Bak proteins are inhibited by the anti-apoptotic Bcl-2 proteins. During apoptosis, Bax/Bak are activated and in turn induce permeabilization of the mitochondria (Green & Kroemer, 2004). The exact mechanism of Bax/Bak activation is not yet clear: two distinct models have been proposed (Figure 1-3). The first model is the "hierarchy model". In this model, the BH3-only proteins are separated into two groups, the inhibitor and the activator. The inhibitors (Bad, Bik, Bmf, Noxa, Bnip3) inactivate the anti-apoptotic Bcl-2 proteins (Galonek & Hardwick, 2006; Kuwana et al, 2005), which relieve the inhibition of the activators Bid and Bim by the Bcl-2. As a result Bax/Bak can now be activated directly by Bid and Bim. The second model is the "indirect activation" model, in which there is only one group of BH3-only proteins and they all directly inhibit the anti-apoptotic Bcl-2 proteins. In this model, unlike the hierarchy model, none of the BH3-only proteins can directly activate Bax/Bak (Adams, 2003; Adams & Cory, 2007; Willis & Adams, 2005). Both of these models are more or less equally supported by results from various cell-free experiments using recombinant proteins/peptides on artificial liposomes or isolated mitochondria (Kim et al, 2006; Kuwana & Newmeyer, 2003). These artificial systems may not faithfully reflect physiological situations and results may vary under different conditions. The critical question is whether these two models can be tested in an *in vivo* setting. In the hierarchy model, since Bim and Bid directly activate Bax/Bak, if both Bim and Bid are absent *in vivo*, the phenotype should be similar to Bax/Bak double knockout (DKO) and the inactivating BH3-only proteins cannot induce cell death. Bim/Bid DKO are viable, unlike Bax/Bak DKO mice which are embryonic lethal. Again unlike Bax/Bak DKO cells which are resistant to cell death, UV and etoposide can induce apoptosis as efficiently in the Bim/Bid DKO as in the wildtype controls. Importantly, the so-called

inactivating BH3-proteins such as Bad and Noxa can still induce cyt c release and apoptosis in these Bim/Bid DKO cells (Willis et al, 2007). This study, therefore, directly shows that Bim/Bid cannot activate Bax/Bak directly, thus suggesting that the indirect model is the more likely scenario. The hierarchy model could still hold true if there are other BH3 proteins such as Puma that can directly activate Bax/Bak. Regardless of the different models proposed, these studies indicate that BH3-only proteins are essential in the activation of Bax/Bak, which are the inducers of MOMP.

3.2: Bax/Bak induced MOMP

The activated Bax/Bak leads to oligomerization that induces MOMP (Antignani & Youle, 2006; Lalier et al, 2007). To investigate the mechanism of Bax/Bak induced MOMP, cell-free systems of isolated mitochondria or artificial liposomes with known amounts of protein and lipids are used. Whether the results from these artificial conditions can translate into the physiological situations are controversial. Nonetheless, two models have been proposed for Bax/Bak induced MOMP, based on whether Bax/Bak alone are sufficient or other proteins are also required. Bax/Bak can modulate MOMP by interacting and controlling VDAC activity at the outer membrane (Shimizu et al, 2000; Shimizu et al, 1999). The BH3-only protein Bim can also interact with VDAC during apoptosis (Shimizu & Tsujimoto, 2000; Sugiyama et al, 2002). The involvement of VDAC in Bax/Bak mediated apoptosis was further shown from the mitochondria of VDAC1$^{-/-}$ yeast (Shimizu et al, 1999), and cells with anti-VDAC antibodies (Shimizu et al, 2001), both of which are resistant to Bax/Bak induced cyt C release. These studies, therefore, suggested that Bax may directly interact with VDAC at the mitochondria for MOMP (Tsujimoto & Shimizu, 2002). All of these VDAC studies, however, did not exclude the possibility that VDAC may act merely as

a receptor for Bax, rather than actively engaging Bax in MOMP. Also, as discussed in section 2, the opening of the MPT pore, in which VDAC is one of the constituents, may not involve Bcl-2 family protein members. In addition, whether this putative VDAC-Bax pore is big enough for other pro-apoptotic proteins such as AIF and Endo G to be released from the mitochondria remains to be shown.

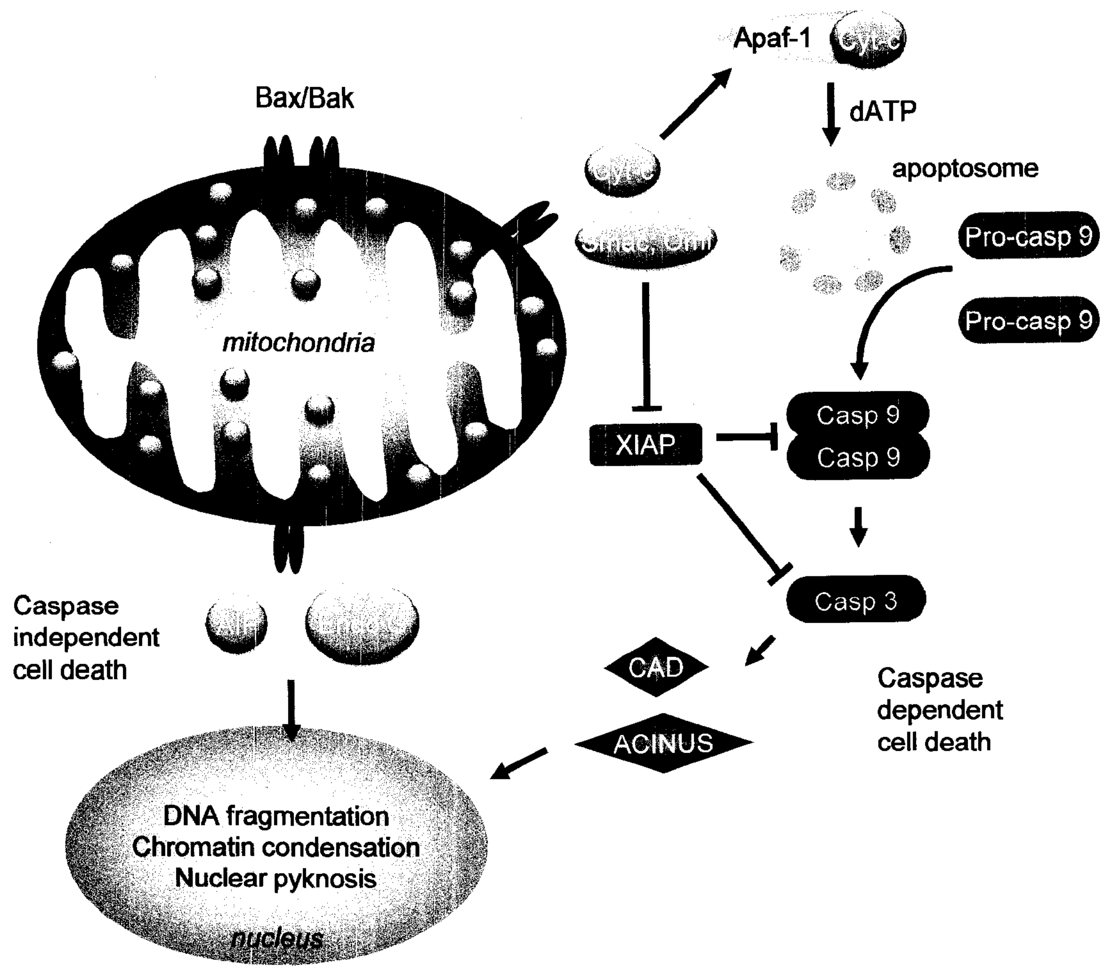
Other studies, in contrast, show that Bax/Bak alone is sufficient in MOMP (Garrido et al, 2006; Kuwana & Newmeyer, 2003; Lalier et al, 2007). This is supported by studies using reconstituted membranes and vesicles with known components (for review see (Newmeyer & Ferguson-Miller, 2003)). For example, a study using progressively simplified vesicular system indicates that Bax with Bid, in the absence of other proteins, can permeabilize the vesicles and release molecules considerably larger than cyt-c (up to 2000 kDa) (Kuwana et al, 2002). This correlates well with the fact that MOMP is non selective such that all the IMS proteins, irrespective of their sizes, are released during apoptosis. One caveat of this study is that only small Bax oligomers are formed (around 100 kDA), in contrast to the large Bax oligomers (more than 200 kDA) seen in whole cells undergoing apoptosis. In addition, these liposomes consist of cardiolipins, which are only abundant in the IM, not in the OM which is the site of Bax induced MOMP. In fact, mitoplasts (IM vesicles) cannot be completely permeabilized by Bax/Bid, in spite of the large amount of cardiolipin. The model that Bax alone can form pores is supported by another study showing that oligomerized Bax alone can form large openings in lipid bilayers (Basanez et al, 1999; Epand et al, 2002b). The large Bax pores were visualized using atomic force microscopy, although the conditions are not physiological (Epand et al, 2002c). The discrepancies between whether Bax/Bak require other proteins or not can be reconciled if Bax/Bak alone can form pores in optimal conditions that may be achieved in some but not all cell-free

systems. The presence of other proteins may significantly increase the efficiency of Bax/Bak in permeabilizing membranes. Regardless of these differences, these studies nonetheless show the importance of activated Bax/Bak in mediating MOMP, which releases the mitochondrial pro-apoptotic proteins such as cyt c and AIF.

4: Release of mitochodrial pro-apoptotic proteins

After the mitochondrial permeabilization by either MOMP and MPT, the pro-apoptotic proteins in the intermembrane space (IMS) of mitochondria are released into the cytosol (Figure 1-4). The kinetics of the release of these proteins can vary among different types of cell death models. In HeLa cells after staurosporine treatment, the release of cyt c is rapid and complete, while other proteins such as AIF and Endo G are released more slowly and often incomplete (Munoz-Pinedo et al, 2006). In neurons after excitotoxic insult, release of cyt c occurs after the relatively rapid AIF release *in vivo* and *in vitro* (Culmsee et al, 2005; Plesnila et al, 2004; Wang et al, 2004; Yu et al, 2002), which underscores a more important role of AIF in cell death during excitotoxicity. In DNA damage induced neuronal cell death, however, release of cyt c occurs prior to the release of AIF, and eventually both proteins are completely released (Cregan et al, 2004; Cregan et al, 2002). Mitochondria from different types of cells have different compositions of lipids and proteins, for example, brain mitochondria are more enriched in cardiolipin, which may explain this late cyt c release since cardiolipin binds to cyt c (Lutter et al, 2000). In any case, not all mitochondria are the same and these differences remind us that, while the canonical cell death model of staurosporine treated HeLa cells is helpful to use as a starting point to investigate the cell death pathway, other models may behave in a very different manner. The following section

Figure 1-4: The release of mitochondrial pro-apoptotic proteins. After the release of cyt c from the mitochondria, it activates the caspase pathway via Apaf-1 and dATP. The activation of caspases activates proteins such as Cad and Acinus that are responsible for the execution of DNA fragmentation and chromatin condensation. Smac and Omi are also released and they assist caspase activation by inactivating XIAP that inhibits caspases. AIF and Endonuclease G (Endo G) are the caspase independent cell death factors that are also released from the mitochondria and translocate into the nucleus. They can also induce DNA fragmentation and chromatin condensation. For details see text.


Apaf-1
dATP
apoptosome
Bax/Bak
Pro-casp 9
Pro-casp 9
mitochondria
XIAP
Casp 9
Casp 9
Casp 3
Caspase
independent
cell death
CAD
ACINUS
Caspase
dependent
cell death
DNA fragmentation
Chromatin condensation
Nuclear pyknosis
nucleus

will focus on the mechanisms of how these mitochondrial pro-apoptotic proteins induce cell death once released from the mitochondria. The release of AIF will be discussed in more detail in section 5.

4.1: Release of cyt c and the activation of caspases

Cyt c, Smac/DIABLO, and Omi/HtrA2, once released from the mitochondria, activate the caspase pathway in the cytosol (Green & Kroemer, 2004; Riedl & Salvesen, 2007; Salvesen & Duckett, 2002). In the presence of ATP/dATP, cyt c mediates the activation and oligomerization of the adaptor molecule apoptosis-protease activating factor 1 (Apaf-1). This caspase activating platform, named the apoptosome, recruits and activates the apical caspase 9 which in turn initiates the executioner caspases such as caspase 3 and 7. Since any accidental activation of these caspases can lead to deleterious damage to the cell, there are additional safety mechanisms that inhibit caspase activation when the cell is healthy. One of these safety mechanisms is the cytosolic inhibitor of apoptosis protein (IAP) that negatively regulates the caspases (Salvesen & Duckett, 2002; Shi, 2002). During cell death, the mitochondria also release the second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point (Smac/DIABLO) and Omi/high temperature requirement protein A 2 (Omi/HtrA2) that can neutralize the IAPs. The release of cyt c and Smac/DIABLO during cell death therefore ensures the complete and rapid activation of caspases which are responsible for the coordinated breakdown of the cell (Timmer & Salvesen, 2007).

The activation mechanism of the apical caspase, caspase 9, is quite different from the activation of the downstream executioner caspases such as caspase 3 and 7, which the latter relies on proteolytic cleavage by the apical caspases. Previously, it has been reported that apical caspases undergo auto-activation by proteolytic cleavage once recruited by the adaptors, such as the apoptosome that activates pro-caspase 9 (Budihardjo et al, 1999; Takahashi & Earnshaw, 1996). This model has been called into question, because pro-caspase 9 with mutated cleavage site can still be activated in the presence of cyt c and cytosolic factors (Rodriguez & Lazebnik, 1999; Stennicke et al, 1999), indicating that proteolytic clevage is not necessary for activation. The activation of apical caspases is instead through homo-dimerization which occurs when they are recruited by the apoptosome. This dimerization event may induce a conformational change that enables exposure of the active sites. In healthy cells, apical caspases, such as caspase 9, primarily exist as inactive monomers in the cytosol. However, they can be activated biochemically by inducing dimerization using high concentration of salts (Boatright et al, 2003; Pop et al, 2006; Renatus et al, 2001). A careful biochemical analysis of the uncleavable mutated form of the apical caspase showed that while most of them exist as inactive monomers, there is a fraction of active dimers (Donepudi et al, 2003). This indicates that the cleavage event is mostly for stability, and not for activation. The cleaved product does not lead to dimerization and does not bind to caspase substrates, indicating that the cleavage event is not required for the dimerization and subsequent activation. Mutation of the dimerization site can abolish the activation and is less effective in cell death induction, which further underscores the importance of dimerization in the activation of the apical caspases (Boatright et al, 2003; Riedl & Salvesen, 2007). The significance of this revised model of apical caspase activation is that, while the executioner caspase activation is through irreversible cleavage, the

activation of apical caspase through dimerization can be reversible which enables therapeutic manipulation. Activation of the apical caspases triggers activation of the executioner caspases (caspase 3 and 7) by cleaving the linker sequences between the two subunits. This cleavage enables the conformational change of the protein and the active site can now be formed between the homodimers. The activated caspases cleaves an array of proteins which contributes to the apoptotic phenotype, including the activation of the nuclease CAD and Acinus that are responsible for the classical apoptotic phenotype: internucleosomal DNA degradation and chromatin condensation, respectively (Enari et al, 1998; Sakahira et al, 1998; Samejima & Earnshaw, 2005; Yasuhara et al, 1997). Cyt c dependent activation of the caspase cascade thus serves as the key executioner of cell death.

While the release of cyt c has been shown to be the key mechanism in activating the caspases, other changes in cells can also activate caspases. During ischemia, glycolysis is activated to maintain ATP production in the absence of an effective oxidative phosphorylation. One byproduct of glycolysis is lactic acid, which reduces the pH inside the cell as it accumulates (Gevers, 1977; Katsura et al, 1993). The acidification occurs before caspase activation, indicating that this is not a secondary effect due to the activation of the cell death machinery. Increasing alkalinity can delay caspase activation and cell death, while decreasing pH by using H^+ ionophore can accelerate caspase activation. More importantly, cell death induced by acidification of the cytosol can be inhibited by a pan-caspase inhibitor z-VAD-fmk, highlighting the role of acidification in caspase activation (Furlong et al, 1997). Although these studies indicate the possibility of caspase activation without cyt c release from the mitochondria, this does not necessarily mean that caspases act upstream of mitochondria to promote cyt c release. It has been suggested that some caspases such as

caspase 2 can act upstream of the mitochondria to promote cyt c release and therefore is required for the activation of other caspases (Lassus et al, 2002; Robertson et al, 2002). Since both of these studies use peptide inhibitors which may not be specific, and proper controls of caspase 2 antisense or siRNA were not performed, it is difficult to conclude that caspase 2 is indeed acting upstream of mitochondria and other caspases. In addition, if caspase 2 is indeed upstream of other caspases, it is hard to reconcile the fact that caspase 2 deficient mice show minimal developmental defect compared to caspase 3 or caspase 9 deficient mice (Bergeron et al, 1998). Nevertheless, activated caspases can still provide a positive feedback to release more cyt c from the mitochondria. Cells with deficient caspase 3 and 7 have delayed Bax translocation, cyt c release and mitochondrial depolarization (Lakhani et al, 2006). Also, mitochondrial function can be disrupted by caspases through cleavage of complex I during apoptosis (Ricci et al, 2004). While the initiation of cyt c release and the subsequent caspase activation requires Bcl-2 induced MOMP or MPT, once caspases are activated after cyt c release they can act back on mitochondria to facilitate the complete release of cyt c and other pro-apoptotic factors from the mitochondria.

4.2: Other roles of Cyt c in cell death

Apart from caspase activation, cyt c release from mitochondria may contribute to cell death by other mechanisms. Since cyt c is an important factor in the electron transport chain for maintenance of mitochondrial membrane potential and ATP production, it is possible that loss of cyt c from the mitochondria can also induce cell death due to loss of energy production and/or loss of mitochondrial membrane potential before caspase activation.

Whether the loss of cyt c from mitochondria is enough to induce mitochondrial dysfunction is highly dependent on the cytotoxic insults. Cell death induced by Fas ligand can decrease mitochondrial membrane potential and respiratory defect which cannot be rescued by the inhibition of caspases, indicating that these defects are not due to activated caspases. Addition of cyt c, however, can partially rescue the loss of mitochondrial function, indicating that the loss of cyt c can lead to loss of mitochondrial function (Mootha et al, 2001). In contrast, cells treated with DNA damaging agents (UV or etoposide) still maintain mitochondrial membrane potential in the presence of caspase inhibitors even though cyt c is completely released. In this case, the cytoplasmic pool of cyt c may be enough to maintain the mitochondrial membrane potential when the mitochondria are permeabilized during cell death (Waterhouse et al, 2001). The differences may be due to the different energy requirement for the cell to survive after different cytotoxic insults. Since neurons require a higher amount of ATP produced from mitochondria, one may envisage that cyt c release from mitochondria may also induce a loss of ATP production even in the absence of caspase activation.

Release of cyt c from mitochondria may also affect how the cell controls Ca^{2+} levels in the cytosol. In a search to find the binding partner of cyt c, it was unexpectedly discovered that cyt c can bind to the ER Ca^{2+} channel inositol (1,4,5) triphosphate receptor (InsP3R) that mediates release of Ca^{2+} from the ER. A high concentration of Ca^{2+} inhibits InsP3R mediated release of Ca^{2+} from the ER which acts as a protective mechanism against accidental Ca^{2+} release. During apoptosis, however, the released cyt c from the mitochondria can bind to InsP3R which relieves the Ca^{2+} induced inhibition of the receptor and as a result Ca^{2+} release from ER is dysregulated (Boehning et al, 2003). Increase of Ca^{2+} ion

concentration in the cytosol, as discussed in section 1.3, can accelerate cell death by increasing ROS productioin, inducing the MPT, and activating endonucleases. The importance of this interaction in cell death has been further demonstrated using a peptide from a small sequence on the InsP3R receptor to displace cyt c binding to the receptor. This small peptide can inhibit cell death induced by either staurosporine or Fas (Boehning et al, 2005). The release of cyt c from the mitochondria therefore engages the Ca^{2+} pathway via ER leading to further release of cyt c from the mitochondria.

Mitochondrial cyt c can also contribute to cell death while it is still in the mitochondria. The 66-kilodalton isoform of the growth factor adapter Shc (p66(shc)) is a cytosolic protein that mediates cell death induced by DNA damage and oxidative stress, and also cellular ageing. p66(shc) deficiency increases longevity, and inhibits p53 response and cell death after UV or H_2O_2 treatment (Migliaccio et al, 1999). After cell death induction, PKCβ phosphorylates p66(shc) and the phosphorylated protein accumulates in the mitochondria via interacting with Pin1 (Pinton et al, 2007). When it is in the mitochondria, p66(shc) can induce MPT by stimulating the production of H_2O_2. Unexpectedly, the production of H_2O_2 does not depend on $O_2^{\cdot-}$, indicating that complex I-III is not involved. Instead, p66(shc) generates H_2O_2 from reduced cyt c that acts as the electron donor to p66(shc). Consequently, mutation of the cyt c redox domain impairs H_2O_2 production and cell death induction, while the ability to activate caspases remains unchanged. This further underscores the role of cyt c in production of H_2O_2 for cell death progression that is independent of its role in caspase activation (Giorgio et al, 2005). In addition to the canonical role of cyt c in caspase activation, it can contribute to cell death via different pathways before and after it is released from the mitochondria.

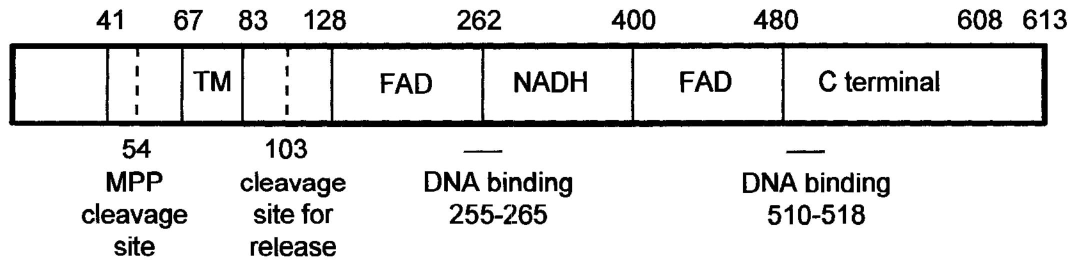
5: Apoptosis-inducing factor (AIF)

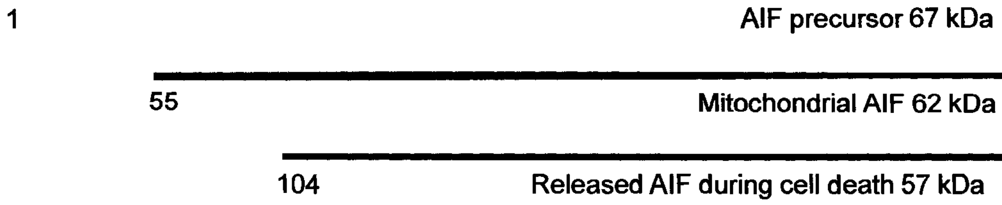
After the loss of mitochondrial outer membrane integrity by either MOMP or MPT, AIF is released from the mitochodrial intermembrane space into the cytosol and is translocated into the nucleus, where it induces large scale DNA fragmentation and peripheral chromatin condensation (Cregan et al, 2004; Daugas et al, 2000a; Hong et al, 2004; Lorenzo et al, 1999). Recent studies in AIF deficient animals have also indicated an essential role of AIF in maintaining cell survival (Krantic et al, 2007; Modjtahedi et al, 2006). Thus similar to cyt c, AIF has dual roles in causing cell death after cytotoxic injury and in maintaining survival in healthy cells. Hence, in order to use AIF as a therapeutic target in acute neuronal injury, we need to understand the dual roles of AIF in cell life and death.

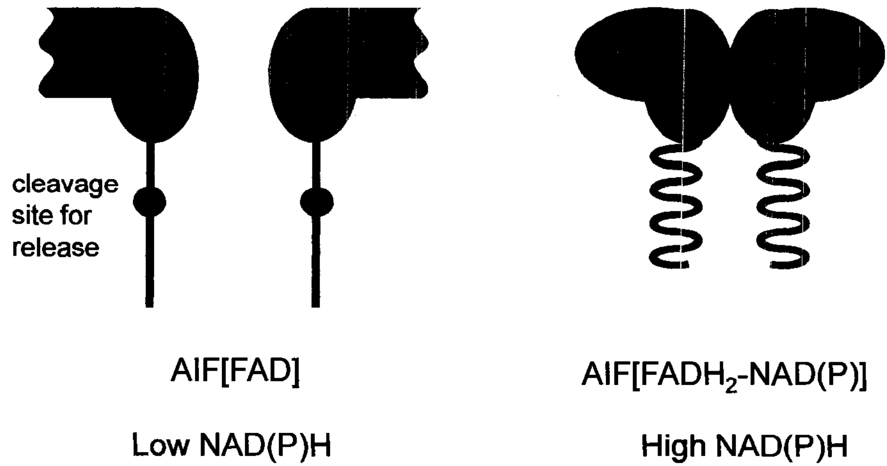
5.1: Biochemical properties of AIF : oxidoreductase activity

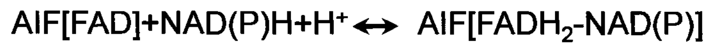
AIF is a 57 kDa (612 aa long) flavoprotein which is similar to the prokaryotic oxidoreductases, especially the NADH-dependent ferredoxin reductases BphA4 from bacteria and archaebacteria (Daugas et al, 2000a; Daugas et al, 2000b; Susin et al, 1999b). AIF contains an FAD binding domain and NADH binding domain, which mediate the oxidoreductase activity of AIF. Unlike the bacterial proteins, AIF has an extra C terminal that contains PEST sequences that could be responsible for protein turnover, and also a proline-rich motif which forms a poly-proline-2-helix (PPII) (Figure 1-5). These sequences have been implicated in interaction with proteins that contain SH3 or WW modules. In addition, unlike the bacterial oxidoreductases, the surface of AIF is positively charged.

Figure 1-5. AIF domains. AIF consists of MLS (mitochondrial localization signal), putative transmembrane (TM) domain, FAD binding domains, NADH binding domain, and the C terminal that may contain PPII and PEST domains that are responsible for protein interaction and protein turnover. After mitochondrial import, the MLS sequence from the AIF precursor (67kDa) is cleaved by MMP (mitochondrial processing peptidase) which gives rise to the 62kDA mitochondrial AIF. During cell death, mitochondrial AIF may be further processed at aa 103 for its release from the mitochondria. The processed AIF (57kDa) is then released from the mitochondria. AIF undergoes conformational change when the level of NAD(P)H is low. At the N-terminal, the cleavage site for the release of AIF during cell death is exposed. In addition, the C-terminal, which contains the putative protein binding domains, also undergoes conformational change and may therefore affect protein interaction. AIF also dimerizes in physiological level of NAD(P)H. This conformational change is reversible if the level of NAD(P)H is returned to normal.


41
67
83
128
262
400
480
608
613
TM
FAD
NADH
FAD
C terminal
54
MPP
cleavage
site
103
cleavage
site for
release
DNA binding
255-265
DNA binding
510-518


1
AIF precursor 67 kDa
55
Mitochondrial AIF 62 kDa
104
Released AIF during cell death 57 kDa


cleavage
site for
release
AIF[FAD]
Low NAD(P)H
AIF[FADH2-NAD(P)]
High NAD(P)H


AIF[FAD]+NAD(P)H+H+ ↔ AIF[FADH2-NAD(P)]

These differences suggest that during the course of evolution, AIF acquired additional functions beyond being merely an oxidoreductase (Mate et al, 2002; Ye et al, 2002). Cell free studies using recombinant human AIF indicate that AIF does not have peroxidase or H_2O_2 scavenging activity, but rather, it can generate superoxide ($O_2^{\cdot-}$) by transferring one electron to molecular oxygen using the FAD moiety. When AIF is released from the mitochondria during cell death, the NADH oxidase activity is retained, although it is not necessary for cell death induction (Miramar et al, 2001). In addition, deletion of the NADH or FAD binding domain does not inhibit the ability of AIF in causing cell death (Cande et al, 2002a; Daugas et al, 2000b; Loeffler et al, 2001; Susin et al, 2000). These experiments suggest that the NADH oxidase activity is not required for cell death induced by AIF.

Recent studies using a different method to generate recombinant AIF have provided a more detailed picture regarding the role of AIF's oxidoreductase activity within the mitochondria. Different from the previous studies on recombinant AIF that used N-terminal affinity tags for purification (Miramar et al, 2001; Susin et al, 1999b), these new experiments used C-terminal tags which were removed after purification to ensure correct folding and incorporation of FAD (Churbanova & Sevrioukova, 2007; Sevrioukova & Poulos, 2002). While these new studies confirm the presence of redox activity of AIF, in contrast to the previous studies (Miramar et al, 2001), the ability of AIF to transfer electrons from NAD(P)H to other proteins/compounds is very low, arguing against a direct role of AIF in the mitochondrial electron transport chain or the production of $O_2^{\cdot-}$. In addition, AIF cannot reduce a number of oxidative species, including $O_2^{\cdot-}$ and H_2O_2 (Churbanova & Sevrioukova, 2007), suggeting that AIF is not a ROS scavenger as previously speculated (Klein & Ackerman, 2003; Klein et al, 2002; Urbano et al, 2005; van Empel et al, 2005; van Empel et

al, 2006). This agrees with results presented in chapter 4, in which the ROS scavenger N acetyl-cysteine (NAC) cannot rescue the defects of AIF deficient neurons (Cheung et al, 2006). AIF reacts with NAD(P)H and undergoes dimerization in healthy cells, whereas in the absence of NAD(P)H, AIF becomes monomeric and undergoes conformational change at the N-terminal that contains the calpain cleavage site and at the C-terminal 509-599 which contains the putative protein binding domain (Churbanova & Sevrioukova, 2007). It is plausible that AIF can sense and transmit the redox changes in the mitochondria through the redox active site to the proteins that are interacting with AIF at the N- and C- terminal. While the binding partners of AIF in the mitochondria is not yet clear, in chapter 4 we will see that one of the interacting proteins could be Opa1 that regulates the mitochondrial structure, which may link mitochondrial metabolic state to mitochondrial structure.

5.2: Isoforms and homologs of AIF

Three other isoforms of AIF has been discovered. AIF-exB is generated by an alternative use of exon 2B instead of exon 2 in AIF, and is of similar length (609 amino acids) compared to full length AIF (Loeffler et al, 2001). AIFsh (AIF short) is generated by exons 10-16, hence it is the only isoform that does not have the oxidoreductase domain and is localized in the cytoplasm in healthy cells (Delettre et al, 2006a). AIFsh2 (AIF short 2) is produced by the alternative use of exon 9b, and has no C-terminal domain (Delettre et al, 2006b). Since only AIFsh2 lacks the C-terminal which has been shown to be essential for cell death induction, overexpression of all the other isoforms except AIFsh2 can induce cell death after translocation to the nucleus (Delettre et al, 2006a; Delettre et al, 2006b; Loeffler

et al, 2001). The contribution of these isoforms during cell death is not clear, since there is no loss-of-function studies that isolate and focus on one particular isoform from the others. The expression levels of AIFsh and AIFsh2 are low in brain tissues, suggesting that their contributions to neuronal survival and cell death may be limited (Delettre et al, 2006a; Delettre et al, 2006b).

Two AIF homologs have been identified in humans: AMID (AIF homologous mitochondrion-associated inducer of death, also called PRG3) on chromosome 10 (Ohiro et al, 2002; Wu et al, 2002), and AIFL on chromosome 22 (Xie et al, 2005). AMID was first identified as a p53 inducible gene that is downregulated in some types of cancer (Ohiro et al, 2002; Wu et al, 2002). This is similar to AIF which is also a transcriptional target of p53, however, unlike AMID, AIF is not upregulated by p53 during cell death (Ohiro et al, 2002; Stambolsky et al, 2006). The exact subcellular location of AMID is not yet clear, as it has been suggested to be a cytosolic protein (Ohiro et al, 2002), an outer membrane protein (Wu et al, 2002), or both (Wada et al, 2005). Similar to AIF, AMID can induce caspase independent cell death at the nucleus, independent of its oxidoreductase domain. AIFL induced cell death, on the other hand, is caspase dependent (Xie et al, 2005). Bcl-2 is not able to inhibit AMID induced cell death (Wu et al, 2002), suggesting that the location of AMID is not in the intermembrane space of the mitochondria. Similar to the AIF isoforms AIFsh and AIFsh2, the expression level of AMID in the brain is low (Wu et al, 2002). In addition, AMID deficient animals do not have significant defects in development and cell death (Mei et al, 2006). Similar to the AIF isoforms, AMID may not be an important contributor to neuronal survival and cell death. Thus this thesis concentrates on AIF and its role in neuronal survival and cell death.

5.3: Mechanisms of AIF release from mitochondria during cell death

Recombinant AIF is able to induce large scale DNA fragmentation and partial chromatin condensation when it is incubated with isolated nuclei (Loeffler et al, 2001; Susin et al, 1999b). The release of AIF from the mitochondria into the nucleus during cell death is well documented in different systems (Table 1-1). In neurons, depending on the particular cell death stimulus, the release mechanism of AIF from the mitochondria can be quite different than other intermembrane proteins such as cyt c. As discussed in section 2 and 3, depending on the cell death stimulus, mitochondria can release their apoptotic proteins via two different (but not mutually exclusive) mechanisms: the MOMP via Bcl-2 family proteins, and the MPT pore due to the increase of Ca^{2+} ion during excitotoxicity. The release of AIF may require extra steps for the complete release, because compared to cyt c sometimes the release of AIF is not complete after MOMP (Munoz-Pinedo et al, 2006). The activation of PARP-1, caspases, and calpain have been suggested to be responsible for the complete release of AIF, which will be discussed in detail below.

5.3.1: PARP-1

PARP-1 is a nuclear protein that catalyses the reaction of NAD^+ into nicotinamide and the addition of poly(ADP-ribose) polymer (PAR polymer) to proteins, which is essential for DNA repair, replication and transcriptional regulation (D'Amours et al, 1999). During apoptosis induced by DNA damage, the activity of PARP-1 may increase substantially and deplete the cell of NAD^+ and ATP (Ha & Snyder, 2000). In PARP-1 knockout neurons,

Type of cell/tissue	Cell death insult	Caspase dependency (method used to inhibit caspases)	Reference
Rat cortex	Traumatic brain injury	NA	(Zhang et al, 2002)
Rat cortical culture	Peroxynitrite	Independent (zVAD)	(Zhang et al, 2002)
Rat CGN	MeHg, H_2O_2, and colchicine	NA	(Fonfria et al, 2002)
Rat hippocampal	Neonatal hypoxia/ischemia	Before caspase activation	(Zhu et al, 2004)
Rat cortex	Global transient ischemia	Independent (zVAD)	(Cao et al, 2003)
Rat cortical culture	OGD	NA	(Cao et al, 2003)
Rat cerebral cortex	MCAO	NA	(Niimura et al, 2006; Zhao et al, 2004)
Mouse cortical culture	NMDA	Independent (BAF)	(Wang et al, 2004)
Dopaminergic cell line	MPP^+	Independent (zVAD, dnApaf1)	(Chu et al, 2005)
Mouse cortex	MCAO	Before cyt c release	(Plesnila et al, 2004)
Mouse spinal motoneurons	Mouse ALS model	NA	(Oh et al, 2006)
Mouse cortical culture, mouse cerebral cortex	OGD, glutamate, MCAO	Independent (zVAD)	(Culmsee et al, 2005)
Mouse cardiomyocytes	Ischemia/Reperfusion injury	Independent (zVAD)	(Kim et al, 2003)
Human b lymphocyte cell line (Raji)	UVA	Independent (zVAD)	(Yuan et al, 2004)
Primary T lymphocytes	Staurosporine	Before cyt c release	(Bidere et al, 2003)
Primary leukemic T cells, Jurkat	cyclophosphamide	Independent (zVAD)	(Strauss et al, 2008)
HeLa, COS	Staurosporine	Independent (zVAD)	(Yuste et al, 2005b)
HeLa, Cos7, 293T	Bax, staurosporine	Dependent (zVAD)	(Arnoult et al, 2003a; Arnoult et al, 2003b)
HeLa, isolated mitochondria	Bax, staurosporine, H_2O_2	Dependent (zVAD), release after cyt c	(Arnoult et al, 2002)

Table 1.1: Translocation of AIF in different cell types and cell death stimuli.

translocation of AIF to the nucleus is absent during excitotoxicity or DNA damage induced cell death, indicating that PARP-1 induces the release of AIF during excitotoxicity (Komjati et al, 2004; Yu et al, 2002). How does PARP-1, a nuclear protein, induce the release of AIF which is a mitochonrdial protein? It has been suggested that the presence of the PAR polymer is sufficient to induce AIF release. Animals with reduced PARG (PAR glycohydroase), the enzyme reponsible for PAR degradation, accumulate a high level of the PAR polymer and as a result have increased AIF release during cell death (Koh et al, 2004). When the PAR polymer is directly injected into the cells, AIF is released and causes cell death that is caspase independent (Andrabi et al, 2006; Yu et al, 2006b). Hence, the presence of PAR polymer produced by PARP may sensitize the release of AIF from the mitochondria. It is however still unknown how the PAR polymer, which is in the cytosol and the nucleus, can act in the intermembrane space of mitochondria. A more likely explanation for the PARP-1 induced AIF release is that, as we have discussed above, the absence of NAD(P)H can induce a conformational change of AIF which enables a more efficient release (Churbanova & Sevrioukova, 2007). As PARP-1 activation depletes NAD^+, the building block for NAD(P)H, it may be the exhaustion of NAD^+, not the increase of PAR polymer, that induces AIF release from the mitochondria during cell death.

5.3.2: Proteases

Recent studies also show that AIF is tightly associated with the mitochondrial inner membrane, and therefore for the complete release of AIF, it has to be proteolytically processed at the N-terminal (around aa 101-102) (Arnoult et al, 2002; Otera et al, 2005).

Proteases such as caspases and/or calpain have been suggested to be responsible for such cleavage (for review see (Cande et al, 2004a; Cregan et al, 2004)). In the presence of the pan-capsase inhibitor zVAD-fmk, AIF release was not observed in HeLa or 293T cells treated with staurosporine or Bax (Arnoult et al, 2003a; Arnoult et al, 2003b; Arnoult et al, 2002). Most of these studies used only one early time point to assess AIF release, but AIF release has been shown to be a late event in HeLa cells (Munoz-Pinedo et al, 2006). In addition, many studies using various cell types (including HeLa cells) and cell death insults indicate that AIF release cannot be repressed by caspase inhibitors (Cande et al, 2004a; Cao et al, 2007; Gallego et al, 2004; Kim et al, 2003; Loeffler et al, 2001; Sanges et al, 2006). Our results in chapter 2 and 3 clearly show that AIF release occurs in a caspase independent manner (Cheung et al, 2006; Cheung et al, 2005). The discrepency in the literature regarding AIF release can be explained by the fact that zVAD-fmk can also inhibit other non-caspase cysteine proteases, especially at a higher concentration (Yuste et al, 2005a). A study using a panel of different protease inhibitors at different concentrations show that AIF release is instead inhibited by calpain or cathepsin inhibitors (Yuste et al, 2005a). Calpain is a non-caspase cysteine protease that is mostly located in the cytosol and can be activated by Ca^{2+} during excitotoxicity (Amadoro et al, 2006; Chan & Mattson, 1999; Kim et al, 2002; Lankiewicz et al, 2000; Takano et al, 2005). Isolated mitochondria incubated with calpain I and tBid exhibited complete AIF release, which can be abrogated by calpain I inhibitor calpeptin (Polster et al, 2005). AIF is cleaved between aa 101-102 by calpain, which is located in the intermembrane space of mitochondria during oxygen-glucose deprivation (OGD) induced neuronal cell death. Most importantly, AIF with its cleavage site mutated (aa101-102) is not released during cell death and cannot resume OGD induced cell death in *Hq* neurons, indicating that this cleavage site is critical for the pro-apoptotic activity of AIF

(Cao et al, 2007). While the exact mechanism of calpain translocation from the cytosol into the mitocondria is unclear, the complete release of AIF from the mitochondria may require calpain mediated AIF cleavage during Ca^{2+} induced cell death.

5.4: Mechanisms of AIF mediated cell death

AIF translocates to the nucleus after its release from the mitochondria, and in the nucleus it can induce chromatin condensation and large scale DNA fragmentation (Cregan et al, 2004; Krantic et al, 2007). While AIF itself does not have any DNase or chromatin condensation activity (Mate et al, 2002; Ye et al, 2002), recombinant AIF can bind to DNA and induce peripheral chromatin condensation and large scale DNA fragmentation in isolated nuclei (Mate et al, 2002; Susin et al, 1999b; Vahsen et al, 2006; Ye et al, 2002). AIF may therefore recruit other proteins, such as EndoG and Cyclophilin A (CypA) to carry out the apoptotic functions once it binds to DNA. CypA is a peptidyl-proyl cis-trans isomerase (for protein refolding and chaperone activity) that resides in the cytosol and the nucleus. It has been shown that CypA can interact with AIF (Cande et al, 2004b). While the exact mechanism of CypA mediated cell death is not known, CypA deficient cells are more resistant to apoptosis induced by staurosporine. CypA knockout animals, while developmentally normal (Colgan et al, 2000), are more resistant to cerebral hypoxia-ischemia (Zhu et al, 2007). These results indicate that CypA may have a role in regulating AIF apoptotic activity, possibly by acting as a chaperone for AIF to efficiently translocate to the nucleus.

Endo G is a caspase independent apoptotic DNase in the mitochondria and is released during cell death (Li et al, 2001; Parrish et al, 2001; Widlak et al, 2001). The *Caenorhabditis elegans* (*C. elegans*) homologs of AIF and Endo G (WAH-1 and CSP-6) can interact with each other to promote DNA degradation (Wang et al, 2002). Translocation studies have shown that similar to AIF, Endo G is also translocated from the mitochondria to the nucleus after various neurological insults such as excitotoxicity, cerebral ischemia, and spinal cord injury (Henne et al, 2006; Lee et al, 2005; Yu et al, 2006a). Endo $G^{+/-}$ adult mice are more resistant to KA induced excitotoxic cell death at the hippocampal CA1 and CA3 region (Wu et al, 2004). While different models with reduced expression of Endo G have suggested a pro-apoptotic role (Bahi et al, 2006; Basnakian et al, 2006; Buttner et al, 2007; Ishihara & Shimamoto, 2006), MEFs and splenocytes from Endo $G^{-/-}$ mice are equally sensitive to cell death compared to wildtype. These mice develop and age normally, suggesting that Endo G is dispensible for cell death and mitochondrial function. (David et al, 2006; Irvine et al, 2005). It is worth nothing that Endo G expression is restricted to adult heart, muscle, brain and kidney - tissues that displayed reduced cell death when Endo G expression is reduced. Adult spleen and all embryonic tissues (including mesenchymal cells for MEF production), in contrary, have no Endo G expression, which may explain the lack of cell death phenotype observed in the knockout studies (Apostolov et al, 2007). The pro-apoptotic activity of Endo G may therefore depend on the type of tissue and the stage of development.

5.5: The vital role of AIF in mitochondria

Apart from its apoptotic role, AIF has recently been shown to have a vital role in cell survival from studies using mice with reduced AIF expression. $AIF^{-/-}$ mice die around E12.5 due to accelerated cell death starting around E9 (Brown et al, 2006; Joza et al, 2005). Muscle or brain specific AIF deficiency also induces cell loss in the tissues with no AIF expression (Cheung et al, 2006; Joza et al, 2005). *Harlequin (Hq)* mice, in which AIF expression is reduced to 20%, exhibit cerebellar and retinal degeneration starting at the age of three months (El Ghouzzi et al, 2007; Klein et al, 2002). All of these mouse models, therefore, indicate an essential vital role of AIF in maintaining cell survival. These animals have defective mitochondrial oxidative phosphorylation, in particular reduction of oxygen consumption and complex I expression (Brown et al, 2006; Cheung et al, 2006; Joza et al, 2005; Vahsen et al, 2004). In some studies, the loss of AIF has also been shown to increase the level of ROS (Apostolova et al, 2006; Klein et al, 2002; Urbano et al, 2005; van Empel et al, 2005; van Empel et al, 2006) which can be reduced by the addtion of antioxidants (Apostolova et al, 2006; Urbano et al, 2005). Based on these studies, AIF has been proposed to act as a stabilizer for complex I or possibly a ROS scavenger (Klein & Ackerman, 2003; Porter & Urbano, 2006). AIF, however, does not seem to be interacting with any redox proteins in the mitochondria (Vahsen et al, 2004), and as we will see in chapter 4, the presence of NAC (a ROS scavenger) cannot rescue AIF deficiency (Cheung et al, 2006). In addition, recent studies have shown that recombinant AIF has very low electron transfer activity and does not have ROS activity (Churbanova & Sevrioukova, 2007). All of these suggest that AIF may utilize its redox domain as a sensor for linking metabolism to other cellular processes, not as an electron carrier or ROS scavenger. In chapter 3 and 4 we have identified a novel role of AIF in maintaining mitochondrial structure through the mitochondrial fusion protein Opa1. Rather than acting as a ROS scavenger or complex I

stabilizer, AIF may use the redox domain to relay signal from the mitochondria to regulate the mitochondrial fusion machinery.

6: Mitochondrial structure: the fission and fusion machinery

Mitochondria are not static isolated bean-shaped elements that are often depicted in the classical cell biology text-books. In fact, the very word "mitochondrion" in greek means "thread" (mitos) and "granule" (khondrion), which alludes to the plastic and complex nature of the mitochondrial structure. Mitochondria undergo fusion to form widespread interconnected structures for energy production and signal transduction across the cell. In order for the mitochondria to travel long distances such as along the axons in neurons, they can be divided into smaller segments for fast and efficient trafficking to areas that require higher energy demand. The delicate balance of fission and fusion, therefore, are critical to maintain the proper structure and function of the mitochondria. Since neurons are highly polarized cells with long axons and high energy demands, the imbalance of fission and fusion caused by mutation of the fusion proteins can induce mitochondrial fragmentation and a variety of neurological disorders (Chan, 2006). Many of these diseases involve neurons that either have very long processes (e.g. the motoneurons) or are under higher levels of stress (e.g. retinal neurons). These neurons are therefore highly dependent on the correct functioning of the mitochondria and have lower tolerance to fission/fusion imbalance than other non-neuronal cells. Chapter 3 describes a similar scenario in which forebrain specific AIF deficient animals also exhibit neurodegeneration *in vivo* and spontaneous cell death *in vitro.* AIF may have a previously undiscovered role in maintaining mitochondrial structure via the fission/fusion proteins. Chapter 4 continues to explore the possible interaction

between the fission/fusion proteins and AIF via the fusion protein Opa1. In order to understand this novel role of AIF in maintaining mitochondrial structure, the following section will serve as an overview of the mitochondrial fission and fusion mechanisms.

6.1: Mitochondrial fission

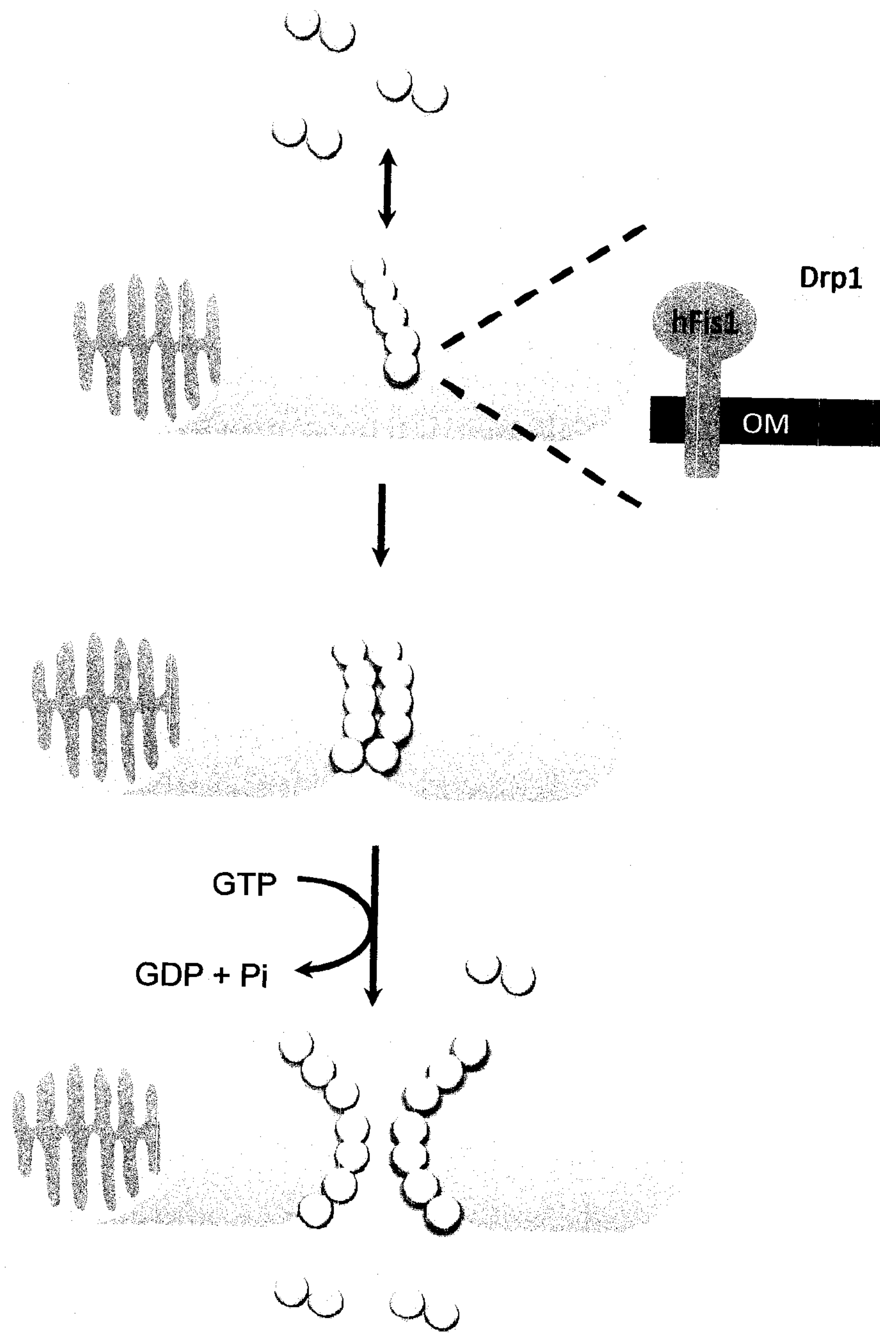
Mitochondrial fission is an ongoing process in healthy cells and is essential for cell division, calcium regulation and development (Chen & Chan, 2005; Jagasia et al, 2005; McBride et al, 2006; Youle & Karbowski, 2005) (Figure 1-6). In neurons, mitochondrial fission is critical for mitochondrial transport along axons and dendrites (Chang & Reynolds, 2006; Li et al, 2004). Immature neurons have smaller, more motile mitochondria; whereas mature neurons with established synaptic connections have longer and more stationary mitochondria which are strategically placed in regions of high energy demand (Chang & Reynolds, 2006). In addition to the physiological role of mitochondrial fission, recently fission has also been implicated in the cell death pathway (Heath-Engel & Shore, 2006; Perfettini et al, 2005; Youle & Karbowski, 2005). The mitochondrial division machinery, therefore, has a key role in the well-being of the cell during physiological situations as well as under stress.

Drp1 and hFis1 are the key proteins for mitochondrial fission in mammals (Hoppins et al, 2007; Okamoto & Shaw, 2005). Other mammalian proteins such as Endophilin B1, DAP3, GDAP1 and MTP18 may also participate in mitochondrial fission, but their exact roles in fission are still not clear (Mukamel & Kimchi, 2004; Niemann et al, 2005; Tondera et al, 2005). hFis1 is a tail-anchored outer mitochondrial protein that has its N terminal domain exposed to the cytoplasm. The N terminal contains a tetratricopeptide repeat (TPR)-like fold that can bind other proteins such as Mdv1/Caf4 in yeast which are the adaptors to

Dnm1 (yeast homolog of human Drp1) (Dohm et al, 2004; Griffin et al, 2005; Tieu & Nunnari, 2000; Zhang & Chan, 2007). Since Mdv1 and Caf4 homolog have not been found in mammalian systems, Drp1 may either interact directly to hFis1 or other adaptor proteins may be responsible for the interaction. Overexpression of hFis1 can induce mitochondrial fragmentation, whereas cells with reduced hFis1 have elongated mitochondria (James et al, 2003; Stojanovski et al, 2004). Drp1 is a cytosolic dynamin related GTPase. Drp1 is mostly cytosolic but it can assemble into punctuate structures on mitochondria, many of which are at the future fission sites (Ingerman et al, 2005; Okamoto & Shaw, 2005). A dominant negative form of Drp1 can inhibit mitochondrial fission, indicating an important role of Drp1 in mediating fission (Ishihara et al, 2003; Santel & Fuller, 2001). In yeast, the formation of Dnm1 is reduced in the absence of Fis1; however, in mammalian systems the lack of hFis1 seems to have no effect on Drp1 recruitment to mitochondria (Jofuku et al, 2005; Lee et al, 2004; Suzuki et al, 2003). In mammalian cells, a weak interaction between hFis1 and Drp1 has been found using a protein cross-linker (Yoon et al, 2003). It is possible that in mammalian systems, there are other proteins unrelated to the yeast Mdv1/Caf4p that act as an adaptor between Drp1 and hFis1 for Drp1 recruitment to mitochondria. Nevertheless, the mammalian Drp1 and hFis1 are the key players in the mitochondrial fission machinery.

Most of the experiments on how these fission proteins perform mitochondrial division were performed in yeast. In yeast, Dnm1 assembles and disassembles on the surface of mitochondria at spots where the future division occurs (Legesse-Miller et al, 2003; Naylor et al, 2006; Otsuga et al, 1998; Sesaki & Jensen, 1999). The targeting of Dnm1 is mediated by Fis1p using Mdv1/Caf4p as adaptors (Griffin et al, 2005; Zhang & Chan, 2007). Dnm1p is able to homo-oligomerize and form spirals around the mitochondria tubule which is driven

Figure 1-6: Mitochondrial fission. hFis1 on the outer membrane of the mitochondria may recruit Drp1 for the fission of mitochondria. The constriction of mitochondria by Drp1 is induced by the hydrolysis of GTP. For details see text.


Drp1
hFis1
OM
GTP
GDP + Pi

by its GTPase activity that is in turn stimulated by the Dnm1 self-assembly as dimers (Ingerman et al, 2005). While the exact mechanism is not yet clear, GTP hydrolysis may induce these spirals to constrict and cleave the mitochondria, which is similar to dynamin-1 mediated endocytosis (Danino et al, 2004; Roux et al, 2006; Zhang & Hinshaw, 2001). In contrast to yeast Dnm1, mammalian Drp1 is mostly diffusely distributed in the cytoplasm, and the self-assembly activity is significantly lower than Dnm1p. Also, Drp1 does not form spirals, but only smaller and simpler rings (Labrousse et al, 1999; Otsuga et al, 1998; Sesaki & Jensen, 1999; Smirnova et al, 2001; Yoon et al, 2001). It is therefore possible that while mammalian Drp1 may act similarly to the yeast Dnm1p in mitochondrial membrane constriction through homo-oligomerization into rings/spirals, the self assembly of Drp1 may be more complex than the yeast Dnm1p.

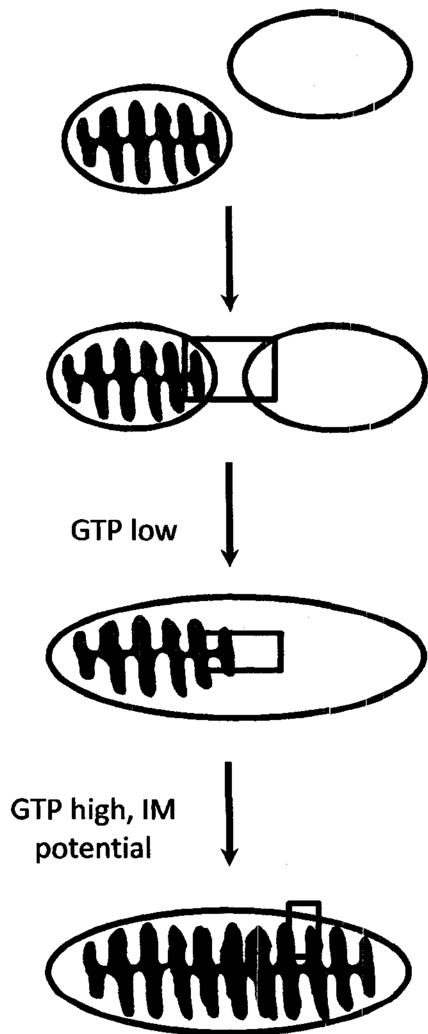
6.2: Mitochondrial fusion

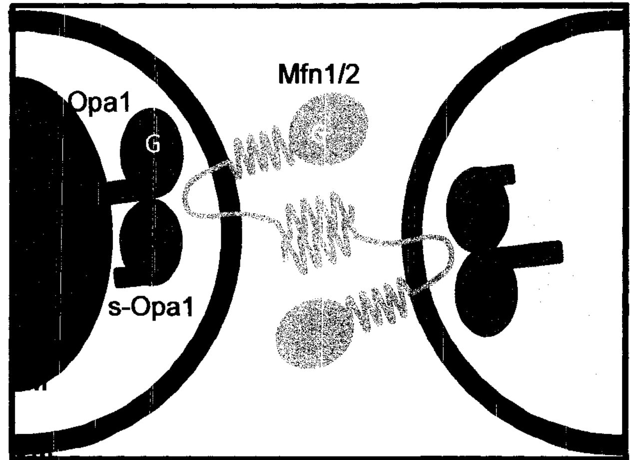
As with mitochondrial fission, the fusion machinery also plays an essential role in development and survival (Chan, 2006; Chen et al, 2005; Chen et al, 2003). Fusion is thought to protect mitochondrial function by allowing rapid mixing of membranes, mitochondrial DNA and soluble contents that may be damaged by localized deficits of substrates as a result of cellular stress (Chan, 2006; Detmer & Chan, 2007b). Recently, several studies indicate that during cell death mitochondrial fusion is inhibited and the activation of the fusion proteins could slow down the rate of cell death (Karbowski et al, 2004; Neuspiel et al, 2005; Sugioka et al, 2004). In this section we will focus on the mitochondrial fusion machinery in regulating mitochondrial structure.

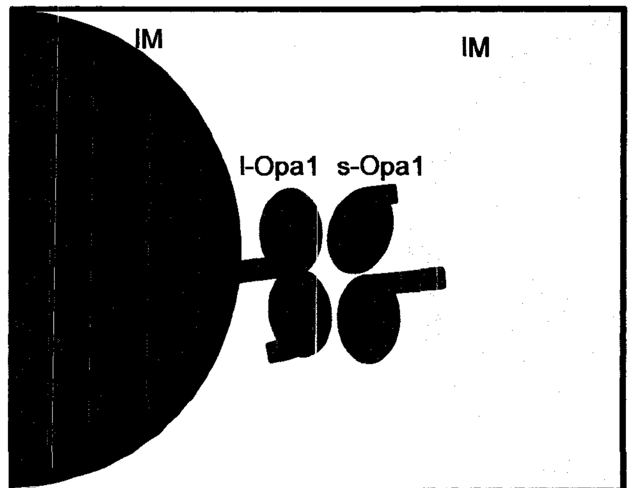
6.2.1: Mfn1 and Mfn2

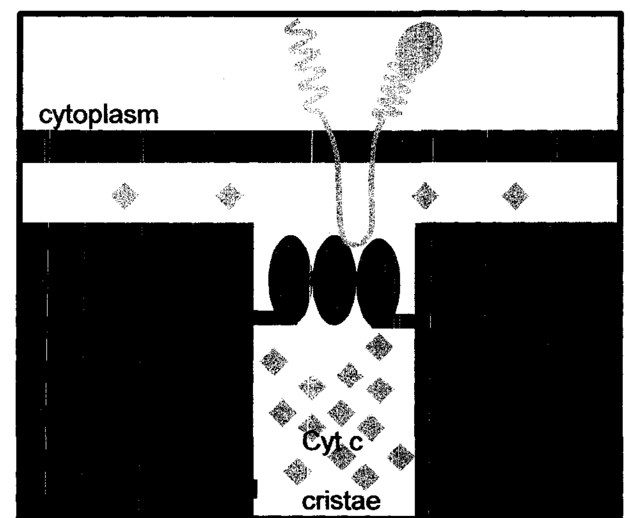
The proteins required for fusion in mammalian cells include Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2), and Optical atrophy 1 (Opa1). Mfn1 and Mfn2 (Fzo1 in yeast) are homologous dynamin related GTPases located on the mitochondrial outer membrane (OM) (Eura et al, 2003; Rojo et al, 2002; Santel et al, 2003) (Figure 1-7). They contain heptad repeats (coiled-coil formation domains) that are responsible for protein interaction, a GTPase domain, and two transmembrane domains (Koshiba et al, 2004; Rojo et al, 2002). The loss of Mfn1 or Mfn2 results in embryonic lethality in mice and the cells have fragmented mitochondria, lost of mitochondrial membrane potential and respiration, and loss of mtDNA (Chen et al, 2005; Chen et al, 2003). Mutations in Mfn2 causes Charcott-Marie Tooth Type 2a (CMT2a) neuromuscular degeneration in human (Zuchner et al, 2004) which underscores the importance of mitochondrial fusion in neuronal survival. Although Mfn1 and Mfn2 are homologues, there are some important functional differences. For example, Mfn1, but not Mfn2 genetically interacts with Opa1 for fusion, as well as having a higher GTPase activity than Mfn2 (Cipolat et al, 2004; Ishihara et al, 2004). Mfn2 deficient mice have a defect in the giant trophoblast layer of the placenta that causes the embryonic lethality whereas the Mfn1 deficient mice do not have this defect. Mfn1 deficient MEF cells also have a more severe mitochondrial defect compared to Mfn2 (Chen et al, 2003). This would suggest that Mfn1 plays a more essential role in mediating fusion than Mfn2 or they may regulate fusion through different pathways. While both Mfn1 and Mfn2 are important for mitochondrial fusion, a GTP hydrolysis deficient form of Mfn2 was shown to stimulate mitochondrial fusion in cultured cells, and protected cells from apoptosis induced by staurosporine (Neuspiel et al, 2005), which may indicate additional regulatory functions for Mfn2. These suggest that apart from the fusion role, Mfn2 may also have additional functions that may impact cellular survival. The roles of Mfn1 and Mfn2 in the survival of neurons have been

Figure 1-7: Mitochondrial fusion. Mitochondrial fusion is mediated by Mfn1/2 (on the outer membrane) and Opa1 (in the intermembrane space). For details see text.


GTP low
GTP high, IM
potential


Mfn1/2
Opa1
G
s-Opa1


IM
IM
l-Opa1
s-Opa1


cytoplasm
Cyt c
cristae

further explored using tissue specific conditional mutants and knock-in mutants using mutated allele that is commonly found in CMT2a patients. As whole embryo knockout causes lethality due to the placental defect, conditional mutants in the embryo but not the placenta were generated. Embryonic (E7) and post-natal (P7) cerebellum specific Mfn2 knockouts have cerebellar degeneration, especially the Purkinje cells, suggesting that Mfn2 is essential in maintaining the survival of cerebellar neurons (Chen et al, 2007). Most of the mitochondria in these neurons are clustered around the cell body and do not extend to the dendritic tracts, indicating that Mfn2 mediated fusion is also important in mitochondrial trafficking along the neurites (Chen et al, 2007). Correct localization of mitochondria is essential for proper dendritic formation (Li et al, 2004), however, the formation of dendrites was not assessed in these Mfn2 mutants. Expression of Mfn1 in these neurons *in vitro* can rescue the mitochondrial defects in the Mfn2 mutants, again highlighting the compensatory and overlapping function between Mfn1 and Mfn2. While the cause of neurodegeneration in these animals is still not clear, these animals have loss of mtDNA due to the lack of fusion and mixing of mitochondria. The lack of mtDNA may have direct impact on survival because most of the mitochondrial respiratory proteins are encoded by mtDNA (Chen et al, 2007). The roles of these Mfn proteins in neurodegeneration are further explored using Mfn2 knock-in mutants using the CMT2a allele Mfn2(R94Q), in which the GTPase domain is mutated (Detmer et al, 2008). Similar to the neurological deficits in CMT2a patients, these animals also have severe movement disorders. The Mfn2(R94Q) MEFs, however, have normal tubular mitochondria with no aggregation, indicating that the presence of wildtype Mfn1 in these MEFs can complement the null Mfn2(R94Q) allele. While the neuronal mitochondria were not observed in this study, it is tempting to speculate that the level of Mfn1 in the neurons are lower than MEFs, therefore hampers the complementation between

wildtype Mfn1 and the mutated Mfn2 (Detmer & Chan, 2007a). These studies highlight the vulnerability of neurons to defects in the fusion machinery and the precise amount of Mfn1 and Mfn2 activity is critical for survival.

6.2.2: Opa1

The third dynamin related GTPase required for mitochondrial fusion is Opa1 (Mgm1 in yeast) which is located in the intermembrane space (IMS) (Alexander et al, 2000b; Delettre et al, 2001b; Delettre et al, 2000b; Olichon et al, 2002b). Opa1 contains an N-terminal presequence for mitochondrial targeting that is cleaved after import, two hydrophobic segments, a GTPase domain essential for its function, and a middle domain (Sesaki et al, 2003b; Shepard & Yaffe, 1999; Wong et al, 2003). Mutations in Opa1 are causal for autosomal dominant optical atrophy, characterized by degeneration of the retinal ganglial cells in the eye resulting in blindness (Delettre et al, 2001a; Delettre et al, 2000a). This has been recently recapitulated in a mouse model that has a mutation in the GTPase domain (Davies et al, 2007). Opa1 deficient cells have dispersed fragmented mitochondria (Griparic et al, 2004; Olichon et al, 2003), indicating a key role of Opa1 in mitochondrial fusion. In addition, the mitochondria of these cells have dilated and disorganized cristae (Frezza et al, 2006; Griparic et al, 2004; Olichon et al, 2003). Opa1 is proteolytically processed into the long and short form, both of which are required for fusion as well as for the cristae formation by oligomerization of the two forms (Frezza et al, 2006; Meeusen et al, 2006; Song et al, 2007). The importance of Opa1 in normal cristae structure and function is further illustrated in yeast deficient in Mgm1 which have defective cristae, ATP synthase assembly and respiratory defects (Amutha et al, 2004). Further studies, however, are required to show that the loss of mitochondrial function in these Mgm1/Opa1 mutants is due

to the defective cristae, and not the loss of mitochondrial fusion which is essential to maintain mitochondrial DNA. In chapter 3 we will see that cells with deficient Opa1 have many similarities with AIF deficient neurons, and in Chapter 4 the novel role of AIF in maintaining mitochondrial structure through Opa1 is further explored.

Mgm1/Opa1 is processed into the long form (l-Mgm1/l-Opa1) and short form (s-Mgm1/s-Opa1) after targeting in the mitochondria. After the translocation of the newly synthesized Mgm1/Opa1 into the IMS of mitochondria, the protein is sorted into the inner membrane (Sesaki et al, 2006). The mitochondrial localization signal (MLS) of Opa1 directs the insertion of the protein into the IM through the TIM complex, until the first hydrophobic transmembrane domain of Mgm1/Opa1 is in the IM which pauses the insertion. At this point, the mitochondrial processing peptidase (MPP) in the mitochondrial matrix cleaves the MLS and forms the long form l-Mgm1/Opa1 (Escobar-Henriques & Langer, 2006). From this point, several proteases have been proposed to produce the short form s-Mgm1/Opa1 from the long form l-Mgm1/Opa1, including the rhomboid proteases, the inner membrane AAA protease, and the matrix AAA proteases.

The first mechanism involves the rhomboid protease Rbd1p/Pcp1p in yeast (McQuibban et al, 2003; Sesaki et al, 2003a). In the presence of high [ATP], the l-Mgm1 is further translocated into the matrix until the second transmembrane domain is in the IM and the cleavage site for Pcp1p is revealed. After this second cleavage by Pcp1p, s-Mgm1 is released into the IMS. Mitochondria of Pcp1p deficient yeast have structural and functional defects, which can be partially rescued by s-Mgm1 (Sesaki et al, 2006; Sesaki et al, 2003a). The partial rescue is possibly because Pcp1p is also required to process other proteins such as Ccp1 (cytochrome c peroxidase) (Michaelis et al, 2005). PARL, the mammalian homolog

of Pcp1p, is also essential for Opa1 processing. PARL$^{-/-}$ cells have a 50% decrease in s-Opa1 and Opa1 oligomers. Surprisingly, even though the level of s-Opa1 is reduced, the mitochondrial structure and respiration is not affected (Cipolat et al, 2006), meaning that other proteases may be responsible for the production of the other 50% of s-Opa1 seen in PARL mutant.

In addition to Pcp1p/PARL, other proteases can also cleave Opa1. The inner membrane AAA protease (i-AAA protease) Yme1 may process Opa1 (Griparic et al, 2007; Song et al, 2007). Similar to PARL knockout studies, siRNA of Yme1L does not induce major changes in mitochondrial structure (Griparic et al, 2007), again indicating that Opa1 processing may require more than one protease. The third protease for Opa1 processing is the hetero/homo-oligomeric matrix AAA protease (m-AAA protease). In humans, the subunits of m-AAA protease are AFG3L2 and Paraplegin, in which Paraplegin has been implicated in hereditary spastic paraplegia (HSP), a neurodegenerative disorder affecting mostly the corticalspinal axons (Casari et al, 1998). Loss of Parapelgin has been implicated in complex I deficiency, increased oxidative stress, and ribosome assembly in mitochondria (Atorino et al, 2003; Nolden et al, 2005). Paraplein interacts with Opa1, and overexpression of Paraplegin causes mitochondrial fragmentation whereas knockdown of paraplegin increases mitochondrial tubulation (Ishihara et al, 2006). Since m-AAA protease can exist either as homo- or hetero- oligomers, depending on the subunit components, the activity of the protease complex towards Opa1 may vary (Duvezin-Caubet et al, 2007). As different mammalian tissues have different m-AAA oligomer compositions (Koppen et al, 2007) and Opa1 splice variants (Delettre et al, 2001a), the importance of paraplegin in Opa1 processing may be highly tissue specific. Interestingly, PARL itself may also be cleaved by m-AAA

protease after the mitochondrial translocation for activation (Pellegrini & Scorrano, 2007). Taken together, the processing of Mgm1/Opa1 is critical in regulating mitochondrial structure and function, and the processing may be regulated by different sets of proteases in different types of tissues and organisms.

6.2.3: Mechanism of mitochondrial fusion

As mitochondria are double-membrane structures, both IM and OM fusions are required and two different mechanisms may be involved (Figure 1-7). *In vitro* fusion assays indicate that while GTP is required for the fusion of both membranes, OM fusion requires the IM proton gradient while IM fusion requires the electrical component of the membrane potential. These also suggest that IM and OM fusions are separable events (Meeusen et al, 2004). The importance of Mgm1/Opa1 in IM fusion is supported by studies involving temperature sensitive yeast Mgm1 mutant (Mgm1(ts)). These mutants undergo normal OM fusion at the nonpermissive temperature, but the IM is not fused, resulting in mitochondria with three or more matrices surrounded by one big OM (Meeusen et al, 2006). EM analysis of Mgm(ts) over a time course showed that IM cristae defects occur before fragmentation of mitochondria, suggesting that the OM fusion may depend on the integrity of the IM cristae structure which is controlled by Mgm1/Opa1 (Meeusen et al, 2006). Mitochondrial fusion therefore, seems to be dependent on both IM and OM fusions which require different sets of protein machineries: Opa1 for IM and Mfn1/2 with Opa1 for OM.

For the OM fusion, Mfn1 and Mfn2 seem to be acting in *trans* for the tethering and the fusion of membranes. Mitochondria are tethered when the heptad domain of Mfn1/2 in one mitochondrion forms an anti-parallel coiled-coil with the heptad domain of another

Mfn1/2 protein on the other mitochondrion (Hoppins et al, 2007; Koshiba et al, 2004; Okamoto & Shaw, 2005). The tethering and fusing requires GTPase activity, however the exact role of GTP hydrolysis remains unclear (Chen et al, 2003; Koshiba et al, 2004). Since the GTPase activity of Mfn1 and 2 are not the same (Ishihara et al, 2004), and they are able to form either homo or heterodimers (Chen et al, 2003), regulation of OM fusion could be complex and may depend on the levels of these proteins in various tissues. Apart from IM fusion, Opa1 may also contribute to OM fusion as Opa1 may cooperate with Mfn1 to fuse mitochondria (Cipolat et al, 2004). Interestingly, the intermembrane domain of Fzo1 (yeast Mfn1/2) is critical for fusion, suggesting that this domain may be responsible for interacting with IMS proteins such as Opa1/Mgm1 (Fritz et al, 2001) or other undiscovered proteins in the IMS. In yeast, the adaptor protein Ugo1 links Mgm1 and Fzo1 for fusion, but the mammalian homolog of Ugo1 has not yet been found (Sesaki & Jensen, 2004; Wong et al, 2003). Taken together, Mfn1 and 2 mediate membrane tethering and fusion in *trans* by homo/heterodimerization of Mfn1/2 between two adjacent mitochondrial membranes. How these complexes mediate the mixing of lipids remains to be discovered.

6.4: Mitochondrial cristae structure and cell death

Apart from the dividing and fusing of the mitochondria, the mitochondrial cristae are remodeled (diulated) during cell death. In healthy mitochondria, the relatively tight cristae junction connects to the tubular and lamellar cristae. The morphological control of this cristae structure may generate a gradient of metabolites and substrates which may regulate the rate of mitochondrial respiration. This tight cristae junction also provides a barrier for cyt c such that approximately 80% is in the cristae (Frey & Mannella, 2000; Mannella, 2006a; Mannella, 2006b). Isolated mitochondria treated with the pro-apoptotic tBid induces

dilation of cristae junction which enables cyt c mobilization from the cristae to the IMS, and thereby assist in Bax/Bak dependent release of cyt c through MOMP (Scorrano et al, 2002). BIK mediated cell death through the ER pathway also exhibits cristae remodeling, which is dependent on Drp1 and Ca^{2+} ions from the ER (Germain et al, 2005). These studies indicate a critical role of mitochondrial cristae remodeling in cell death, particularly the release of cell death factors.

Mitochondrial cristae remodeling during cell death may be regulated by Opa1 (Cipolat et al, 2006; Frezza et al, 2006). Opa1 oligomers between the long and short form are essential for the formation of the tight cristae junction. After tBid treatment, these Opa1 oligomers were lost and as a result the tight tubular cristae are dilated and cyt c is mobilized, which accelerates cell death progression. This is in agreement with previous Opa1 siRNA studies showing dilated cristae junction in mitochondria (Griparic et al, 2004). Opa1 overexpression therefore can reduce cell death by maintaining the tight cristae junctions, which is independent of Mfn1/2 (Frezza et al, 2006). The role of Opa1 in cell death is further supported by studies in PARL. $PARL^{-/-}$ MEFs are more sensitive to cell death due to the lack of s-Opa1 and Opa1 oligomers. After tBid treatment, $PARL^{-/-}$ mitochondria have faster cristae remodeling and cyt c release, again suggesting that Opa1 oligomer is essential for maintaining the tight cristae as a barrier of cyt c. It is not clear how tBid can disassemble Opa1 oligomers. It has been suggested that tBid interacts with cardiolipin at contact sites between IM and OM, and this interaction induces cristae morphological changes (Kim et al, 2004), possibly by tBid's lipid transfer activity (Degli Esposti & Dive, 2003) or tBid induced alteration of membrane curvature (Epand et al, 2002a). Opa1 release from mitochondria has also been observed, and this may lead to the disruption of Opa1 oligomers (Arnoult et al, 2005). Other proteins that regulate cristae morphology, such as Mitofilin which can form

oligomers (John et al, 2005) and ATP synthase subunit e/g (Arselin et al, 2004; Paumard et al, 2002) may also be involved. Taken together, mitochondrial cristae undergo extensive morphological changes during cell death and this facilitates the complete release of pro-apoptotic proteins in the IMS. In chapter four we will see that the interaction between AIF and Opa1 to control mitochondria cristae structure may indicate a signaling role of AIF to induce cristae remodeling via Opa1 during cell death induction.

7: Hypothesis

As discussed above, the pro-apoptotic role of AIF has been demonstrated in various systems, including acutely induced neuronal cell death. The recent studies of AIF deficient animals and drug induced caspase inhibition during cell death, however, have suggested alternative views regarding the importance of AIF's cell death role. The direct role of AIF in executing caspase independent cell death was questioned because in some situations caspase inhibitor can inhibit AIF release, and cells lacking AIF exhibit spontaneous cell death. The vital role of AIF in the mitochondria is also not yet clear, since no AIF binding partners in the mitochondria have been found, and also no direct mechanism has been determined. Hence, with these questions in mind, I have proposed the following hypotheses which will be tested in this thesis:

Hypothesis 1: AIF has a critical pro-apoptotic role in executing caspase dependent and caspase independent neuronal cell death in vitro and in vivo. (Chapter 2)

Hypothesis 2: The pro-apoptotic role of AIF is direct, not due to the loss of its mitochondrial function during cell death. (Chapter 3)

Hypothesis 3: The vital role of AIF is to control mitochondrial structure and function through interacting with the mitochondrial fusion protein Opa1. (Chapter 3 and 4)

Chapter II

Cheung et al. (2005) AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. **Journal of Neuroscience**. 25(6): 1324-34.

This first manuscript established the critical role of AIF in DNA damage and excitotoxic neuronal cell death *in vitro*. The importance of AIF in executing excitotoxic neuronal cell death is further underscored by an *in vivo* model of excitotoxicity. This paper is the first to directly demonstate the role of AIF in neuronal cell death using animals with reduced AIF.

The experiments in this manuscript were designed and performed by E.C. Cheung, assisted by J.L. Vanderluit and K.L. Ferguson for the *in vivo* studies. W.C. McIntosh provided technical assistance for the kainic induced seizure model. Dr. S.P. Cregan performed the Bax experiments. L. Melanson-Drapeau from the laboratory of Dr. S.A. Bennett demonstrated and provided advice for the *in vivo* experiments. Dr. D.S. Park provided feedback. Reagents were provided as outlined in the text. This manuscript was written under the guidance and editorial assistance of Dr. R.S. Slack.

AIF IS A KEY FACTOR IN NEURONAL CELL DEATH PROPAGATED BY BAX-DEPENDENT AND BAX-INDEPENDENT MECHANISMS

Eric C. C. Cheung[1], Lysanne Melanson-Drapeau[2], Sean P. Cregan[1v], Jacqueline L. Vanderluit[1], Kerry L. Ferguson[1], William C. McIntosh[1], David S. Park[1], Steffany A. L. Bennett[2], and Ruth S. Slack[1*]

[1]Ottawa Health Research Institute – Neuroscience Center and Department of Cellular and Molecular Medicine; [2]Neural Regeneration Laboratory, Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5.

***Correspondence:**
Ruth S. Slack, Ph.D.
Ottawa Health Research Institute,
University of Ottawa,
451 Smyth Road,
Ottawa, Ontario,
Canada K1H 8M5
Email: rslack@uottawa.ca

[v]Current Address: Robarts Research Institute, Cell Biology Group, PO Box 5015,
100 Perth Drive London, ON, N6A 5K8

Key words

AIF, excitotoxicity, neuron, caspase independent, kainic acid, apoptosis.

Running Title

AIF in neuronal cell death

Acknowledgements

We would like to thank Nicole Arbour and André Fortin for critical reading of this manuscript. This work is supported by grants from the Canadian Institutes of Health Research (CIHR), the Heart and Stroke Foundation of Canada (HSFC) and the Canadian Stroke Network (CSN) to R.S.S, and CIHR and NSERC to S.A.L.B.. The adenovirus core facility is supported by a grant from CSN to R.S.S. and D.S.P. L.M.D. is supported by a Canadian Graduate Scholarship. S.P.C. was supported by a CIHR postdoctoral fellowship. J.L.V. is supported by a HSFC fellowship. K. L. F. is supported by a CIHR studentship. D.S.P. is a Glaxo-Welcome Professor. S.A.L.B. is an Ontario Mental Health Foundation Intermediate Investigator and a CIHR new investigator.

Abstract

Mitochondria release proteins that propagate both caspase dependent and caspase independent cell death pathways. Apoptosis inducing factor (AIF) is an important caspase independent death regulator in multiple neuronal injury pathways. Presently there is considerable controversy as to whether AIF is neuroprotective or proapoptotic in neuronal injury such as oxidative stress or excitotoxicity. To evaluate the role of AIF in BAX dependent (DNA damage induced) and BAX independent (excitotoxic) neuronal death, we used Harlequin (*Hq*) mice, which are hypomorphic for AIF. Neurons carrying double mutations for *Hq/Apaf1*$^{-/-}$ are impaired in both caspase dependent and AIF mediated mitochondrial cell death pathways. These mutant cells exhibit extended neuroprotection against DNA damage as well as glutamate-induced excitotoxicity. Specifically, AIF is involved in NMDA and kainic acid but not AMPA induced excitotoxicity. *In vivo* excitotoxic studies using kainic acid induced seizure showed that *Hq* mice had significantly less hippocampal damage than wild-type littermates. Our results demonstrate an important role for AIF in both Bax dependent and Bax independent mechanisms of neuronal injury.

Introduction

Mitochondria relay signals for both caspase dependent and caspase independent death pathways. The caspase dependent pathway is initiated by release of cytochrome c which associates with APAF1 to activate caspases (Danial & Korsmeyer, 2004). One key mitochondrial protein in caspase-independent death pathway is the flavoprotein apoptosis inducing factor (AIF) (Cregan et al, 2004). In healthy cells, mitochondrial AIF may protect against oxidative stress (Klein et al, 2002). Once cell death is initiated, however, AIF translocates to the nucleus to execute DNA fragmentation that culminates in cell death (Cande et al, 2002a; Cande et al, 2002b; Lorenzo et al, 1999).

Previous studies suggested that AIF may be important in neuronal injury. AIF translocates to the nucleus following DNA damage, excitotoxicity and *in vivo* ischemia (Joza et al, 2001; Plesnila et al, 2004; Susin et al, 1999b). Microinjection of AIF neutralizing antibodies decreased BAX-mediated neuronal apoptosis when caspases are inhibited (Cregan et al., 2002). PARP1 activation is required for AIF translocation in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induced damage or NMDA induced excitotoxicity as AIF fails to translocate in *Parp1*$^{-/-}$ neurons (Wang et al, 2003; Yu et al, 2003; Yu et al, 2002). PARP1 is over-activated in ischemia-reperfusion injury (Eliasson et al, 1997; Endres et al, 1997) and excitotoxicity (Mandir et al, 2000; Zhang et al, 1994). Consistently, the absence of PARP1 protects against excitotoxicity and cerebral ischemia (Moroni et al, 2001; Ying et al, 2001). These studies suggest that AIF is important in several models of acute neuronal injury.

A recent study with mice having greater than 80% reduced AIF expression, the "Harlequin" (*Hq*) mice, suggest that AIF may also protect against oxidative stress (Klein et

al, 2002). Presently the role of AIF as a death executor or as an oxidative stress scavenger is controversial. There are limited studies examining loss of AIF since *Aif*$^{-/-}$ mice die at E3 (Joza et al, 2001). In *Hq* mice, instead of supernumerary cells due to failed apoptosis, surprisingly their cerebellum degenerates progressively and *Hq* neurons are more sensitive to oxidative stress. While AIF has been suggested as a death inducer in excitotoxicity, *Hq* neurons are more sensitive to 2 mM glutamate treatment (Klein et al, 2002). This suggests that in addition to inducing apoptosis, AIF may also protect against several types of neuronal injury.

To resolve the role of AIF in different mechanisms of neuronal injury, we asked whether AIF reduction in *Hq* mice could protect neurons against: (a) BAX mediated, DNA damage induced apoptosis; and, (b) BAX independent, NMDA receptor mediated excitotoxicity (Dargusch et al, 2001; Miller et al, 1997). Using *Hq/Apaf1*$^{-/-}$ mice, we show that reduced AIF confers sustained protection in DNA damage-induced neuronal cell death when caspase activity was inhibited. Furthermore, *Hq* neurons exhibit a striking resistance specifically to NMDA and kainic acid induced excitotoxicity. A reduced AIF level also protects hippocampal neurons *in vivo* against kainic acid induced seizure. Our results demonstrate that AIF plays an important proapoptotic role in neuronal injury involving both BAX dependent and BAX independent cell death pathways.

Materials and methods

Mice and primary neuronal cultures

Hq, *Apaf1*$^{-/-}$ and *Bax*$^{-/-}$ mice were maintained and genotyped as previously described (Fortin et al, 2001; Klein et al, 2002). Cortical neurons and cerebellar granule neurons (CGNs) were cultured from cortices of E15.5 mice and cerebellums of P7 mice respectively as previously described (Fortin et al, 2001). Recombinant adenoviral vectors carrying human AIF or LacZ expression cassettes were prepared and used at 50 multiplicity of infection (MOI) as previously described (Cregan et al, 2002).

Camptothecin treatment and cell viability assays

Neurons were treated with 10 μM camptothecin with or without 10 μM Boc-aspartyl (Ome)-fluoromethylketone (BAF, Enzyme System Products) after 2 days *in vitro* (DIV). Cell survival was measured by: Live/Dead staining (Molecular Probes Inc.), Hoechst staining, MTT assay (Cell Titer Kit, Promega, Madison, WI), TUNEL assay, and PI staining, as previously described (Cregan et al., 2002). Caspase activity assay was performed as previously described (Cregan et al, 2002). Active caspase 3 immunohistochemistry (BD Biosciences, 1:250) was performed according to manufacturer's instructions. AIF immunohistochemistry was performed as previously described (Cregan et al, 2002). For Live/Dead assay, survival was determined as the fraction of cells exhibiting positive staining for calcein-AM over the total cell number per field. For Hoechst staining, dead cells were indicated by the characteristic nuclear morphology of chromatin condensation. Representative samples were photographed using ZEISS Axiovert 100 with a Northern Eclipse Sony Power HAD 3CCD color video camera.

Mitochondrial membrane potential

Loss of mitochondrial membrane potential was determined in unfixed cells using the membrane potential–dependent dye Mitotracker CMX-Ros and TMRE (Molecular Probes Inc.) as previously described (Cregan et al, 2002; Susin et al, 1999b). These two types of fluorescent dyes are selectively incorporated into mitochondria with an intact transmembrane potential and therefore serve as an indicator of mitochondrial depolarization. Unfixed cells were incubated with CMX-Ros at 0.25 μM and TMRE at 50nM in fresh media for 30 min at 37°C, washed in fresh media, and images were captured as described above. The fraction of cells maintaining mitochondrial transmembrane potential was determined by counting CMX-Ros positive or TMRE positive cells and expressed as a percentage of total cell number in corresponding phase images.

Excitotoxicity

Cortical neurons were used in excitotoxic experiments after 14 DIV. After 2 DIV cultures were treated with 10μM Ara-C to inhibit glial growth, and at 7 DIV received a half change of the neuralbasal media prepared as previously described (Fortin et al, 2001). At 14 DIV, sister cultures were treated under the following conditions: a) NMDA (N-methyl-D-aspartic acid): 100 μM NMDA and 10 μM glycine; b) for AMPA (alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid): 100 μM AMPA, 100 μM cyclothiazide (6-chloro-3,4-dihydro-3-(5-norbornen-2-yl)-2H-1,2,4-benzothiazidiazine-7-sulfonamide-1,1-dioxide), and 10 μM MK-801 ((5S,10R)-(1-5-methyl-[3,7]-10,11,-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine); c) for kainic acid ((2S,3S,4R)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid): 100μM kainic acid, 10 μM Con A, 20 μM GYKI 52466 (4-(8-methyl-9H-1,3-dioxolo

[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride), and 10 μM MK 801; d) for glutamate: 100 μM glutamate. After one hour the medium was completely removed and replaced with conditioned medium from untreated cells. Cell viability was assessed as described above. All reagents, unless otherwise specified, were purchased from Sigma-Aldrich.

Kainic acid induced seizure in vivo

Male *Hq* and wild-type littermates of at least two months of age were injected i.p. 30 mg/kg kainic acid (OPIKA-1™, Ocean Produce, Nova Scotia, Canada) which was dissolved in 10mM PBS (Phosphate Buffer Saline) in a total volume of 10 ml/kg. Seizure intensity was quantified using a previously published 5 point behavioral scale (Bennett et al, 1995; McIntyre et al, 1982). To minimize suffering and prevent mortality, animals were sedated with 5 mg/kg diazepam once they had reached stage 4 criteria for 5 min. Mice were euthanized 4 or 7 days (d) after kainic acid injection and their brains cut into 14 μm sections as previously described (Ferguson et al, 2002). To evaluate apoptotic and necrotic neuronal death, FluoroJade staining (Schmued et al, 1997), methyl green pyronine Y (MGPY) staining (Moffitt, 1994), and cresyl violet (Bennett et al, 1995) were used. Fluorojade staining was performed as previously described (Ferguson et al, 2002). In MGPY staining sections, DNA is stained blue and RNA is stained pink. Viable cells exhibited a light blue nucleus, confirming nuclear integrity, and a pink cytoplasm, indicative of RNA transcription. Damaged cells exhibited hyperchromatic nucleus and/or lack of cytoplasmic (RNA) staining (Al-Hazzaa & Bowen, 1998). In cresyl violet staining, damaged cells appeared pyknotic, eosinophilic or hyperchromatic with amorphous or fragmented nuclei.

Healthy cells had with oval nuclei, prominent nucleoli lacking eosinophilic cytoplasm and homogenously staining (Bennett et al, 1995; Bennett et al, 1998). All reagents, unless specified otherwise, were purchased from Sigma-Aldrich.

Quantification and Statistical Analysis

For *in vitro* studies, a minimum of 500 cells per field were scored for each treatment at indicated time points. For *in vivo* studies, 10 sections of similar level were counted for the fluorojade and MGPY staining. The authors were blinded to the experiments they performed. The data represented the mean and standard deviation from at least three independent experiments (n=3). p values were obtained using two way ANOVA and Fisher's post hoc tests. A p value < 0.05 was considered significant.

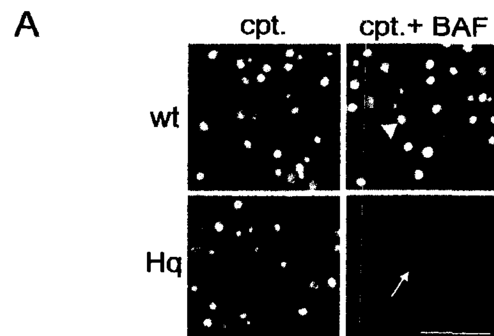
Results

Simultaneous inhibition of AIF and caspases provides extended neuroprotection against BAX-mediated apoptotic pathways

We have previously shown that AIF is involved in neuronal cell death using microinjection of AIF neutralizing antibodies (Cregan et al, 2002). To ask if AIF is required for BAX dependent and BAX independent mechanisms of neuronal cell death, we utilized primary neuronal cultures from *Hq* mice, which have less than 20% of AIF expression (Klein et al, 2002). We first assessed the requirement of AIF in BAX dependent apoptosis induced by DNA damage. Cortical neurons cultured from *Hq* and wild-type littermates were treated with 10 μM camptothecin and cell survival was assessed by nuclear morphology (Fig. 1A). Beginning at 12 hours (h) after treatment, a similar loss of survival was observed in both *Hq* and wild-type littermates (Fig. 1B). To ask whether inhibition of both mitochondrial pathways would provide added protection, total caspase activity was blocked using the broad-spectrum caspase inhibitor BAF. In the presence of BAF, *Hq* cortical neurons were more resistant to camptothecin-induced apoptosis relative to *Hq* alone or BAF treated wild type neurons (Fig. 1A and B). Significantly, BAF treated *Hq* neurons were still present in culture even after 48 h exhibiting drastically increased number of surviving neurons relative to wild-type (Fig. 1B, *Hq* neurons: 60% survival versus wild-type: 3%). A similar trend was also found with cerebellar granule neurons (CGNs) exposed to DNA damage (data not shown).

To assess the apoptotic pathways involved, we used a genetic approach whereby *Hq/Apaf1*$^{-/-}$ double mutant mice were generated. In these mutant neurons both

Figure 2-1: Reduced AIF in *Hq* mice protects neurons from camptothecin-induced neuronal cell death in the absence of caspase activity. Cortical neurons from wild-type and *Hq* littermates were treated with camptothecin (10 μM) and with or without BAF (10 μM). A) Photomicrographs of neurons after 36 h of treatment. Bar = 50μm. Neurons were stained with Hoechst 33258 to assess nuclear morphology. Arrow indicates morphologically healthy nuclei and arrowhead indicates pyknotic condensed nuclei that denote cell death. (B) Quantitative analysis of neuronal survival rate at the indicated time points after camptothecin treatment with or without BAF. Survival as determined by nuclear morphology is reported as a percentage of live cells over the total number of cells (n=3).


A
cpt.
cpt.+ BAF
wt
Hq

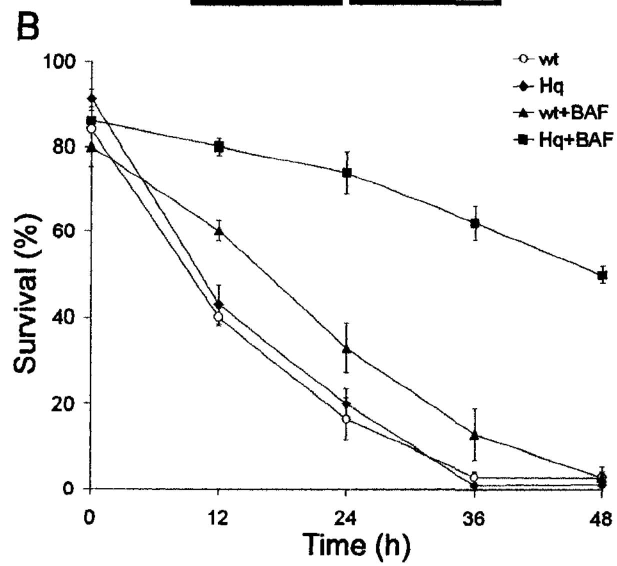
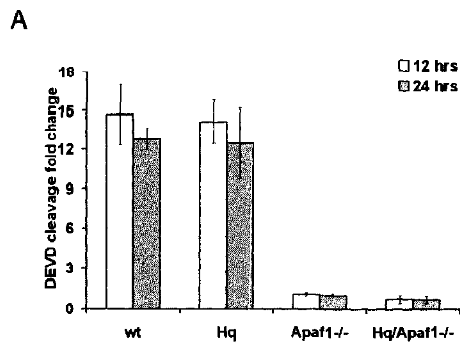
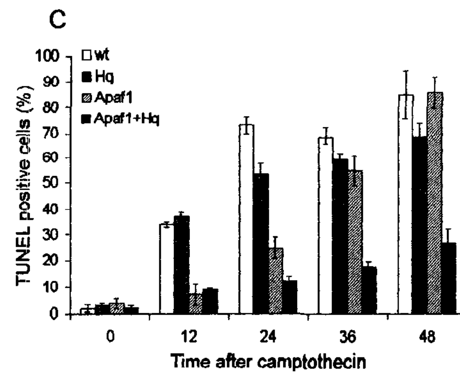

B
Survival (%)
100
80
60
40
20
0
0
12
24
36
48
Time (h)
wt
Hq
wt+BAF
Hq+BAF

Figure 2-2: Caspase activation and DNA fragmentation induced by camptothecin is reduced in *Apaf1*$^{-/-}$ and *Hq/Apaf1*$^{-/-}$ neurons. Cortical neurons cultured from wild-type, *Hq*, *Apaf1*$^{-/-}$ and *Hq/Apaf1*$^{-/-}$ littermates were treated with camptothecin (10 μM). (A) Caspase activity was measured by DEVD-AFC cleavage (n=3). (B) Immunocytochemistry of active caspase 3 and nuclear staining by Hoechst at 24 h. (C) Quantification of TUNEL staining (n=3). (D) TUNEL staining and nuclear staining by Hoechst at 24 h. Bar = 50μm.

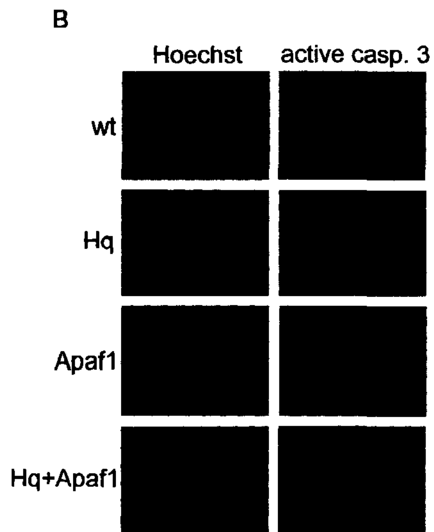
A



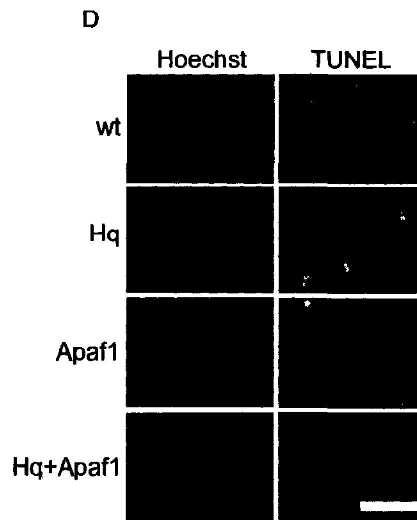
C



B

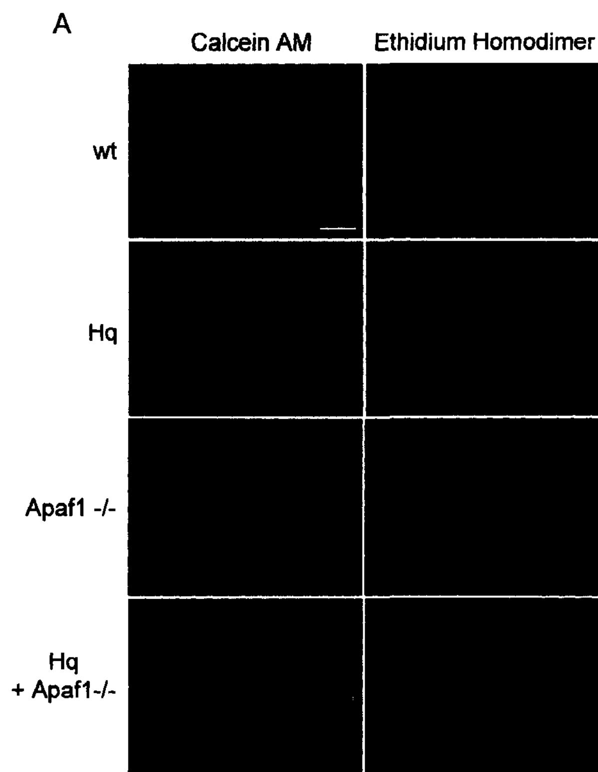


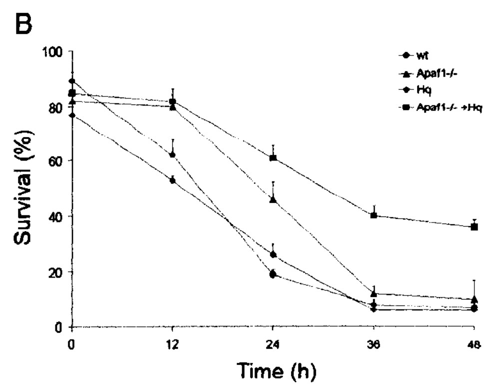
D

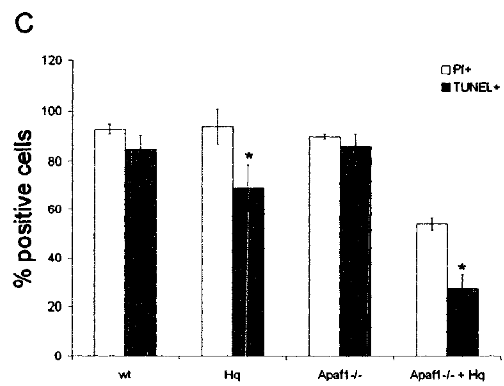


mitochondrial-initiated pathways, caspase dependent and caspase independent, were simultaneously impaired. We have previously shown that *Apaf1*$^{-/-}$ neurons remained viable only in the first 24 h following camptothecin treatment, after which survival rapidly decreased. This delayed death in *Apaf1*$^{-/-}$ cells was caspase independent and could not be inhibited by BAF (Cregan et al, 2002). Neurons were cultured from *Hq/Apaf1*$^{-/-}$ double mutant embryos that were viable up to E15.5, similar to that seen with *Apaf1*$^{-/-}$ alone. As previously described, neurons from *Hq* mice revealed an approximate 80% reduction in AIF protein expression (result not shown). In wild-type and *Hq* neurons, the DNA damaging agent, camptothecin, induced caspase activation as indicated by DEVD cleavage (Fig. 2A) and active caspase 3 immunohistochemistry (Fig. 2B). Consistent with our previously published work (Fortin et al, 2001), no significant caspase activation could be detected in *Apafl*$^{-/-}$ and *Hq/Apaf1*$^{-/-}$ neurons (Fig. 2A,B). Following a similar trend, TUNEL staining was similar in wild type and *Hq* neurons while Apafl deficiency resulted in a transient reduction in TUNEL staining evident at 12 and 24 h. Previously we have shown that the level of DNA fragmentation in *Apaf1*$^{-/-}$ neurons revealed by TUNEL staining is similar with or without the addition of the pan-caspase inhibitor, BAF. This suggests that DNA fragmentation revealed by TUNEL staining in *Apaf1*$^{-/-}$ neurons results from caspase-independent mechanisms (Cregan et al, 2002). The double *Hq/Apaf1*$^{-/-}$ mutants exhibited a sustained delay in apoptotic DNA fragmentation such that only 27% of cells were TUNEL positive relative to 85-86% seen in wild type and *Apaf1*$^{-/-}$ neurons (Fig. 2C). These data demonstrate a striking and sustained reduction in apoptotic DNA fragmentation in neurons in which both mitochondrial apoptotic pathways are impaired.

Figure 2-3: Inhibition of caspases and AIF in *Hq/Apaf1*$^{-/-}$ double mutants increases the survival of cortical neurons against camptothecin induced cell death. Cortical neurons from wild-type, *Apaf1*$^{-/-}$, *Hq* and *Hq/Apaf1*$^{-/-}$ littermates were treated with camptothecin (10 μM). Neuronal survival was measured by Live/Dead assay. Live cells exhibit staining for calcein AM activity (green fluorescence), whereas dead cells stain positive for ethidium homodimer (red fluorescence). (A) Neurons after 36 h of camptothecin treatment with Live/Dead staining. Bar = 100μm. (B) Quantitative analysis of survival rate measured by Live/Dead assay after camptothecin treatment (n=3). (C) Quantitative analysis of propidium iodide positive (PI^+) and TUNEL positive neurons 48h following camptothecin treatment (n=3). * $P<0.01$ compared to PI^+.


A
Calcein AM
Ethidium Homodimer
wt
Hq
Apaf1 -/-
Hq
+ Apaf1-/-


B
Survival (%)
Time (h)
wt
Apaf1-/-
Hq
Apaf1-/- +Hq


C
% positive cells
PI+
TUNEL+
wt
Hq
Apaf1-/-
Apaf1-/- + Hq

Previous studies have suggested that AIF may play a key role in facilitating DNA fragmentation during apoptosis. Since TUNEL staining and DNA condensation was severely reduced in *Hq/Apaf1*$^{-/-}$ neurons, cell viability was evaluated by two alternative methods that do not rely on DNA fragmentation. First, Live/Dead staining was used which measures: (a) cell viability, by the enzymatic conversion of the nonfluorescent calcein-AM to the fluorescent calcein in healthy cells; simultaneously with (b) cell death by propidium iodide (PI) uptake to evaluate loss of membrane integrity during apoptosis. Consistent with DNA fragmentation assays, cell viability was highest in *Hq/Apaf1*$^{-/-}$ double mutants and this enhanced viability was sustained for at least 48 h (*Hq/Apaf1*$^{-/-}$ 36% survival at 48 h versus 6-10% for all other genotypes; Fig. 3A,B). Since AIF through association with endonucleases is proposed to be responsible for DNA fragmentation during apoptosis (Parrish et al, 2003; Wang et al, 2002), we asked the question as to what proportion of the *Hq* neurons that go on to die exhibit DNA fragmentation. To assess this, we compared the number of cells exhibiting DNA fragmentation with the number of dying cells as determined by PI staining. Our results revealed that cells lacking AIF exhibited a defect in DNA fragmentation during apoptosis. Specifically, *Hq/Apaf1*$^{-/-}$ double mutants revealed a 54% loss of viability based on PI staining but only 27% of total cells exhibited DNA fragmentation (TUNEL) at 48 hours (Fig. 3C). Thus, of the dying cells, only 50% (27% TUNEL : 54% PI) exhibited DNA fragmentation relative to 91% (85% TUNEL : 93% PI) seen in wild type neurons. Indeed, neurons carrying the *Hq* mutant alone exhibited reduced TUNEL staining (60 % of dying cells) despite the presence of intact caspase pathways (Fig. 3C and 2B). These results demonstrate that: (a) based on PI staining neurons impaired in both mitochondrial pathways exhibit sustained viability even after 48 hrs following camptothecin treatment; and (b) of the cells that go on to die in *Hq/Apaf1*$^{-/-}$ neurons, there is a striking reduction of DNA

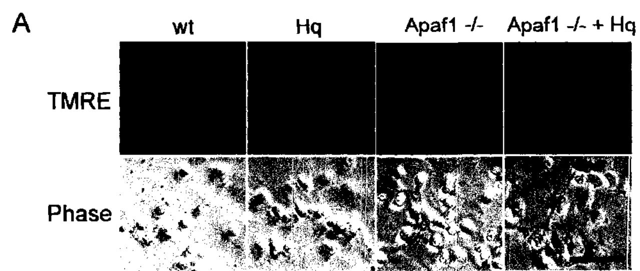
fragmentation during cell death. These results strongly support a function for AIF in caspase independent DNA fragmentation during the execution phase of cell death.

As a second measure of cell viability, independent of DNA fragmentation we evaluated the retention of mitochondrial membrane potential. The indicator dye TMRE, which can only be taken up by mitochondria with an intact membrane potential, was used. At time 0, neurons from all the genotypes exhibited similar levels of TMRE staining (83%-86%) indicating that mitochondrial function of the different genotypes is similar. Consistent with our results obtained by PI staining, many of these double mutant neurons were found to retain their mitochondrial membrane potential for as long as 48 h following camptothecin treatment. Neurons derived from the other genotypes (wild-type, *Hq* and $Apaf1^{-/-}$) showed a complete loss of mitochondrial polarization by this time (Fig. 4A and B). A similar trend was also observed using Mitotracker Red CMX-Ros, another dye for intact mitochondrial potential (data not shown). These results demonstrate that by blocking both mitochondrial pathways, the caspase dependent (by BAF treatment or $Apaf1^{-/-}$) and the AIF pathway (by *Hq* mutation), neurons can be significantly protected against DNA damage-induced cell death.

Neurons with reduced AIF are more resistant to glutamate induced excitotoxic cell death.

Excitotoxic neuronal cell death is one of the mechanisms that causes major damage following acute brain injury such as stroke or trauma (Arundine & Tymianski, 2003; Yuan et al, 2003). Excitototoxicity can occur independent of BAX and caspase activation (Dargusch et al, 2001; Miller et al, 1997). To confirm that BAX is not involved in excitotoxic cell death, $Bax^{-/-}$ and wild type neurons were exposed to 100μM glutamate or 200μM NMDA.

Figure 2-4: A higher proportion of *Hq/Apaf1*$^{-/-}$ double mutant neurons retain mitochondrial membrane potential after campothecin treatment. Cortical neurons from wild-type, *Apaf1*$^{-/-}$, *Hq* and *Hq/Apaf1*$^{-/-}$ littermates were treated with camptothecin (10 μM) and mitochondrial membrane potential was assessed by TMRE, which only incorporate into mitochondria with intact membrane potential. (A) Neurons 36 h after camptothecin treatment. Bar = 50μm. (B) Quantitative analysis of neurons with positive TMRE staining which indicates intact mitochondrial membrane potential at the indicated times. TMRE positive cells are reported as a percentage of stained cells over the total number of cells (n=3).


A
wt
Hq
Apaf1 -/-
Apaf1 -/- + Hq
TMRE
Phase

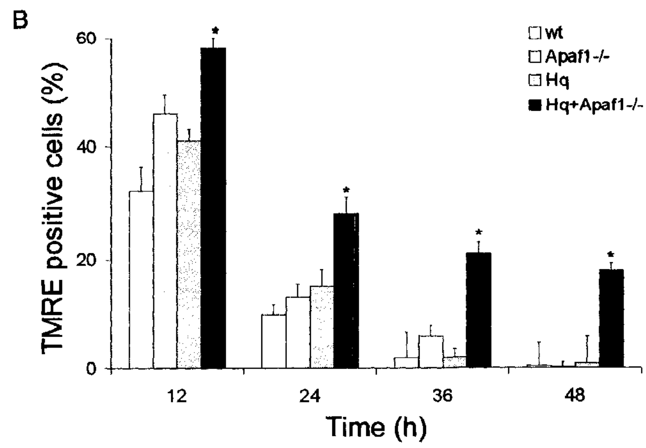
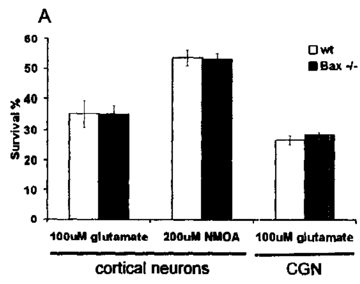
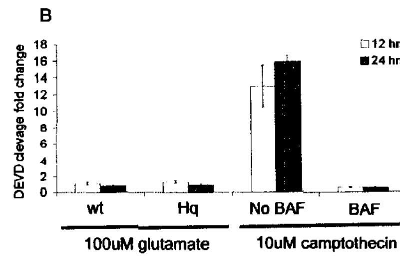
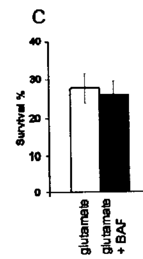
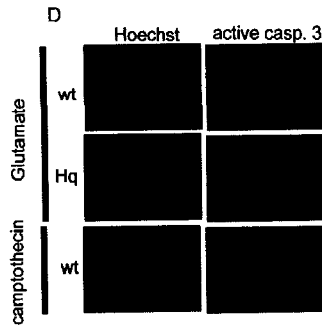

B
TMRE positive cells (%)
0
20
40
60
wt
Apaf1-/-
Hq
Hq+Apaf1-/-
*
12
24
36
48
Time (h)

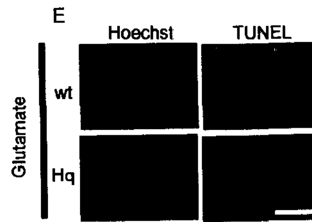
Figure 2-5: Excitotoxicity induced neuronal cell death is BAX and caspase independent. (A) Quantification of survival by MTT assay in cortical neurons and CGNs from BAX deficient and wild-type littermates after 24 h of treatment with 100μM glutamate and 200μM NMDA for cortical neurons and 100μM glutamate for CGNs (n=4). (B) Caspase 3 activity is measured by DEVD-AFC cleavage in wild-type and *Hq* cortical neurons after 24 h of treatment with 100uM glutamate. Camptothecin treated wild-type neurons with or without BAF are used as positive and negative controls, respectively (n=3). (C) MTT assay for wild-type neurons treated with 100μM glutamate in the presence or absence of 50μM BAF (n=3). Note that BAF does not provide protection after exposure to glutamate. (D) Active caspase 3 immunohistochemistry 24 h after 100μM glutamate treatment, and camptothecin treated wild-type cells were used as a control. (E) TUNEL staining of cortical neurons 24 h after 100μM glutamate treatment indicating DNA fragmentation in the absence of caspase. Bar = 50μm.


A
Survival %
60
50
40
30
20
10
0
wt
Bax -/-
100uM glutamate
200uM NMDA
100uM glutamate
cortical neurons
CGN


B
DEVD clevage fold change
18
16
14
12
10
8
6
4
2
0
12 hrs
24 hrs
wt
Hq
No BAF
BAF
100uM glutamate
10uM camptothecin


C
Survival %
40
30
20
10
0
glutamate
glutamate + BAF


D
Hoechst
active casp. 3
Glutamate
wt
Hq
camptothecin
wt

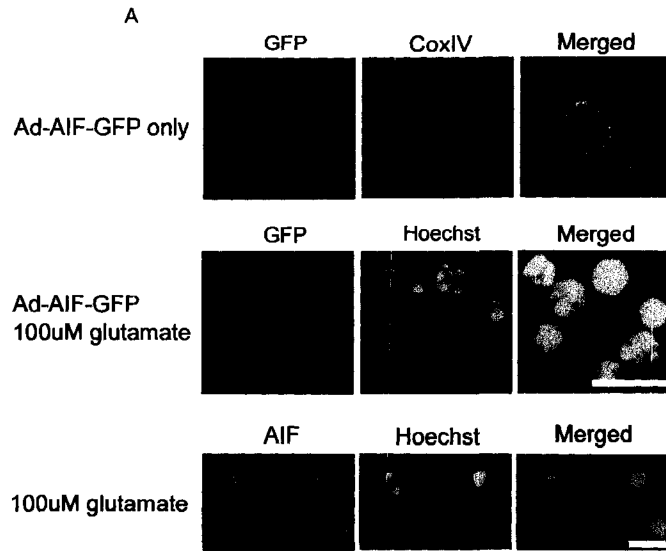

E
Hoechst
TUNEL
Glutamate
wt
Hq

Following 24 h cell viability was measured by MTT assay. BAX deficiency did not protect cortical neurons against excitotoxicity induced by 100μM glutamate or 200μM NMDA (Fig. 5A). Similarly, BAX deficiency had no effect against glutamate induced cell death in CGNs (Fig. 5A). To determine whether caspases are activated during glutamate mediated cell death, DEVD cleavage was measured at 12 and 24 h following treatment. No significant DEVD cleavage was detected during excitotoxic cell death compared to camptothecin treatment (Fig. 5B). Similarly, immunohistochemistry using active caspase 3 antibody did not reveal caspase 3 activation. This is in contrast to the robust staining observed following treatment of these same neurons with camptothecin (Fig. 5D). Consistent with the absence of caspase involvement, treatment of cortical neurons with BAF provided no protection against cell death induced by glutamate receptor activation (Fig. 5C). Despite the lack of BAX and caspase involvement in glutamate-mediated cell death, wild type neurons undergoing this caspase independent cell death exhibited DNA fragmentation as determined by TUNEL staining. This was drastically reduced in *Hq* neurons (Fig. 5E) suggesting that the absence of AIF may protect against this mechanism of cell death.

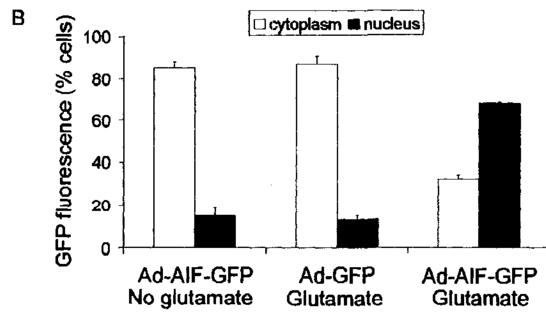
Next we asked whether AIF is translocated to the nucleus during glutamate induced cell death. Wild-type cortical neurons and CGNs were infected with a recombinant adenoviral vector expressing GFP tagged AIF, and treated with 100 μM glutamate. AIF localization was assessed after 24 h using confocal microscopy to assess double labeling with the mitochondrial marker CoxIV. In healthy cells GFP-tagged AIF was mitochondrial (Fig. 6A), however, 24 h following glutamate exposure over 70% of GFP positive cortical neurons exhibited nuclear translocation of AIF (Fig. 6A and B). Translocation was also observed in CGNs (Fig. 6C). A similar trend was also observed by immunostaining for endogenous AIF after glutamate treatment in cortical neurons (Fig. 6A). To assess viability

Figure 2-6: AIF translocates to the nucleus during glutamate induced cell death. (A) Cortical neurons from wild-type mice were infected with recombinant adenoviral vector containing GFP tagged AIF and were treated with glutamate (100μM) for one hour. After 24 h cells were fixed and stained with Hoechst for nucleus and CoxVI antibody for mitochondria and GFP fluorescence were observed. Translocation of endogenous AIF was also assessed using AIF antibody and Hoechst staining. Bar = 10μm. (B) Quantitative analysis of GFP fluorescence in nucleus in (B) cortical neurons and (C) CGNs (n=3).

A



B



C

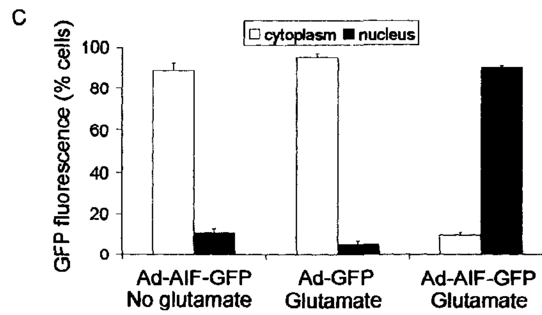
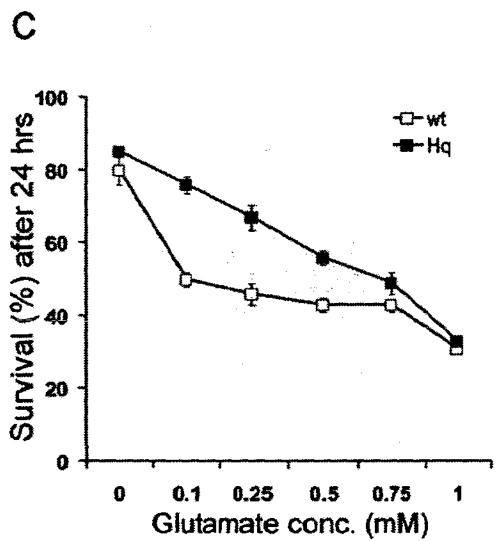
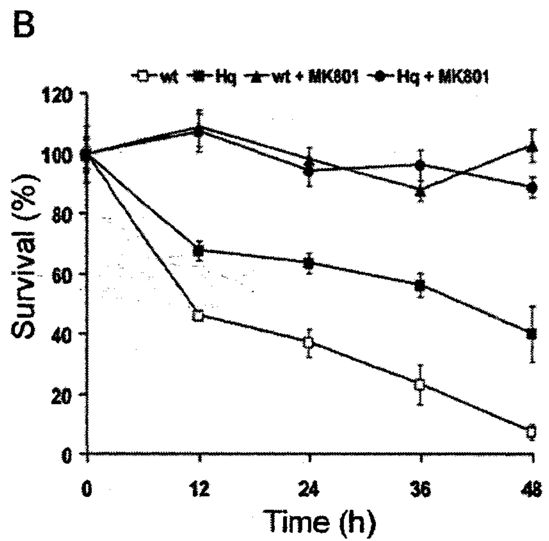
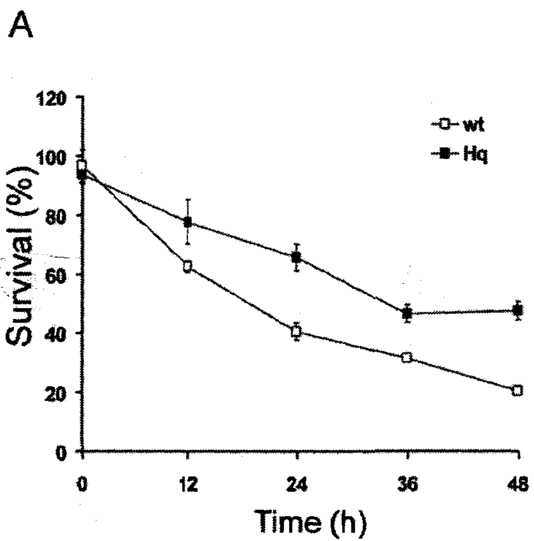


Figure 2-7: *Hq* cortical neurons are more resistant to excitotoxicity induced by glutamate. Cortical neurons (14 DIV) from wild-type and *Hq* littermates were treated with glutamate (100μM) for one hour. (A) Quantitative analysis of neuronal survival rate by Live/Dead assay at the indicated time points after glutamate treatment (n=3). (B) Quantitative analysis of neuronal survival rate by nuclear morphology revealed by Hoechst staining at the indicated time points after glutamate treatment (n=3). Survival is reported as a percentage of cells with morphologically healthy nuclei over the total number of cells. (C) Quantitative analysis of cortical neurons survival with increasing glutamate concentration after 24 h (n=3).


A
Survival (%)
120
100
80
60
40
20
0
0
12
24
36
48
Time (h)
wt
Hq

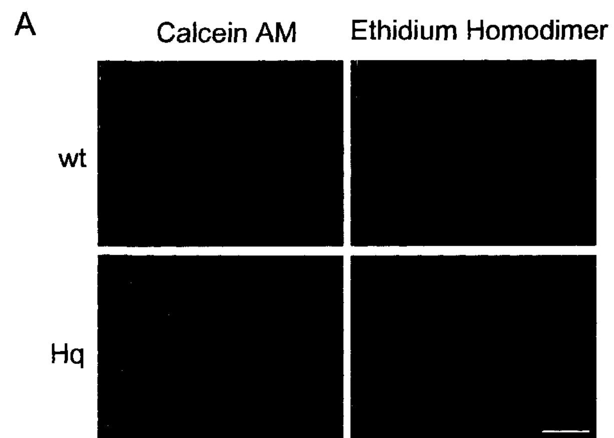

B
wt
Hq
wt + MK801
Hq + MK801
Survival (%)
120
100
80
60
40
20
0
0
12
24
36
48
Time (h)


C
Survival (%) after 24 hrs
100
80
60
40
20
0
0
0.1
0.25
0.5
0.75
1
Glutamate conc. (mM)
wt
Hq

of *Hq* and wild type neurons after glutamate exposure, nuclear condensation was evaluated by Hoechst (Fig. 7B) and survival measured by Live/Dead assay (Fig. 7A). At all time points after transient application of 100μM glutamate, *Hq* cortical neurons exhibited significantly higher survival rates relative to neurons cultured from wild-type littermates. Specifically, at 48 h 40.3% of *Hq* cortical neurons were still viable relative to only 7.6% of wild-type littermates. This glutamate-induced neurotoxicity was completely inhibited by MK-801, demonstrating that toxicity occurs specifically through activation of NMDA receptors (Fig. 7B). Since previous studies by Klein et al. 2002 indicated that the sensitivity to glutamate following AIF depletion differed between cortical and cerebellar neurons we also conducted this experiment in CGN's. Our results show a similar trend regardless of the neuronal cell type (CGN's 45% versus 24%) as indicated by Live/Dead assay (Fig. 8A and B) and nuclear morphology (data not shown).

Previous studies examining high dose treatment with glutamate (1mM) revealed that *Hq* CGNs were not resistant, but instead exhibited increased sensitivity, while *Hq* cortical neurons were not different from controls (Klein et al., 2002). To ask whether AIF deficiency renders neuron more sensitive to high levels of glutamate treatment, a dose-response curve was conducted (0.1mM to 1.0mM glutamate) with both CGN and cortical neurons. Using either the Live/Dead assay or nuclear condensation, the reduction of AIF resulted in protection against glutamate-induced cell death up to 0.75mM. There was no difference between *Hq* and wild-type littermate controls at concentrations higher than 0.75mM (Fig. 7C). Similar trend is also observed using CGNs (data not shown). Taken together, our results reveal AIF as a cell death executor in mechanisms involving acute neuronal injury such as excitotoxicity.

Figure 2-8: *Hq* CGNs are more resistant to excitotoxicity induced by glutamate. CGNs (14 DIV) from wild-type and *Hq* littermates were treated with glutamate (100μM) for one hour. Neuronal survival was determined with Live/Dead assay. (A) Photomicrographs of neurons 36 h after glutamate treatment. Bar = 250μm. (B) Quantitative analysis of survival rate at the indicated time points after glutamate treatment (n=3).


A
Calcein AM
Ethidium Homodimer
wt
Hq

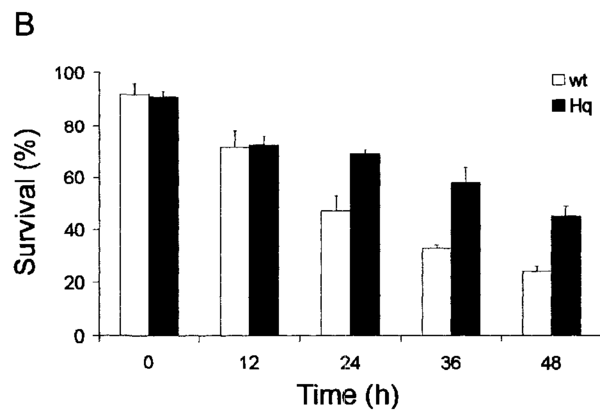
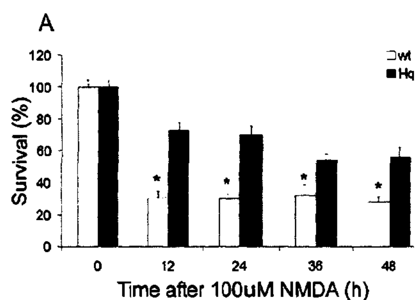
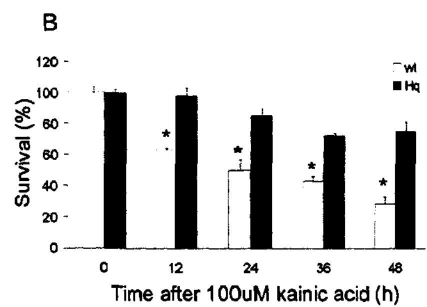
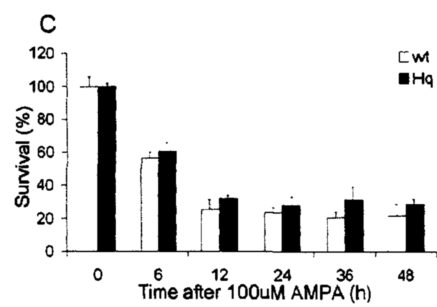
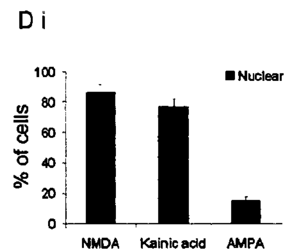

B
Survival (%)
100
80
60
40
20
0
wt
Hq
0
12
24
36
48
Time (h)

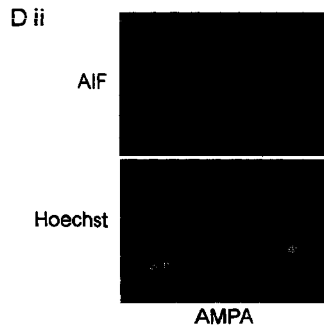
Figure 2-9: *Hq* cortical neurons are more resistant to excitotoxicity induced by NMDA and KA, but not AMPA. Cortical neurons (14 DIV) from wild-type and *Hq* littermates were treated with (A) 100μM NMDA (B) 100μM KA and (C) 100μM AMPA for one hour with the appropriate co-agonists, desensitization blockers and specific receptor blockers (see materials and methods) (n=3). Survival was determined by nuclear morphology using Hoechst stain. (D) i) Nuclear translocation of AIF assessed by AIF immunhistochemistry after NMDA, KA and AMPA treatment (n=3). ii) Photomicrographs of AIF immunohistochemistry show the lack of nuclear translocation after AMPA treatment. Bar = 10μm. (E) Quantitative analysis of propidium iodide (PI) positive and TUNEL positive neurons 36 h after NMDA and kainate treatment (n=3). * $P<0.01$ compared to wild-type (A and B) or PI^{+} (C).

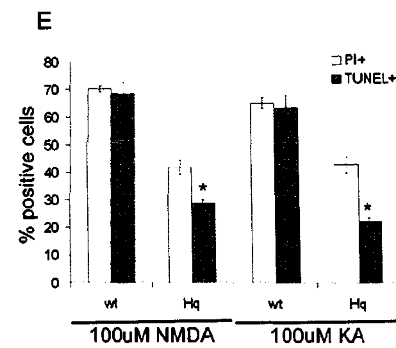

A
Survival (%)
120
100
80
60
40
20
0
wt
Hq
0
12
24
36
48
Time after 100uM NMDA (h)


B
Survival (%)
120
100
80
60
40
20
0
wt
Hq
0
12
24
36
48
Time after 100uM kainic acid (h)


C
Survival (%)
120
100
80
60
40
20
0
wt
Hq
0
6
12
24
36
48
Time after 100uM AMPA (h)


D i
% of cells
100
80
60
40
20
0
Nuclear
NMDA
Kainic acid
AMPA

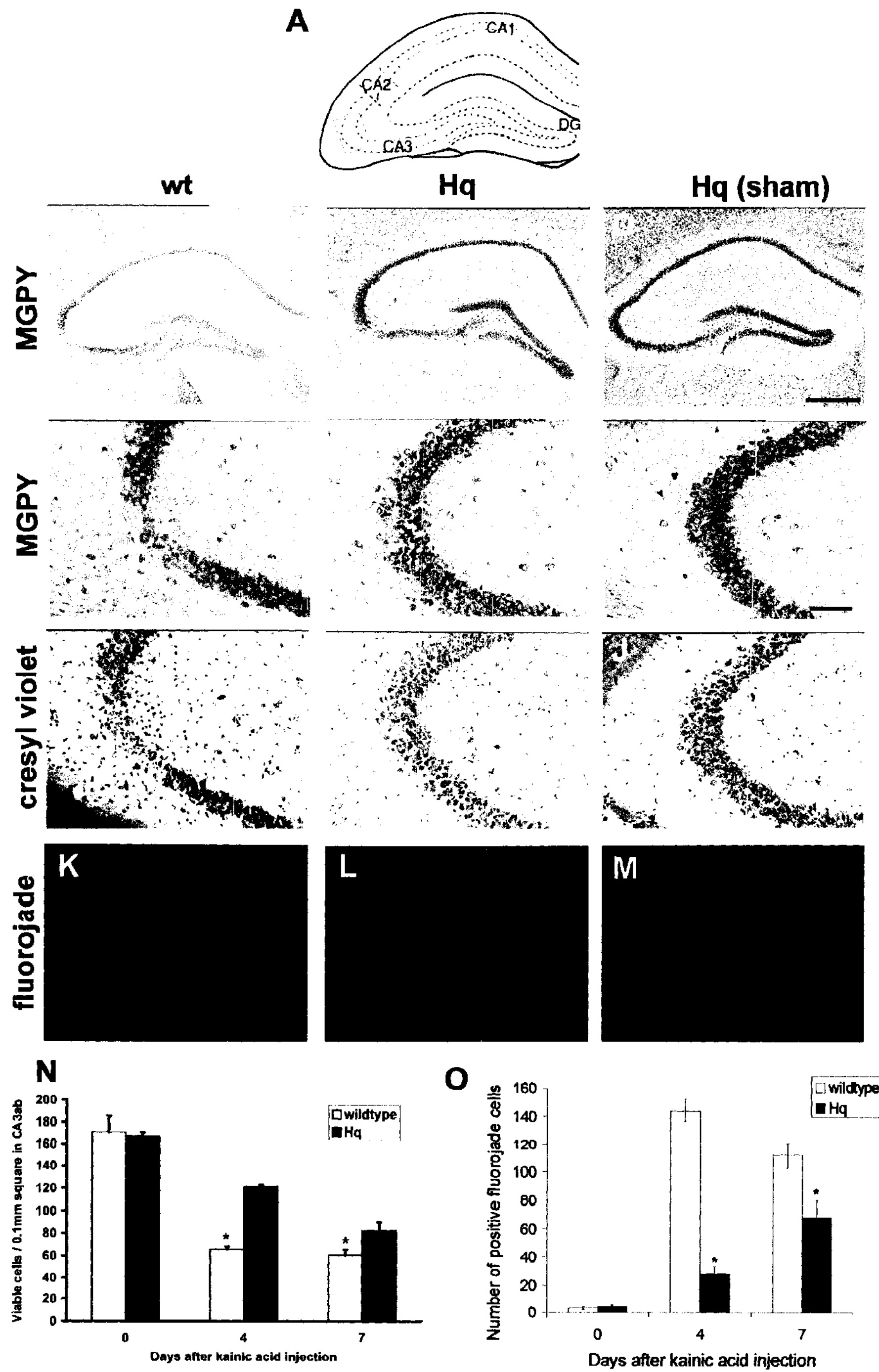

D ii
AIF
Hoechst
AMPA


E
% positive cells
80
70
60
50
40
30
20
10
0
PI+
TUNEL+
wt
Hq
wt
Hq
100uM NMDA
100uM KA

***Hq* neurons are more resistant to NMDA and kainic acid induced cell death but not AMPA.**

To determine which receptors recruit AIF function during excitotoxicity, specific agonists for the different glutamate receptors were assessed over a time course of 48 h in both cortical neurons and CGNs. Agonists to the three ionotrophic glutamate receptors were tested including NMDA, AMPA, and kainic acid (Dingledine et al, 1999). Here, we asked whether AIF recruitment to the death cascade occurred with all or only a subset of ionotrophic glutamate receptors. Cortical neurons from *Hq* and wild-type littermates were treated with AMPA, kainic acid and NMDA with the appropriate co-agonist (glycine for NMDA treatment) and desensitizing inhibitors (cyclothiazide for AMPA and Con A for kainic acid) (Huettner, 1990; Wong & Mayer, 1993). For AMPA and kainic acid treatments MK-801 was added to prevent NMDA receptor activation, and for kainic acid treatment GYKI 52466 was added to prevent AMPA receptor activation (Huettner & Bean, 1988; Paternain et al, 1995). *Hq* cortical neurons were more resistance to 100μM NMDA treatment (Fig. 9A) and 100μM kainic acid treatment (Fig. 9B), which was similar to the response obtained with 100μM glutamate treatment (Fig. 7A and B). The NMDA induced neurotoxicity, as in the case of glutamate, was inhibited by MK 801 confirming NMDA receptor specificity (data not shown). Reduced level of AIF did not protect against all ionotrophic glutamate receptor agonists, since *Hq* neurons exposed to AMPA exhibited a similar rate of cell death as that seen with wild-type littermates (Fig. 9C). Lower dosages of 50μM AMPA were also tested and, unlike NMDA and kainic acid treatment, *Hq* neurons were not protected against exposure to AMPA (data not shown). Supporting the lack of AIF involvement in AMPA induced cell death, AMPA did not induce AIF translocation (Fig.

Figure 2-10: *Hq* neurons are more resistant to excitotoxicity *in vivo* induced by KA seizure in adult mice. Animals were injected with KA (30mg/kg) and sacrificed 4 days or 7 days afterwards. (A) Cartoon of coronal section of hippocampus. The box indicates the CA3ab region. (B-D) Methyl Green/Pyronin-Y (MGPY) staining were used to assess viability Bar = 500μm. (E-G) MGPY staining at higher magnification. Bar = 100μm. (H-J) Cresyl Violet staining. (K-M) Fluorojade staining were used to assess cell death. Sham is *Hq* mice. (N) Quantification of viable cells per 0.1 mm^2 in CA3ab area by MGPY stain (n=3). * $P<0.01$ compared to wild-type. (O) Quantification of cell death in the CA3ab area by fluorojade staining (n=3). * $P<0.05$ compared to wild-type.


A
CA1
CA2
CA3
DG
wt
Hq
Hq (sham)
MGPY
MGPY
cresyl violet
fluorojade
K
L
M
N
Viable cells / 0.1mm square in CA3ab
wildtype
Hq
Days after kainic acid injection
O
Number of positive fluorojade cells
wildtype
Hq
Days after kainic acid injection

9D), in contrast to that seen following glutamate exposure (Fig. 6). This was consistent with a recent study examining AIF translocation in PARP deficient mice (Wang et al, 2004). Of the dying *Hq* neurons, only 69% for NMDA (29% TUNEL:47.78% PI), and 52% for kainic acid (22.3% TUNEL: 43% PI) exhibited DNA fragmentation relative to 98% for NMDA (68.6% TUNEL:70.45% PI) and 97% for kainic acid (63.3% TUNEL:65.2% PI) seen in wild-type neurons (Fig. 9E). This suggests that, similar to camptothecin treated *Hq/Apaf1*$^{-/-}$ neurons (Fig. 3C), there is a drastic reduction of DNA fragmentation in excitotoxin treated *Hq* neurons. These results strongly support that AIF is a key factor in DNA fragmentation and neuronal cell death during excitotoxicity.

Reduced AIF levels confers increased resistance to hippocampal damage resulting from kainic acid induced seizure

Since *Hq* cortical and CGNs are more resistant to excitotoxic injury *in vitro*, we next asked whether *Hq* neurons are also more resistant to excitotoxicity *in vivo* using a kainic acid seizure model. In these experiments CA3 hippocampal damage was assessed after kainic acid induced seizure (Strain & Tasker, 1991). Adult mice received i.p. injections of 30mg/kg kainic acid and were allowed to develop stage four seizures (Bennett et al, 1995). Both *Hq* mice and wild-type littermate controls were tested in pairs and equally developed stage 4 seizures. Damage in the hippocampal CA3ab region was assessed by methyl green pyronin Y (MGPY), cresyl violet and fluorojade staining at 4 and 7 days following treatment. *Hq* mice exhibited significantly less damage in the CA3ab region after 4 days following kainic acid induced seizure (Fig. 10A). Resistance to injury in *Hq* mice was indicated by increased numbers of healthy cells shown by MGPY staining in the CA3ab

region (Fig. 10A and B), and significantly fewer cells with apoptotic morphology as revealed by cresyl violet staining (Fig. 10A). *Hq* mice also exhibited five fold less fluorojade positive cells relative to littermate controls (Fig. 10A and 10C). To ask whether *Hq* mice exhibited long-term protection, hippocampal neuron survival was also assessed one week following kainic acid injection. While neuroprotection in *Hq* mice was still significant at 7 days, the number of viable cells was decreased with a corresponding increase in the number of fluorojade positive cells (Fig. 10B and C). This gradual decrease in cell viability was likely due to the residual AIF expression (20%) in *Hq* neurons that may allow the death pathway to proceed at an attenuated rate. These *in vivo* results support our *in vitro* findings that a reduction of AIF expression in neurons provides protection against excitotoxic injury.

In summary, using mice carrying the *Hq* mutation we have shown that AIF plays a crucial role in both the BAX dependent and BAX independent cell death cascades involved in neuronal injury. Following BAX recruitment after DNA damage, AIF is involved in the caspase independent death cascade and functions to facilitate DNA fragmentation. Furthermore, AIF is also directly involved in Bax independent death pathways such as excitotoxicity. In excitotoxic neuronal cell death, AIF is specifically recruited following activation of NMDA and kainic acid receptors. These results demonstrate that AIF plays a key role in executing cell death in multiple mechanisms of acute neuronal injury.

Discussion

In this study we used *Hq* mice that have severely reduced AIF levels to study the role of AIF in neuronal injury. First, we showed that in the absence of caspase activity, *Hq* neurons exhibited extended protection against camptothecin induced DNA damage well beyond that seen by caspase inhibition alone. The surviving *Hq* neurons with caspase inhibition retained their mitochondrial membrane potential relative to wild-type, *Apaf1*$^{-/-}$ and *Hq* mutants. Second, *Hq* neurons were more resistant to glutamate-induced excitotoxicity, particularly through NMDA and kainic acid receptors but not AMPA receptors. Consistent with AIF recruitment in excitotoxicity, adult *Hq* mice exhibited less damage in the hippocampus after kainic acid induced seizure *in vivo*. These studies demonstrate an important role for AIF in multiple cell death pathways and suggest that AIF may provide a valuable therapeutic target for the treatment of acute brain injury.

Apaf1 deficient Hq neurons exhibit sustained protection against BAX-mediated apoptosis.

Previous studies have shown that inhibition of caspase activity can delay the onset of neuronal cell death. Specifically, studies using animal models of stroke have revealed a striking short-term protection following caspase inhibition. This protection is only transient, however, since at extended time points neuronal death still ensues at a delayed rate despite inhibition of caspase activity (Cregan et al, 2002; Cregan et al, 1999; Fortin et al, 2001). Similarly, the use of caspase inhibitors in an animal model of stroke have again shown only short term protection against apoptosis but after extended time courses death occurs nonetheless (Endres et al, 1998). It is therefore evident that caspase independent pathways can compensate when the caspase cascade is blocked. Consistent with this, we have previously shown that AIF is the key factor in neuronal apoptosis when caspase is inhibited,

either by BAF or in *Apaf1*$^{-/-}$ deficient animals (Cregan et al, 2002). Microinjection of AIF antibodies can significantly rescue this form of caspase-independent cell death. AIF has also been shown to translocate in in vivo animal models of neurodegeneration such as focal ischemia (Komjati et al., 2004; Plesnila et al., 2004; Zhu et al., 2003). Since *Aif*$^{-/-}$ animals are early embryonic lethal, the role of AIF in neuronal cell death has not been directly examined. In this report, we studied the role of AIF using Hq mice in which AIF expression is reduced by over 80% (Klein et al, 2002). This provides the opportunity to directly examine the role of AIF in neuronal cell death and the extent of protection when both caspase dependent and independent pathways are blocked. In the present studies, we first show that in the absence of caspases (by BAF application), *Hq* neurons were more resistant to camptothecin induced DNA damage apoptosis. More significantly, during DNA damage induced apoptosis, we also demonstrate extended protection in *Hq/Apaf1*$^{-/-}$ double mutant neurons in which the mitochondrial initiated caspase dependent and independent pathways are blocked (Fig. 3A and B). Importantly, of the *Hq/Apaf1*$^{-/-}$ neurons that go on to die, there is dramatically less DNA fragmentation as determined by TUNEL staining relative to that seen in neurons with *Apaf1*$^{-/-}$ mutations alone or in wild-type littermates. These results demonstrate that AIF plays an important role in the regulation of DNA degradation during injury-induced apoptosis (Fig. 3C and 2D).

Our data also indicate that *Hq/Apaf1*$^{-/-}$ mutant cells reveal a higher percentage of cells containing mitochondria with intact membrane potential, suggesting that they are still viable and metabolically active (Fig. 4A and B). These results show that by inhibiting both caspase-dependent and caspase-independent death pathways, neurons are provided sustained protection against Bax-mediated cell death that extends well beyond that resulting from inhibition of caspase alone. This protection was particularly striking considering that these

neurons retain about 20% of the wild type AIF activity, which should still allow some residual apoptotic signaling to occur. It is unlikely that this protection is due to other unknown genetic defects in the *Hq* mice since a similar level of protection against camptothecin-induced death was observed in double mutant neurons cultured from mice carrying conditional Aif^{Δ} mutations on an $Apaf1^{-/-}$ background (Cheung and Slack, unpublished data). Although mice with *Hq* alone exhibited reduction in DNA fragmentation, there is no long-term protection compared to $Hq/Apaf1^{-/-}$ double mutants. This may due to the fact that the presence of caspases in these cells overwhelms the protection conferred by AIF reduction alone. Recently, other studies in non-neuronal cell line (Arnoult et al, 2003a; Arnoult et al, 2002) and rat cortical neurons (Lang-Rollin et al, 2003) suggested that AIF requires caspases to be released from mitochondria, in contrast to other studies (Cregan et al, 2002; Daugas et al, 2000b; Susin et al, 2000; Susin et al, 1999a; Susin et al, 1999b; Yu et al, 2002) that clearly indicates AIF translocation can occur independent of caspases. This discrepancy may due to the fact that different cells types recruit different mechanisms of cell death depending on the type of injury sustained. Our previous (Cregan et al, 2002) and present studies directly demonstrate that the caspase-independent signaling cascade, in which AIF is a key determinant, clearly plays a role in BAX-mediated neuronal cell death.

The importance of AIF in excitotoxic neuronal injury

Excitotoxicity is a major contributor to many neurodegenerative diseases such as ischemia and acute neurotrauma (Arundine & Tymianski, 2003; Yuan et al, 2003). During stroke or trauma, excitotoxic neuronal damage results from an excessive release and impaired reuptake of the neurotransmitter glutamate from synapses. There are several receptors for glutamate but the key mediators in terms of excitotoxic cell death is the NMDA

receptor (Arundine & Tymianski, 2003) which, in this system, functions in a BAX independent (Dargusch et al., 2001; Miller et al., 1997) and caspase independent manner (Fig. 5). Once activated by excessive levels of glutamate, the NMDA receptors allow excessive calcium ion influx and elevated cytoplasmic calcium ion levels. This influx of calcium ions activates nitric oxide synthase (nNOS) and unleashes the subsequent overactivation of PARP1 due to oxidative DNA damage (Cregan et al, 2004; Yu et al, 2003). Recently, it was demonstrated that PARP1 induces AIF translocation in NMDA induced cell death. In PARP1 deficient neurons AIF does not translocate to nucleus and cell death is abolished after NMDA application (Yu et al, 2002). These studies implicate AIF as a key death effector in glutamate induced excitotoxicity. Surprisingly, a previous study with *Hq* mice revealed that neurons with reduced AIF were found to be more sensitive to high doses of glutamate (Klein et al, 2002). In the present study, we used a transient and lower concentration (100μM) of glutamate treatment and showed that *Hq* neurons, similar to the knockout neurons of its upstream regulator PARP1 (Eliasson et al., 1997), are more resistant to excitotoxicity for up to at least 48 h. Importantly, we also show that at this concentration, glutamate toxicity is NMDA receptor specific since the toxicity is inhibited completely by MK-801.

The discrepancy between our studies and those previously reported by Klein et al., (2002) regarding the role of AIF in glutamate-mediated cell death is due to the fact that distinct death signaling pathways are being examined. We are studying glutamate receptor mediated signaling specifically through the NMDA, kainic acid and AMPA receptors. These experiments are done according to well established protocols (Aarts et al, 2002; Sattler et al, 1999). In these experiments cells are exposed to glutamate or agonist transiently for 1 h which results in activation of receptors that can be specifically inhibited

by MK-801. Thus, all death signaling induced by glutamate in the present study is through specific glutamate receptors. We show that AIF deficiency in *Hq* mice results in protection against this mode of cell death. Our *in vitro* results are supported by our *in vivo* experiments using a model of excitotoxicity involving kainic acid induced seizure. At 4 days after treatment, *Hq* adult mice were more resistant to excitotoxic brain damage relative to their wild-type littermates. *Hq* mice sustained similar seizure symptoms as wild-type mice since AIF does not contribute to the electrical excitability of these neurons. All of these results demonstrate that AIF is important in inducing cell death after excitotoxicity and by reducing its expression neurons are protected against NMDA and kainic acid receptor mediated injury. In contrast, studies by Klein et al. (2002), focus on non-excitotoxic cell death involving chronic glutamate exposure at relatively high concentrations. With this type of exposure multiple pathways including those involving oxidative stress are evoked. This type of cell death is non-excitotoxic and cannot be inhibited by MK-801 (data not shown) and is not protected by the *Hq* mutation. Consistent with the results of Klein et al. (2002), we have obtained similar results showing that *Hq* neurons are more sensitive to oxidative stress (data not shown). Thus, exposure to glutamate can evoke multiple death pathways and the results of our experiments show that AIF reduction is protective to the excitotoxic mode of cell death specifically involving activation of NMDA and kainic acid glutamate receptors. This type of death pathway is directly involved in seizure, trauma and stroke induced acute brain damage.

In summary, the results of our studies demonstrate that the reduction of AIF combined with caspase inhibition provides extended neuroprotection against BAX-mediated apoptosis. Also, neurons with reduced AIF are protected against excitotoxicity specifically through the activation of NMDA receptors and kainic acid receptors. Consistent with our *in vitro*

results, in an *in vivo* model of excitotoxicity, *Hq* adult mice exhibited less hippocampal damage induced by kainic acid treatment. Our results show that AIF is a key determinant in caspase independent death cascades involving both BAX dependent and BAX independent death pathways. Due to its activity in multiple death pathways, AIF may serve as a promising therapeutic target for the treatment of acute brain injury and neurodegenerative diseases.

Chapter III

Cheung et al. (2006) Dissociating the dual roles of Apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. **EMBO Journal.** 25(17):4061-73.

This manuscript is the first to directly establish the pro-apoptotic role of AIF in the nucleus using mitochondrially anchored AIF that cannot be released from the mitochondria during apoptosis. In addition to the cell death role, the novel physiological role of AIF in maintaining mitochondrial structure was also revealed using the forebrain specific AIF mutant.

E.C. Cheung designed and conducted the experiments in this manuscript. The *in vivo* analysis was assisted by K.A. McClellan and S. McNamara. N. Joza from the laboratory of Dr. J. Penninger provided the floxAIF mice. M. Neuspiel and P. Rippstein from the laboratory of Dr. H.M. McBride provided assistance in mitochondrial imaging. Dr. N.A. Steenaart and Dr. G. Shore provided assistance in the mitochondrial studies. J.G. MacLaurin provided technical assistance in molecular cloning. Dr. D.S. Park provided feedback. E.C. Cheung wrote the manuscript with the guidance and editorial assistance from Dr. R.S. Slack.

DISSOCIATING THE DUAL ROLES OF AIF IN MAINTAINING MITOCHONDRIAL STRUCTURE AND APOPTOSIS

Eric C.C. Cheung[1], Nicholas Joza[3], Nancy A.E. Steenaart[5], Kelly A. McClellan[1], Margaret Neuspiel[2], Stephen McNamara[1], Jason G. MacLaurin[1], Peter Rippstein[2], David S. Park[1], Gordon C. Shore[4], Heidi M. McBride[2], Josef M. Penninger[3*], and Ruth S. Slack[1*]

[1]Ottawa Health Research Institute-Department of Cellular and Molecular Medicine, [2]Ottawa Heart Institute, University of Ottawa, Ottawa, Ontario, Canada; [3]IMBA, Institute of Molecular Biotechnology of the Austria Academy of Sciences, Vienna, Austria, [4]Department of Biochemistry, McGill University, Montreal, Quebec, Canada [5]Gemin X Biotechnologies Inc., Montreal, Quebec, Canada

*Correspondence should be addressed to:

Dr. Josef M. Penninger, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohr-gasse 3, A-1030 Vienna, Austria.
e-mail: josef.penninger.@imba.oeaw.ac.at

or

Dr. Ruth S. Slack, Ottawa Health Research Institute, University of Ottawa, 451 Smyth Road Room 2452, Ottawa, Ontario, Canada K1H8M5.
e-mail: rslack@uottawa.ca

Running Title
Dissociating AIF dual roles in cell life and death

Keywords
Apoptosis, apoptosis inducing factor (AIF), mitochondria, neuron, DNA damage.

Acknowledgements
We would like to thank Drs. Valina Dawson and Seong Woon Yu for advice in subcellular fractionation, and to Carl McIntosh for assistance in the in vivo studies. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to R.S.S.; and a Marie Curie Excellence Grant and the Austrian National Bank to J.M.P. The viral vector facility is supported by a grant from Canadian Stroke Network (R.S.S. and D.S.P.). E.C.C.C. and KAM are supported by a CIHR studentship.

Abstract

The mitochondrial protein Apoptosis Inducing Factor (AIF) translocates to the nucleus and induces apoptosis. Recent studies, however, have indicated the importance of AIF for survival in mitochondria. In the absence of a means to dissociate these two functions, the precise roles of AIF remain unclear. Here we dissociate these dual roles using mitochondrially anchored AIF that cannot be released during apoptosis. Forebrain-specific AIF null (tel. Aif^{Δ}) mice have defective cortical development and reduced neuronal survival due to defects in mitochondrial respiration. Mitochondria in AIF deficient neurons are fragmented with aberrant cristae, indicating a novel role of AIF in controlling mitochondrial structure. While tel. Aif^{Δ} $Apaf1^{-/-}$ neurons remain sensitive to DNA damage, mitochondrially-anchored AIF expression in these cells significantly enhanced survival. AIF mutants that cannot translocate into nucleus failed to induce cell death. These results indicate that the pro-apoptotic role of AIF can be uncoupled from its physiological function. Cell death induced by AIF is through its pro-apoptotic activity once it is translocated to the nucleus, not due to the loss of AIF from the mitochondria.

Introduction

Mitochondria are the central relaying stations for apoptotic signals. After the induction of apoptosis, cyt c is released from the mitochondria that interacts with Apafl and pro-caspase 9, which in turn activates the caspase cascade (reviewed in (Danial & Korsmeyer, 2004; Yuan et al, 2003)). Apart from the caspase dependent pathway, mitochondrial factors also initiate a caspase independent apoptotic signaling cascade (reviewed in (Cregan et al, 2004; Hong et al, 2004). This pathway is initiated by the release of the mitochondrial protein, Apoptosis Inducing Factor (AIF), which translocates to the nucleus and induces DNA fragmentation through interactions with factors including EndoG in *C. elegans*, CypA in mice, and others such as FEN-1 (Cande et al, 2004b; Daugas et al, 2000b; Parrish & Xue, 2003; Parrish et al, 2003; Susin et al, 1999b; Wang et al, 2002). The significance of these interactions, however, are not yet clear, as EndoG and CypA null animals have no apparent defect in apoptosis (Colgan et al, 2000; Irvine et al, 2005).

The role of AIF in neuronal cell death was first suggested from the observation that AIF translocates to nucleus after the induction of various types of acute neuronal injury *in vitro* and *in vivo* (Cao et al, 2003; Cregan et al, 2002; Fonfria et al, 2002; Plesnila et al, 2004; Wang et al, 2004; Zhang et al, 2002; Zhu et al, 2004). Mitochondrial release of AIF has been shown to depend on PARP activity (Wang et al, 2004; Yu et al, 2002). We have previously demonstrated that AIF translocation following neuronal injury is caspase independent (Cheung et al, 2005; Cregan et al, 2002). Using *Apafl*$^{-/-}$ neurons, we have shown that AIF is translocated to the nucleus upon induction of apoptosis and this can be inhibited by microinjecting AIF neutralizing antibodies (Cregan et al, 2002). Depending on the cell type and death stimulus, the release of AIF may also be caspase dependent, as studies using *C. elegans* with BH-3 only protein EGL-1 (Wang et al, 2002), HeLa cells with

staurosporine (Arnoult et al, 2003a), and rat cortical neurons (Lang-Rollin et al, 2003) have previously shown. We have used *Harlequin* (*Hq*) mice, which exhibit only 20% AIF expression (Klein et al, 2002), to directly investigate the role of AIF in various models of neuronal cell death. Using *Hq/Apaf1*$^{-/-}$ double mutant mice we have shown that reduced levels of AIF, along with inactivation of caspase activity, can sustain neuronal survival after DNA damage and excitotoxic induced cell death. These results revealed that AIF is involved in both Bax dependent and Bax independent mechanisms of cell death (Cheung et al, 2005). In mammalian systems, therefore, AIF is a key death inducer that functions in multiple mechanisms of neuronal cell death; thus understanding its mechanism of action is crucial.

Apart from the apoptotic role of AIF, studies with AIF depleted cells have indicated that AIF also has a physiological role in the mitochondria. Studies using *Hq* mice, which exhibit cerebellar degeneration and increased sensitivity to oxidative stress, (Klein et al, 2002) suggesting that AIF acts as an oxidative radical scavenger in the mitochondria (Lipton & Bossy-Wetzel, 2002). A recent study, however, revealed that AIF depleted cells (*Aif*$^{-/-}$ ES and *Hq* cells) have defective oxidative phosphorylation and reduced expression of Complex I and III in the electron transport chain of the mitochondria. AIF, however, was not found to be associated with either Complex I or III (Vahsen et al, 2004), therefore the mechanism by which AIF stabilizes complex I remains unknown.

Since AIF has dual roles, dissociating its functions in each of these cellular events has been difficult. For example, it remains unknown whether cell death is triggered by the loss of AIF from mitochondria. This would argue that AIF's pro-apoptotic role is not essential to the induction of apoptosis. Here, we resolve this controversy by dissociating the physiological role and the apoptotic role of AIF. To this end, we have constructed a mitochondrial inner membrane anchored form of AIF (anchored AIF) that cannot be released

from the mitochondria during apoptosis and thus maintains its physiological role. These constructs were then introduced in AIF deficient neurons from a telencephalon specific conditional mutant of AIF. Here we show that: (a) AIF plays an important role in neuronal survival by maintaining mitochondrial structure; and, (b) AIF has a major role in proapoptotic signaling following nuclear translocation. Expression of anchored AIF in cells with endogenous AIF can offer protection only during the initial stages of apoptosis by maintaining the pool of AIF in mitochondria. At longer time points, however, the cells still succumb to death even when AIF is present in the mitochondria. This demonstrates that reconstitution of mitochondrial AIF is not sufficient to rescue cell death and that AIF plays an active role in pro-apoptotic signaling in the nucleus. In conclusion, we dissociated the dual functions of AIF and directly demonstrate the importance of the pro-apoptotic role of AIF, apart from its novel role in maintaining mitochondrial structure.

Materials and Methods

Mice and primary neuronal cultures

The floxed AIF mice have been previously described (Joza et al, 2005). To generate telencephalon-specific AIF conditional mutants, floxed AIF homozygous female mice were bred with Foxg1-cre mice (Hebert & McConnell, 2000), to generate mutant Foxg1-cre:AIF $^{flox/Y}$ or female Foxg1-cre:AIF$^{flox/flox}$, (both indicated as tel. *Aif* $^{\Delta}$) mice. *Apaf1*$^{-/-}$ mice were obtained from Cecconi et al. (Cecconi et al, 1998). To detect the presence of cre-mediated recombination, PCR analysis of AIF exon 7 was performed on DNA extracted from the telencephalon of mutant and control embryos. Primers for 1303f (5'-GTAGATCAGGTTGGCCAGAAACTC -3'), 1903r (5'-GGATTAAAGGCATGTGCCAACACG-3') and 659r (5'-GAATCTGGAATATGGCACAGAGG-3') yielded 700 and 600 bp products for the unrecombined floxed and wild-type alleles, respectively, and a 350 bp band for the recombined floxed allele. Western blot analysis was performed as described (Cregan et al, 1999), with antibodies against AIF (D-20, 1:500, Santa Cruz Biotechnology) and the 39kDa subunit of complex I (A21344, 1:500, Molecular Probes). Mice were maintained on FVB/N and C57/BL6 mixed genetic backgrounds and littermates were used in all experiments. Cortical neurons were cultured as described previously (Fortin et al, 2001).

ATP production and oxygen consumption assays

At indicated time points, ATP levels were determined using a luciferase-based CellTitre-Glo assay kit (Promega) with a PolarStar plate reader (BMG). Data were collected

from multiple replicate wells in each of the three experiments (n=3). For oxygen consumption measurements, cells were plated in the fluorescent dye-embedded 96-well microplate of the BD oxygen biosensor system (BD Biosciences). Results were read at indicated times with a fluorescent microplate reader. The data were normalized according to the manufacturer's protocol.

Subcellular fractionation

Subcellular fractionation of neurons was performed as described previously (Yu et al, 2002). Antibodies against histone (US Biological H5110-10, 1:500), mtHsp70 (Affinity BioReagents MA3-028, 1:500), and lactate dehydrogenase (Sigma L7016, 1:500), were used to detect nuclear, mitochondrial, and cytoplasmic fractions, respectively. GFP and cytochrome C were detected using antibodies against GFP (Abcam Ab6556, 1:500), and cyt c (BD Pharmingen 556433, 1:500), respectively.

Tissue fixation, cryoprotection and immunohistochemistry

Tissue fixation, cryoprotection and immunohistochemistry of cortical tissues using active caspase 3 (BD Pharmingen, 559565, 1:100) and phospho-histone H3 (PH-3) (Upstate Biotechnology, 06-570, 1:500) antibodies were performed as described previously (Ferguson et al, 2002).

AIF constructs

Anchored AIF constructs (N-AID, D-AIF) and AIF with a nuclear export signal (AIF-NES) were generated by standard subcloning procedures. Briefly, for N-AIF, the

mitochondrial localization sequence (MLS) of the protein pOSA-141I4, an inner membrane anchored protein (Steenaart & Shore, 1997), was cloned in frame to mouse AIF as follows. The DFHR moiety was removed and two restriction sites (Pst1 and Kpn1) and a stop codon were introduced. A second pOCT cleavage site was introduced into the encoded protein so that MPP will process the protein such that only the amino acids SQVAR will remain N-terminal to the transmembrane domain after import into the mitochondrial inner membrane. For D-AIF, the MLS domain of D-lactate dehydrogenase (Flick & Konieczny, 2002; Rojo et al, 1998) was inserted at the N-terminus of ΔMLS AIF-GFP (Δ1-120), with the proper in frame start codon. AIF-NES was constructed by adding the NES of HIV Rev (LPPLERLTL) (Fischer et al, 1995) to the N terminal of AIF-GFP. Constructs were confirmed by DNA sequencing. Recombinant adenoviral vectors carrying these constructs were then prepared and used as described (Cregan et al, 2002).

Digitonin permeabilization

Immunocytochemical analysis on digitonin permeabilized neurons was performed as described previously (Otera et al, 2005). Western analysis on digitonin permeabilized isolated mitochondria was performed as follows. Briefly, cells expressing GFP tagged wildtype AIF, and GFP tagged N-AIF were collected and mitochondria were isolated in isolation buffer (220mM mannitol, 68mM sucrose, 80mM KCl, 0.5mM EGTA, 2mM magnesium acetate, 1X protease inhibitor, and 10mM Hepes at pH7.4). The mitochondria were resuspended in succinate buffer (1mM ATP, 5mM sodium succinate, 0.08 mM ADP, 2mM K_2HPO_4, pH 7.4). A gradient of digitonin (0 to 2 mg digitonin / mg mitochondrial protein) was then added with typsin. 1% TritonX-100 plus trypsin was used as a positive

control. After 30 min. on ice, the reaction was stopped by adding soybean trypsin inhibitor for 10 min. The samples were then subjected to western blot analysis.

Camptothecin treatment, cell viability assays, and caspase activity assay

Camptothecin treatment (10μM) and AIF immunocytochemistry were performed as described previously (Cregan et al, 2002). Cell death was determined by the characteristic nuclear morphology of chromatin condensation revealed by Hoechst staining. Caspase activity assay was performed as described previously (Cregan et al, 2002).

Mitochondrial membrane potential and length measurements

For live mitochondrial imaging and electrochemical potential determination, neurons were plated on poly D lysine coated glass coverslips. 36 hours after seeding, cells were incubated with 50 nM TMRE at 37°C for 20 minutes and the coverslips were mounted in live-cell chambers and visualized as described (Neuspiel et al, 2005). Total fluorescence arbitrary units were recorded for the whole field. The total fluorescence intensity was quantified as the sum of the values of each pixel within the field minus the average background signal per pixel. The average intensity per cell was calculated by dividing the total fluorescence intensity by the number of cells in that field. Mitochondrial length was measured by tracing mitochondria using Northern Eclipse software.

Electron Microscopy and intra cristae cross sectional distance measurements

Electron microscopy was performed as described (Neuspiel et al, 2005). Briefly, after 2 days of culture, neurons were isolated, washed with PBS, fixed in 1.6% glutaraldehyde and

embedded in SPURR resin (Mariva, Québec). Thin sections were cut with a Leica Ultracut E ultramicrotome and counterstained with lead citrate and uranyl acetate. Digital images were taken using a JEOL 1230 TEM at 60 kV adapted with a 2K x 2K bottom mount CCD digital camera (Hamamatsu, Japan) and AMT software. Intra cristae cross sectional distance was measured using Northern Eclipse software.

Quantifications and statistical analysis

For cell death studies, a minimum of 500 cells per field was scored for each treatment at the indicated time points. For mitochondrial membrane potential measurements, a minimum of 100 cells for each treatment was scored. For mitochondrial length measurements, a minimum of 1000 mitochondria for each treatment was scored. For intracristal cross-sectional distance measurements, a minimum of 100 mitochondria for each treatment was measured. The data represents mean values +/- standard deviation from three independent experiments (n = 3) unless otherwise noted. p values were obtained using two-way ANOVA and Fisher's post hoc tests.

Results

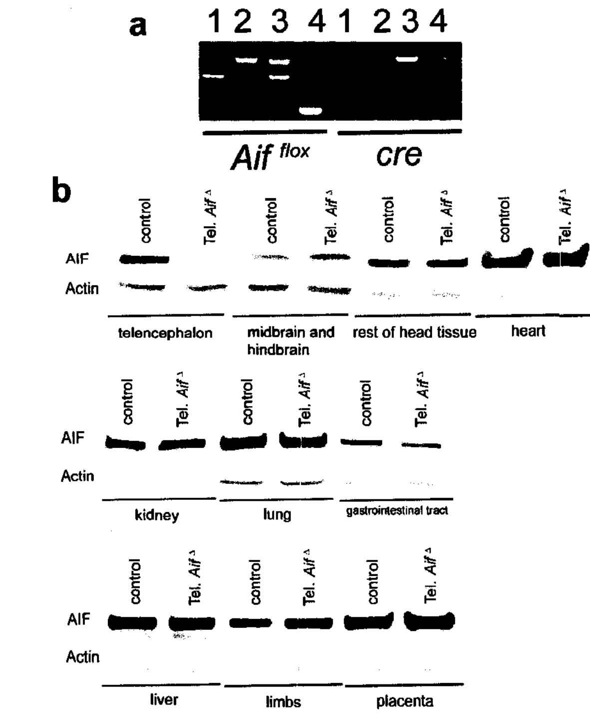
Generation of telencephalon conditional Aif $^{\Delta}$ mice

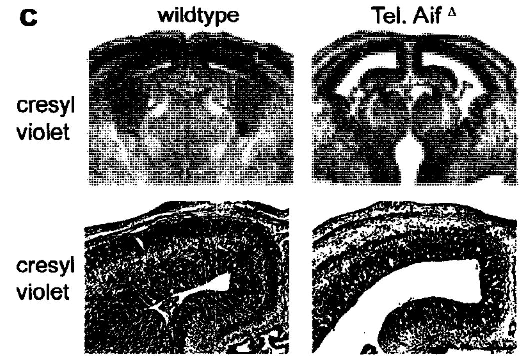
AIF null embryos die around embryonic day (E) 12, and muscle-specific loss of AIF leads to mitochondrial dysfunction, skeletal muscle atrophy and dilated cardiomyopathy (Joza et al, 2005). To study the function of AIF in neurons, we generated telencephalon-specific AIF mutant mice (tel. *Aif* $^{\Delta}$) by crossing *Aif* $^{flox/flox}$ mice with mice carrying Cre driven by the promoter Foxg1. Cre mediated excision of the floxed allele occurs in neuronal precursors of the telencephalon at E9 (Hebert & McConnell, 2000), resulting in deletion of the targeted gene in all cortical neurons. Deletion of the floxed AIF allele was assessed by PCR and Western blot analysis. In the absence of Cre, the floxed allele was present (Fig. 1a, lanes 1, 2). Cre expression resulted in deletion of AIF (Fig. 1a, lane 4). Western blot analysis confirmed absence of AIF only in the mutant telencephalon (Fig. 1b).

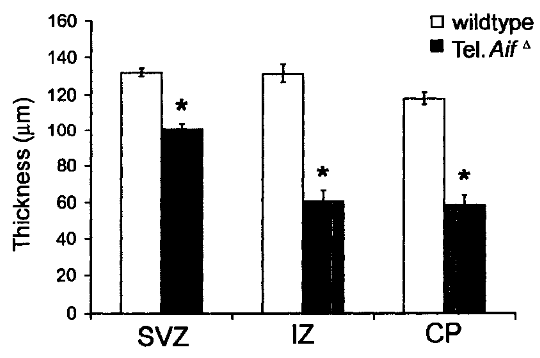
AIF is required for neuronal survival during cortical development

We next examined the telencephalon of the conditional mutants to investigate the role of AIF in cortical development. Tel. *Aif* $^{\Delta}$ conditional mutants die by E17 (data not shown). The tel. *Aif* $^{\Delta}$ mice exhibited reduced cortical thickness at E15.5 compared to control littermates (Fig. 1c). This reduction of thickness occurs mostly at the cortical plate (CP) and intermediate zone (IZ), and to a lesser extent at the subventricular zone (SVZ) (Fig. 1c). To address whether this reduction in size is due to increased apoptosis or reduced numbers of progenitor cells, active caspase 3 and phospho-histone H3 (PH3) staining were used to assess cell death and progenitor proliferation, respectively (Ferguson et al, 2002). Active caspase 3 staining revealed a marked increase in cell death in the tel. *Aif* $^{\Delta}$ in regions of post-

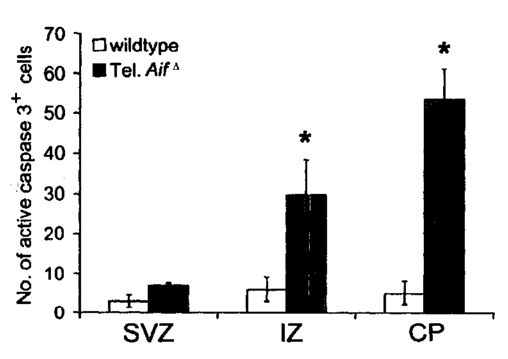
Figure 3-1: AIF is essential for neuronal survival during cortical development. a, PCR analysis of E15.5 telencephalon tissue. Lane 1: $Aif^{+/+}$ $cre^{+/+}$; lane 2: $Aif^{flox/Y}$ $cre^{+/+}$; lane 3: $Aif^{flox/+}$, $cre^{+/-}$, lane 4: $Aif^{flox/Y}$, $cre^{+/-}$ (tel. Aif^{Δ}). **b,** Western blot analysis for AIF and control β-actin expression of various tissues from tel. Aif^{Δ} and wild type littermates at E15.5. **c,** Cresyl violet staining of control and tel. Aif^{Δ} mice coronal forebrain sections at E15.5. SVZ = subventricular zone, IZ = intermediate zone, CP = cortical plate. Bar = 250μm. n=3. **d,** Active caspase 3 immunohistochemistry of control and tel. Aif^{Δ} coronal forebrain sections at E15.5. n=3. **e,** Phospho-histone H3 (PH-3) immunohistochemistry of control and tel. Aif^{Δ} coronal forebrain sections at E15.5. n=3.

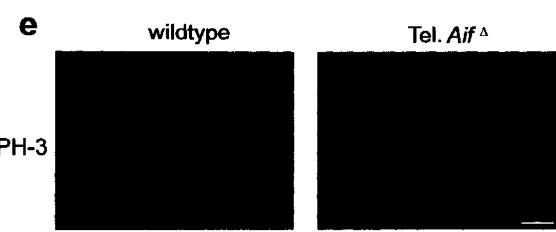

a
1 2 3 4 1 2 3 4
Aif flox
cre
b
control
Tel. Aif Δ
AIF
Actin
telencephalon
midbrain and hindbrain
rest of head tissue
heart
kidney
lung
gastrointestinal tract
liver
limbs
placenta

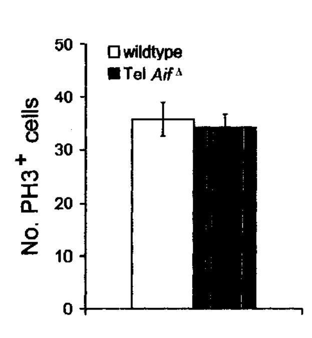

c
wildtype
Tel. Aif Δ
cresyl violet


cresyl violet
wildtype
Tel. Aif Δ
Thickness (μm)
SVZ
IZ
CP

d
wildtype
Tel. Aif Δ
active caspase 3


wildtype
Tel. Aif Δ
No. of active caspase 3+ cells
SVZ
IZ
CP


e
wildtype
Tel. Aif Δ
PH-3


wildtype
Tel Aif Δ
No. PH3+ cells

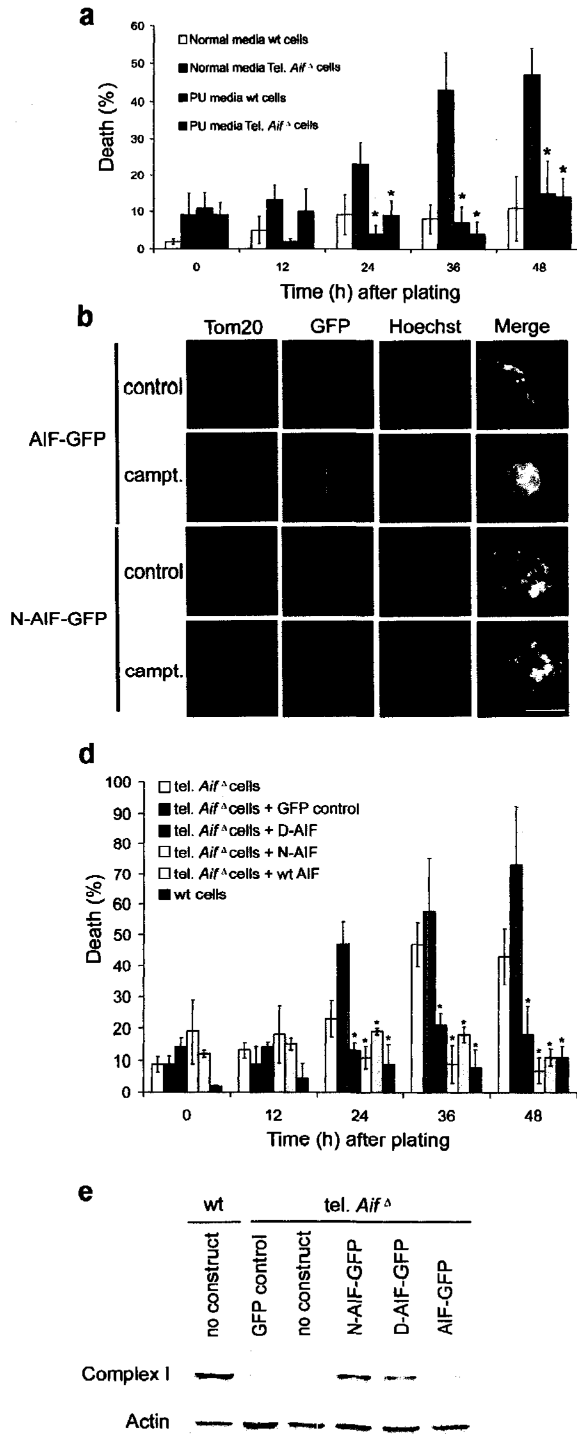
mitotic cells (Fig. 1d), whereas PH3 staining indicated similar numbers of proliferating progenitor cells along the ventricles in both the conditional mutants and their control littermates (Fig 1e). These data indicate that AIF is essential for survival of maturing neurons during cortical development, but AIF expression is dispensable for the proliferation of neuronal progenitors.

To assess whether cell death due to AIF depletion was cell autonomous, primary neuronal cultures were examined. After 2 days of culture, primary neuronal cells from E14.5 tel. Aif^{Δ} cortices exhibited increased cell death relative to neuronal cells from wild type controls (Fig. 2a). Consistent with previous reports on $Aif^{-/Y}$ ES cells (Vahsen et al, 2004), we found that expression of respiratory chain complex I was abrogated in mutant neurons compared to control neurons (Fig. 2e). Importantly, the reduced viability of tel. Aif^{Δ} neurons can be rescued when cells were cultured in media enriched with pyruvate, uridine, and additional glucose (PU media) to bypass defects in mitochondrial respiration (Fig. 2a). These supplements were used previously to culture cells lacking cyt c (Li et al, 2000) or mtDNA (King & Attardi, 1989), which exhibit defective mitochondrial respiration. These results show that AIF is required for neuronal cell survival and normal mitochondrial respiration in neurons.

Construction of AIF anchored to the inner membrane of the mitochondria

During apoptosis, AIF is released from mitochondria and translocates to the nucleus, inducing chromatin condensation and degradation. Since AIF depletion causes early lethality in neurons, the dual roles of AIF in mediating apoptosis and cellular homeostasis are difficult to resolve. In order to dissect the potential role of AIF in apoptosis from its role

Figure 3-2: Mitochondrially anchored AIF rescues reduced survival of tel. *Aif* $^{\Delta}$ neurons. a-c, Cortical neurons were isolated from E15.5 tel. *Aif* $^{\Delta}$ and wild type littermates and cultured in normal media or enriched PU media containing 50mg/L pyruvate, 110mg/L uridine, and 5mM glucose. **a**, Quantitative analysis of cell death of tel. *Aif* $^{\Delta}$ and wild type neurons cultured in normal and PU media (n=3). **b**, Wild type cortical neurons were infected with recombinant adenoviral vector containing GFP-tagged wild type AIF (AIF-GFP) or GFP-tagged N-AIF (N-AIF-GFP) and were treated with or without campthothecin. After 36 h, cells were fixed and stained with Hoechst to visualize nuclei and an anti-Tom20 antibody (red) to detect mitochondria. Green GFP fluorescence indicates AIF localization. **c**, Western analysis on subcellular fractionation of neurons infected with GFP-tagged wildtype AIF, N-AIF and D-AIF. Upper panel: no camptothecin, Lower panel: 12 hours after camptothecin treatment. m=mitochondrial fraction, n=nuclear fraction, and c=cytoplasmic fraction. The experiment was repeated at least three times with similar results. **d**, Quantitative analysis of spontaneous cell death of cortical neurons isolated from tel. *Aif* $^{\Delta}$ and wild type littermates infected with control virus, wild type AIF (wt AIF), or mitchondrially anchored AIF (N-AIF and D-AIF) at 50 MOI in normal media. Cell death was quantified by apoptotic nuclear morphology using Hoechst (n=3). * $p < 0.05$ compared to tel. *Aif* $^{\Delta}$ in normal media. **e**, Western analysis of complex I (39kDa subunit) expression in tel. *Aif* $^{\Delta}$ neurons expressing N-AIF and D-AIF compared to wild type and control tel. *Aif* $^{\Delta}$ neurons. The experiment was repeated at least three times with similar results. **f**, ATP production of the tel. *Aif* $^{\Delta}$ neurons expressing either N-AIF, D-AIF, or GFP as control (n=3). * $p < 0.05$ compared to tel. *Aif* $^{\Delta}$ neurons with GFP control. **g**, Oxygen consumption of the tel. *Aif* $^{\Delta}$ neurons expressing either N-AIF, D-AIF, or GFP as control (n=3). * $p < 0.05$ compared to tel. *Aif* $^{\Delta}$ neurons with GFP control.



a
Normal media wt cells
Normal media Tel. Aif cells
PU media wt cells
PU media Tel. Aif cells
Death (%)
Time (h) after plating
b
Tom20
GFP
Hoechst
Merge
AIF-GFP
N-AIF-GFP
control
campt.
d
tel. Aif cells
tel. Aif cells + GFP control
tel. Aif cells + D-AIF
tel. Aif cells + N-AIF
tel. Aif cells + wt AIF
wt cells
Death (%)
Time (h) after plating
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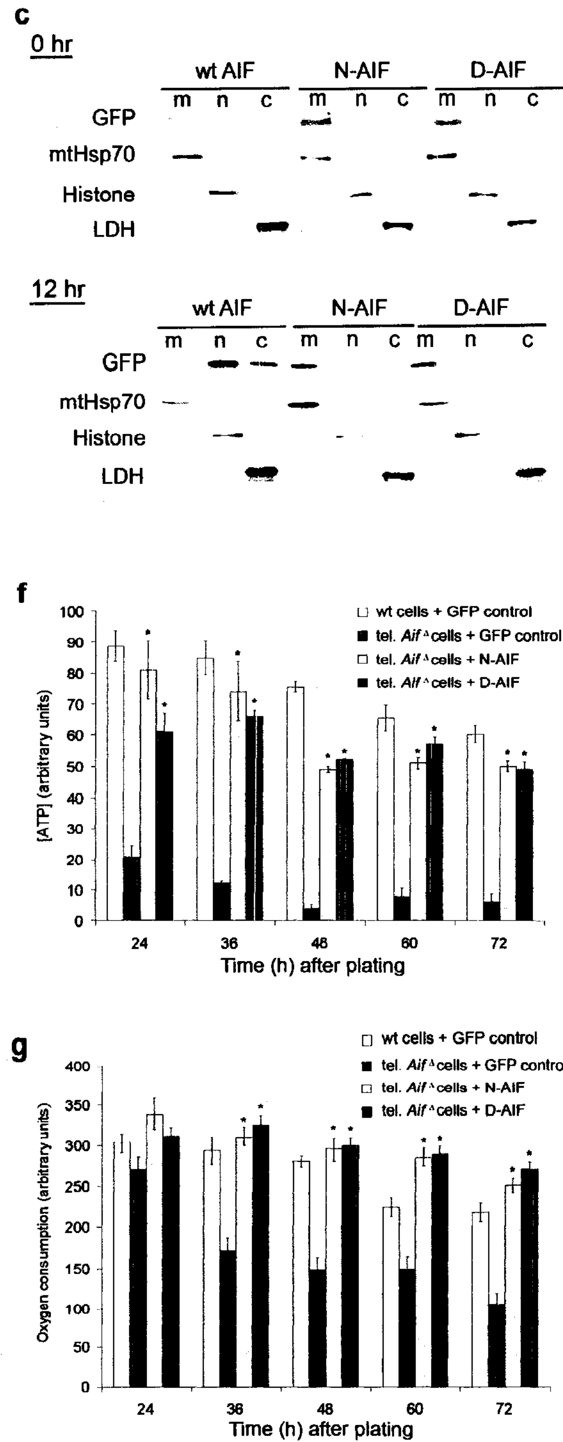
wt
tel. Aif
no construct
GFP control
no construct
N-AIF-GFP
D-AIF-GFP
AIF-GFP
Complex I
Actin



c
0 hr
12 hr
wt AIF
N-AIF
D-AIF
m n c
GFP
mtHsp70
Histone
LDH
f
wt cells + GFP control
tel. Aif cells + GFP control
tel. Aif cells + N-AIF
tel. Aif cells + D-AIF
[ATP] (arbitrary units)
Time (h) after plating
g
wt cells + GFP control
tel. Aif cells + GFP control
tel. Aif cells + N-AIF
tel. Aif cells + D-AIF
Oxygen consumption (arbitrary units)
Time (h) after plating

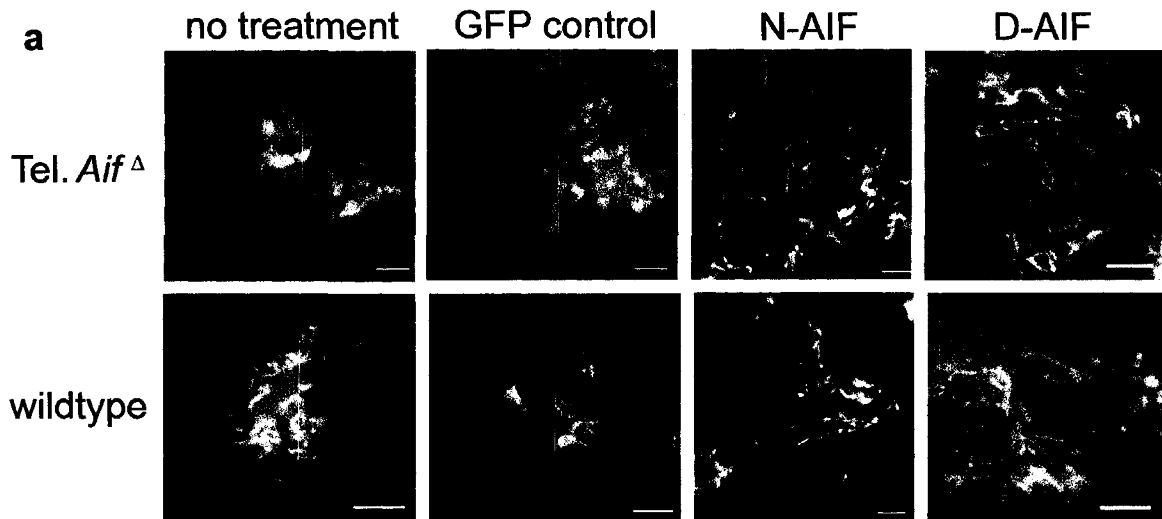
in mitochondria, we reconstituted AIF deficient neurons with mitochondrially-anchored AIF constructs such that AIF was permanently tethered to the inner mitochondrial membrane. To this end, we exchanged the mitochondrial localization sequence (MLS) of AIF with the MLS of two proteins (D-lactate dehydrogenase (Flick & Konieczny, 2002; Rojo et al, 1998) for D-AIF, and a modified form of pOSA-141I4 for N-AIF (Steenaart & Shore, 1997)) that are anchored to the outer leaflet of the inner membrane of mitochondria (Supp. Fig. 1a). Quantification of GFP fluorescence and western blot analysis of infected cells revealed similar expression of these constructs, comparable to endogenous level (Supp. Fig. 1b and 1c). Western analysis of digitonin gradient treatment on isolated mitochondria with anchored AIF mutant, as well as immunocytochemical analysis on digitonin treated neurons with anchored AIF mutant, showed that the anchored AIF mutant is located in the intermembrane space, similar to wildtype AIF (supp. fig. 1d and e). These results indicate that the anchored AIF constructs maintained the normal orientation of AIF in the mitochondria. GFP fluorescence from these anchored AIF constructs showed that both D-AIF and N-AIF were localized in mitochondria. Importantly, in contrast to wildtype AIF, both D-AIF and N-AIF remained associated with mitochondria after an apoptotic insult at all time points, as shown by immunocytochemistry and subcellular fractionation followed by western blot analysis (Fig. 2b and 2c). Expression of respiratory chain complex I (Fig. 2e) was restored in AIF deficient neurons expressing mitochondrially-anchored AIF, indicating that these constructs are functional. We next assessed survival of tel. Aif^{Δ} neurons expressing mitochondrially-anchored AIF. After two days under normal non-enriched culture conditions, tel. Aif^{Δ} neurons with either one of the anchored AIF constructs maintained the same level of cell survival as wild type neurons, which was not rescued by GFP control vector (Fig. 2d). ATP

production and oxygen consumption were also restored in AIF deficient neurons expressing the anchored AIF constructs compared to GFP control vector (Fig. 2f and 2g). These experiments demonstrate that expression of mitochondrially anchored AIF rescues the cell death of tel. *Aif*$^{\Delta}$ neurons.

AIF is required for maintaining mitochondrial morphology and cristae structure

We next asked whether there was perturbation of mitochondrial morphology in tel. *Aif*$^{\Delta}$ neurons. The mitochondrial membrane potential sensitive dye TMRE was used to visualize mitochondria in cells cultured in enriched media. Wild type neurons have elongated and tubular mitochondria which often spread along neurites (Fig. 3a). In contrast, tel. *Aif*$^{\Delta}$ neurons exhibited short and fragmented mitochondria that are often perinuclear (Fig. 3a). Few mitochondria were observed in the neurites of tel. *Aif*$^{\Delta}$ neurons. Mitochondrial membrane potential of tel. *Aif*$^{\Delta}$ neurons was hyperpolarized relative to wild type cells (Supp. Fig 2a), which can be dissipated using FCCP, the mitochondrial potential uncoupler (Supp. Fig 2b). The hyperpolarized membrane potential as well as altered mitochondrial morphology were restored to normal by the expression of either anchored AIF or wild type AIF in tel. *Aif*$^{\Delta}$ neurons (Fig. 3a and Supp. Fig 2). Interestingly, expression of anchored AIF in wild type cells resulted in increased mitochondrial length compared to controls (Fig. 3a,b), suggesting a role for AIF in maintaining mitochondrial morphology. Next we asked if the mitochondrial ultrastructure is also disrupted in these cells. We used transmission electron microscopy to visualize mitochondria from neurons cultured in enriched PU media to eliminate secondary effects due to reduced survival in tel. *Aif*$^{\Delta}$ neurons. In wild type cells, mitochondrial cristae are shaped as compact tubules in an

Figure 3-3: AIF controls mitochondrial structure. Cortical neurons from E15.5 tel. Aif^{Δ} and wild type littermates were infected at time of plating with mitochondrially anchored N-AIF and D-AIF and a GFP control virus at 50 MOI in enriched PU media. **a-b**, After 36 hours 50nm TMRE was added to media and live cell images were taken. **a**, Representative images of tel. Aif^{Δ} and wild type mitochondria infected with the indicated constructs. Bars = 1 μm. **b**, Average length of mitochondria of neurons. The cell types and treatments are as indicated (n = 4). * $p < 0.05$ compared to wild type with no virus; + $p < 0.05$ compared to tel. Aif^{Δ} infected with the GFP control virus; ** $p < 0.05$ compared to wild type with GFP control.


a
no treatment
GFP control
N-AIF
D-AIF
Tel. *Aif* Δ
wildtype

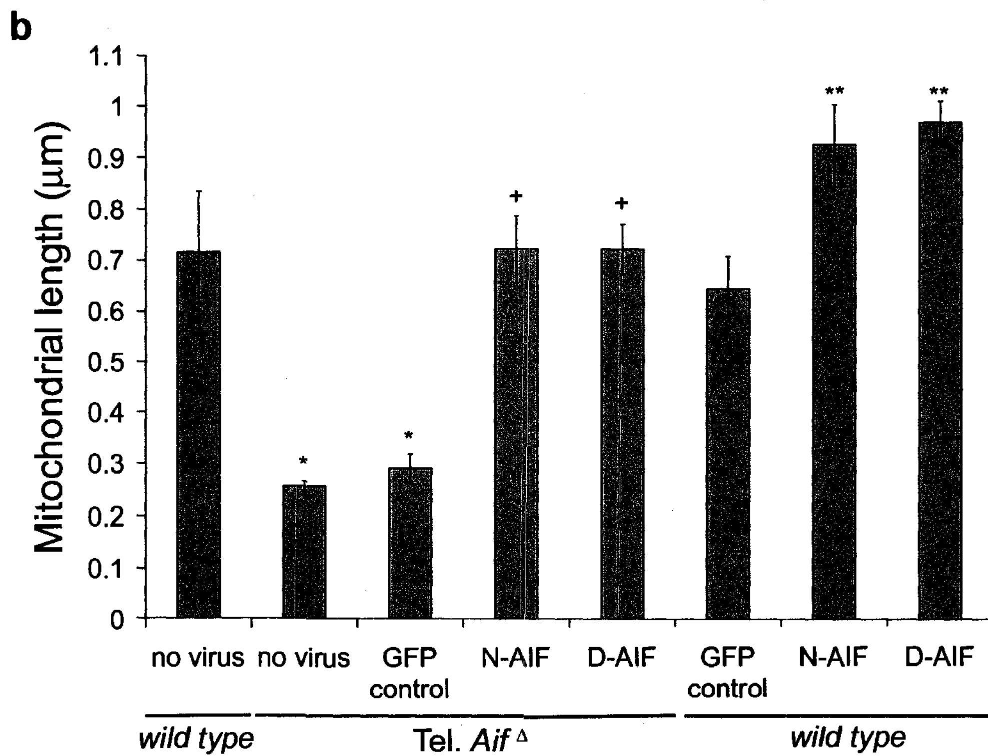
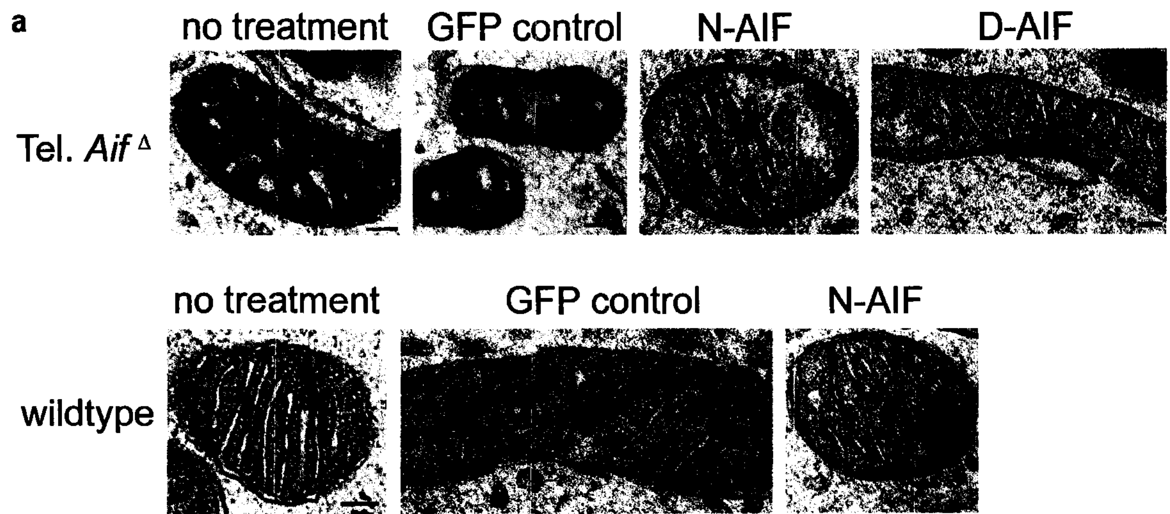
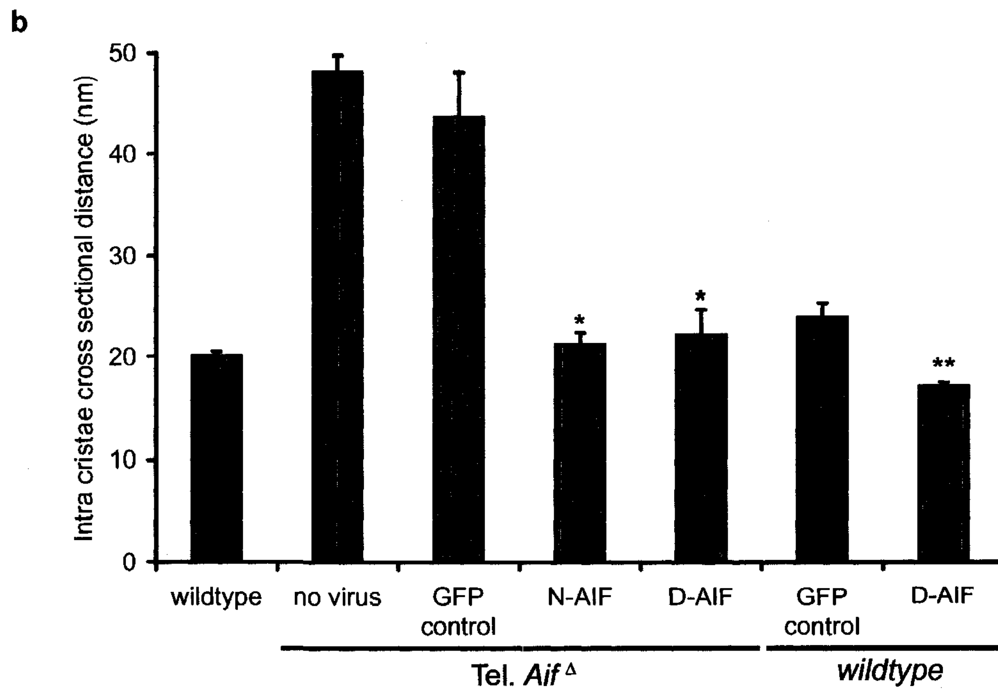

b
Mitochondrial length (μm)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
no virus
no virus
GFP control
N-AIF
D-AIF
GFP control
N-AIF
D-AIF
wild type
Tel. *Aif* Δ
wild type

Figure 3-4: AIF depleted neurons have perturbed mitochondrial cristae structure. a-b, Transmission electron microscopy of mitochondria. **a**, Representative images of mitochondria of tel. *Aif* $^{\Delta}$ and wildtype mitochondria infected with the indicated constructs. Bars = 100nm **b**, Quantification of the intracristal cross-sectional distances (n = 4). * $p < 0.05$ compared to tel. *Aif* $^{\Delta}$ infected with the GFP control virus; ** $p < 0.05$ compared to wild type neurons infected with the GFP control virus.


a
no treatment
GFP control
N-AIF
D-AIF
Tel. Aif Δ
no treatment
GFP control
N-AIF
wildtype

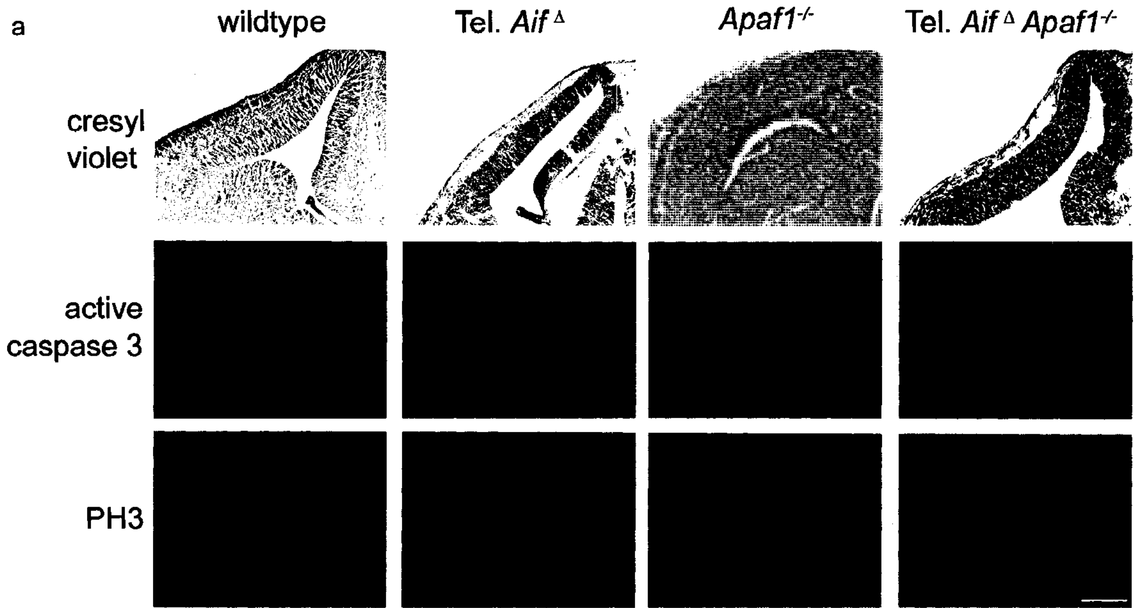

b
Intra cristae cross sectional distance (nm)
50
40
30
20
10
0
*
*
**
wildtype
no virus
GFP control
N-AIF
D-AIF
GFP control
D-AIF
Tel. Aif Δ
wildtype

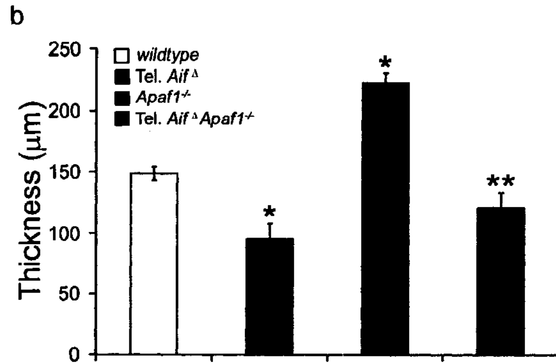
orderly fashion (Fig. 4a). Tel. *Aif* $^{\Delta}$ neurons, on the other hand, displayed aberrant cristae morphology. The cristae are dilated and do not orient in an orderly fashion (Fig. 4a). The cross sectional distance of tel. *Aif* $^{\Delta}$ mitochondrial cristae is ~ 2.5 times wider than in control wild type mitochondria (Fig. 4b). Expression of anchored AIF in wild type cells again reduced intra-cristae distances from 20nm to 17nm (Fig. 4b), further supporting the notion that the defect seen in tel. *Aif* $^{\Delta}$ neurons does not result from secondary effects. A detailed analysis of intracristal cross-sectional distance is shown in Supp. fig. 3a and b. These studies demonstrate a novel role for AIF in regulating mitochondrial structure and cristae morphology.

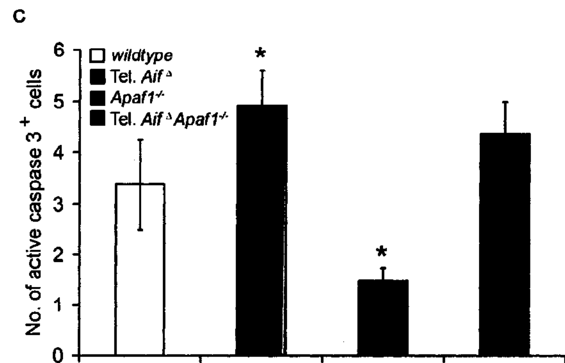
Mitochondrially anchored AIF revealed critical role of AIF during apoptosis

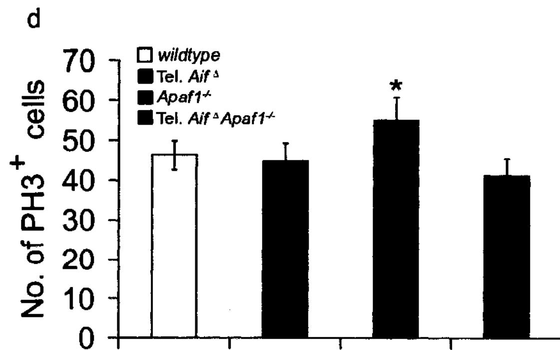
Defining the proapoptotic function of AIF has been confounded by recent findings demonstrating an essential physiological role for AIF in the mitochondria. Presently, it is unknown if mitochondrial release of AIF in itself induces apoptosis due to loss of AIF mitochondrial function, or whether AIF plays a proapoptotic role following nuclear translocation. First we generated double tel. *Aif* $^{\Delta}$ *Apaf1* $^{-/-}$ animals in which both caspase dependent and independent pathways have been inactivated. Double mutant embryos exhibit some increase in cortical thickness relative to tel. *Aif* $^{\Delta}$ animals (Fig 5a,b), possibly due to the increased survival of cells in *Apaf1* $^{-/-}$ background. As reported previously (Cozzolino et al, 2004), *Apaf1* $^{-/-}$ mice have increased numbers of progenitor cells compared to wildtype and tel. *Aif* $^{\Delta}$ mice (Fig. 5a,d). Next we asked if the double mutant neurons exhibit protection against DNA damage induced cell death. After inducing cell death with camptothecin, the tel. *Aif* $^{\Delta}$ *Apaf1* $^{-/-}$ double mutant neurons cultured in pyruvate

Figure 3-5: Apafl deficiency can partially compensate neuronal loss due to AIF deficiency during development. a. Cresyl violet staining, active caspase 3 staining, and PH-3 staining of coronal telencephalon sections from control wild type, tel. Aif^{Δ}, $Apafl^{-/-}$, and tel. Aif^{Δ} $Apafl^{-/-}$ double mutant mice (E14.5). Bar = 250μm. **b-d**. Quantitative analysis of **b.** cortical thickness (n = 3), **c.** active caspase 3 positive cells (n = 3), and **d**. phospho-histone H3 (PH-3) positive cells (n = 3); * $p < 0.05$ compared to wild type; ** $p < 0.05$ compared to tel. Aif^{Δ}.


a
wildtype
Tel. Aif Δ
Apaf1-/-
Tel. Aif Δ Apaf1-/-
cresyl violet
active caspase 3
PH3


b
wildtype
Tel. Aif Δ
Apaf1-/-
Tel. Aif Δ Apaf1-/-
Thickness (μm)
250
200
150
100
50
0
*
*
**

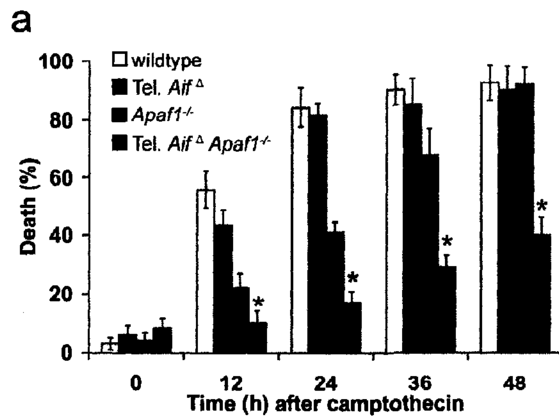

c
wildtype
Tel. Aif Δ
Apaf1-/-
Tel. Aif Δ Apaf1-/-
No. of active caspase 3+ cells
6
5
4
3
2
1
0
*
*

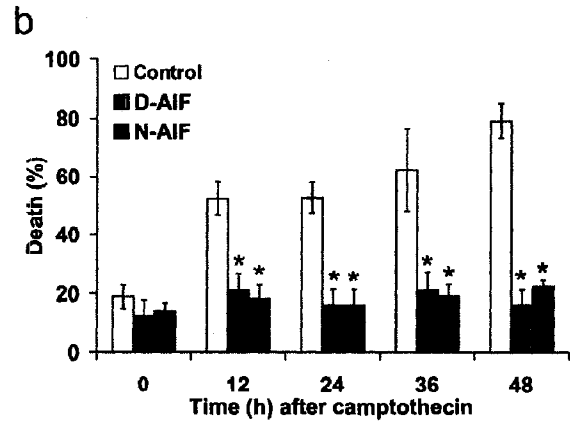

d
wildtype
Tel. Aif Δ
Apaf1-/-
Tel. Aif Δ Apaf1-/-
No. of PH3+ cells
70
60
50
40
30
20
10
0
*

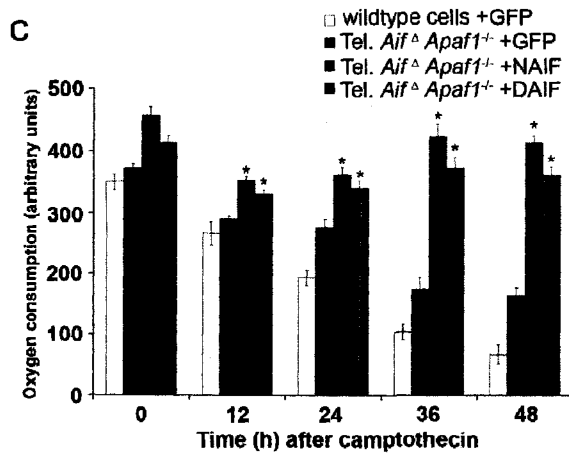
supplemented media, exhibited increased survival relative to single mutants and wildtype control neurons (Fig. 6a). AIF deficiency alone can offer transient but significant protection at 12 hours (Fig. 6a, ~45% for tel. Aif^{Δ} versus ~55% for wildtype), and at later time points the percentage of cell death of tel. Aif^{Δ} cells becomes similar to wildtype cells due to the presence of caspases. This is in agreement with *Hq* neurons which also showed a transient delay in chromatin condensation compared to wildtype during cell death (Cheung et al, 2005). Cytochrome C release in these mutants is not affected, suggesting that mitochondrial permeablization is not affected by the deletion of Apafl and AIF (supp. fig. 4). AIF release in $Apafl^{-/-}$ neurons is similar to wildtype cells (Supp. Fig 5a and b), which is in agreement with our previous results showing AIF release in $Apafl^{-/-}$ neurons is similar to wildtype after p53 and camptothecin induced cell death (Cregan et al. 2002). Caspase activity was not detected in $Apafl^{-/-}$ and tel. $Aif^{\Delta}Apafl^{-/-}$ compared to wildtype and tel. Aif^{Δ} (Supp. Fig. 6a).

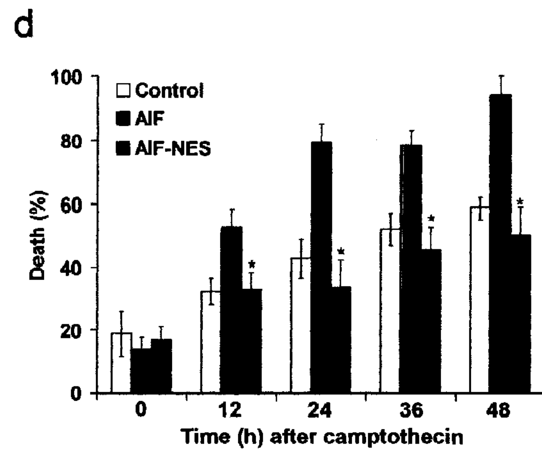
Using the anchored AIF mutants and tel. $Aif^{\Delta}Apafl^{-/-}$ neurons, we asked whether AIF is indeed an apoptotic effector after induction of cell death. We first addressed if anchored AIF can provide further protection against cell death in tel. $Aif^{\Delta}Apafl^{-/-}$ neurons. Western analysis revealed that anchored AIF is not released during cell death but is retained in the mitochondria (Supp. Fig. 7), and similar to tel. $Aif^{\Delta}Apafl^{-/-}$ neurons, caspase was not activated (Supp. Fig. 6b). Strikingly, expression of either of the anchored AIF constructs in the double mutant neurons in enriched media, could provide further protection against cell death for an extended time after insult (Fig. 6b). Anchored mutants could also maintain oxygen consumption following camptothecin treatment (Fig. 6c). This indicates that by retaining AIF in the mitochondria during cell death, survival can be sustained first by inhibiting AIF's apoptotic role in the nucleus, and secondly by maintaining AIF's

Figure 3-6: Dissociation of the dual roles of AIF in tel. *Aif* $^{\Delta}$ *Apaf1*$^{-/-}$ neurons revealed AIF's proapoptotic role at the nucleus apart from its physiological role in the mitochondria. **a**, Cortical neurons cultured from E14.5 wild type, tel. *Aif*$^{\Delta}$, *Apaf1*$^{-/-}$, and tel. *Aif*$^{\Delta}$ *Apaf1*$^{-/-}$ double mutant embryos were treated with camptothecin in enriched media and cell death were assessed at the indicated time points (n = 3). **b**, Cortical neurons from E14.5 tel. *Aif* $^{\Delta}$*Apaf1*$^{-/-}$ double mutant embryos were infected at time of plating with mitochondrially anchored D-AIF and N-AIF and a GFP control virus at 50 MOI in enriched media. Campthothecin was then added and cell death was assessed by Hoechst staining (n = 3). **c**, Oxygen consumption of tel. *Aif*$^{\Delta}$ *Apaf1*$^{-/-}$ neurons expressing D-AIF and N-AIF after camptothecin treatment (n = 3). $*p<0.05$ compared to tel. *Aif*$^{\Delta}$ *Apaf1*$^{-/-}$ neurons expressing GFP control. **d**, Cortical neurons from tel. *Aif*$^{\Delta}$*Apaf1*$^{-/-}$ double mutant were infected at time of plating with AIF, NES-AIF, or a GFP control at 50 MOI in enriched media. Camptothecin was then added, and cell death was assessed at the indicated time points (n = 3). $*p < 0.05$ compared to GFP control.


a
wildtype
Tel. Aif^Δ
Apaf1-/-
Tel. Aif^Δ Apaf1-/-
Death (%)
Time (h) after camptothecin


b
Control
D-AIF
N-AIF
Death (%)
Time (h) after camptothecin


c
wildtype cells +GFP
Tel. Aif^Δ Apaf1-/- +GFP
Tel. Aif^Δ Apaf1-/- +NAIF
Tel. Aif^Δ Apaf1-/- +DAIF
Oxygen consumption (arbitrary units)
Time (h) after camptothecin


d
Control
AIF
AIF-NES
Death (%)
Time (h) after camptothecin

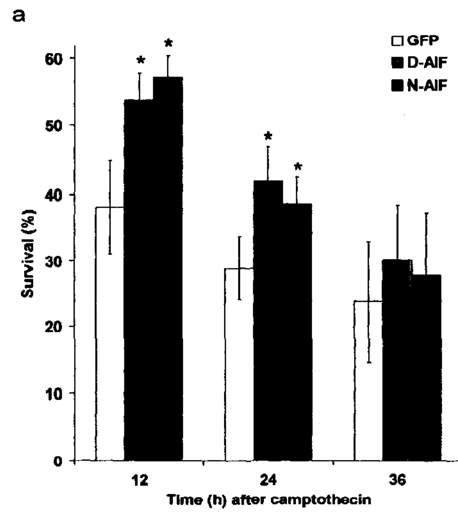
physiological function in the mitochondria. These data show that AIF is an important contributor to the execution of cell death following DNA damage.

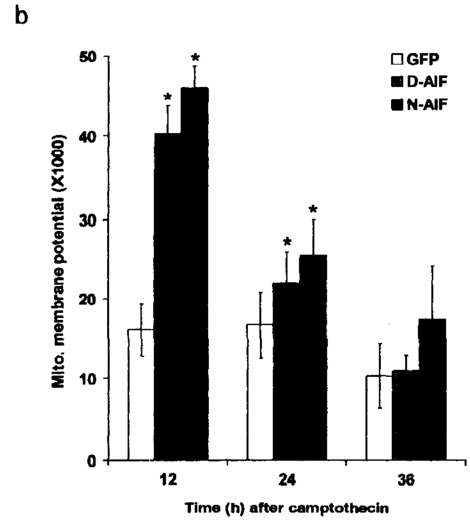
We next asked whether the effects of AIF in the nucleus are crucial to its pro-apoptotic function. To address this, we generated an AIF construct harbouring a nuclear export signal (NES) (Fischer et al, 1995), which prevents AIF from accumulating in the nucleus. After induction of apoptosis, tel. *Aif* $^{\Delta}$ *Apaf1* $^{-/-}$ double mutant neurons reconstituted with wildtype AIF exhibited markedly increased cell death compared to neurons expressing control GFP (Fig. 6d). Importantly, expression of AIF-NES failed to restore AIF-mediated cell death, as shown by comparable survival rates between tel. *Aif* $^{\Delta}$ *Apaf1* $^{-/-}$ neurons expressing AIF-NES and control GFP (Fig. 6d). These studies indicate that a major part of the pro-apoptotic function of AIF is mediated in the nucleus.

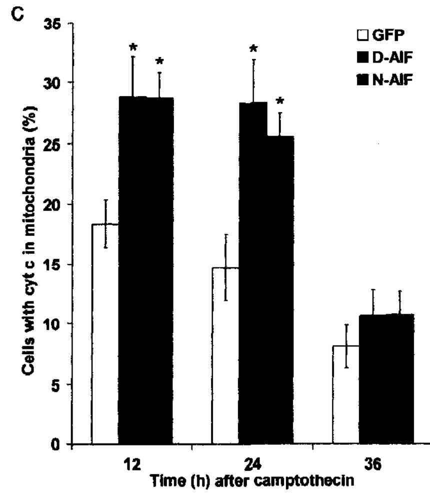
Overexpression of anchored AIF in wildtype cells only transiently delayed cell death after apoptosis induction

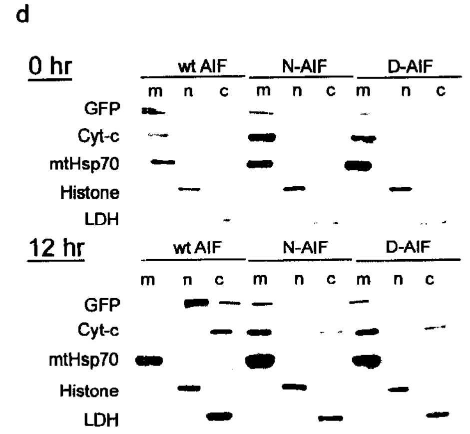
Since AIF has dual functions in apoptosis and cell survival, we next asked which of the following is the cause of cell death after apoptosis induction: (a) the loss of AIF from the mitochondria, (b) the pro-apoptotic action of AIF in the nucleus, or (c) both as equally important. We answer this question first by looking at apoptosis of wildtype neurons expressing anchored AIF constructs. The release and loss of AIF in the mitochondria of wildtype cells during apoptosis, therefore, will be reconstituted by the anchored AIF. At the same time the endogenous wildtype pool of AIF will still translocate to the nucleus. If the loss of AIF from mitochondria is indeed sufficient to induce apoptosis, then replenishing anchored AIF in the mitochondria should rescue cell death in these wildtype cells. At 12

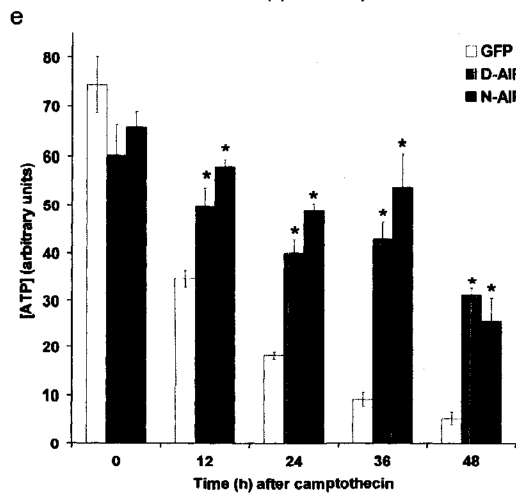
Figure 3-7: Anchored AIF can transiently protect wildtype neurons against DNA damage induced apoptosis. Cortical neurons from E14.5 wild type mice were infected at time of plating with the anchored D-AIF and N-AIF constructs and a GFP only control at 50 MOI. Camptothecin were then added. **a**, Survival of the cells was measured by nuclear morphology revealed using Hoechst staining (n=5) *$p<0.05$. **b**, Mitochondrial membrane potential was measured by TMRE intensity (n=5) *$p<0.05$. **c**, Percentages of cells with Cyt c retained in the mitochondria (n=5). Cyt c retention in mitochondria was determined using anti cyt-c immunohistochemistry. * $p<0.05$. **d**, Western analysis on subcellular fractionation of neurons infected with N-AIF, D-AIF, and GFP control, to show cytochrome c release. The experiment was repeated at least three times with similar results. Upper panel: no camptothecin, Lower panel: 12 hours after camptothecin treatment. m=mitochondrial fraction, n=nuclear fraction, and c=cytoplasmic fraction. **e**, ATP concentration of the neurons expressing either N-AIF, D-AIF, or GFP as control. * $p < 0.05$ compared GFP control. **f**, O_2 consumption of neurons expressing either N-AIF, D-AIF, or GFP as control. * $p < 0.05$ compared to GFP control.

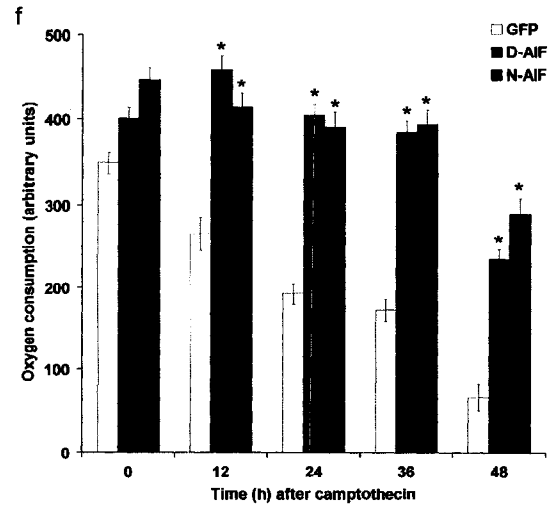

a
GFP
D-AIF
N-AIF
Survival (%)
Time (h) after camptothecin


b
GFP
D-AIF
N-AIF
Mito. membrane potential (X1000)
Time (h) after camptothecin


c
GFP
D-AIF
N-AIF
Cells with cyt c in mitochondria (%)
Time (h) after camptothecin


d
0 hr
wt AIF
N-AIF
D-AIF
m n c
GFP
Cyt-c
mtHsp70
Histone
LDH
12 hr

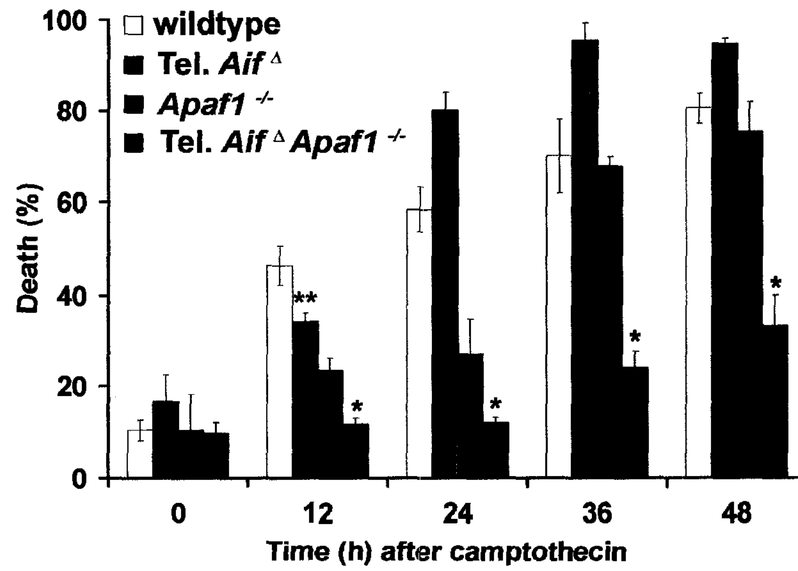

e
GFP
D-AIF
N-AIF
[ATP] (arbitrary units)
Time (h) after camptothecin


f
GFP
D-AIF
N-AIF
Oxygen consumption (arbitrary units)
Time (h) after camptothecin

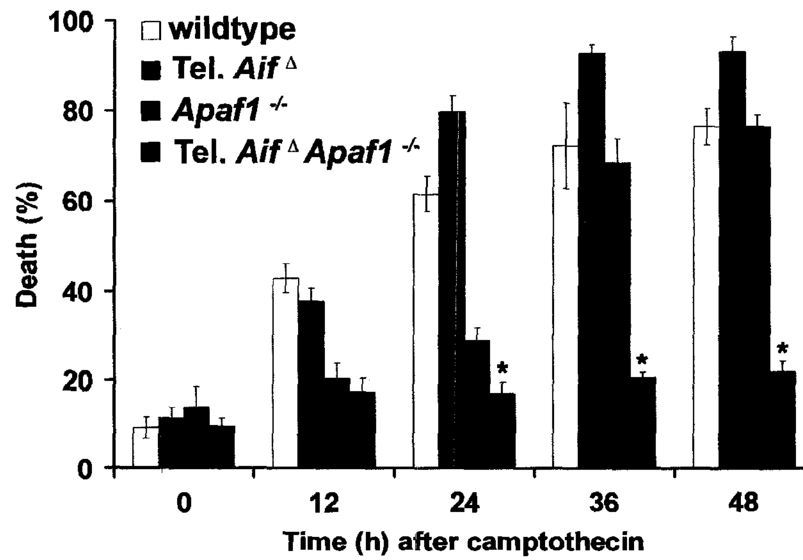
hours and 24 hours after camptothecin treatment, wildtype cells expressing the anchored AIF constructs exhibited less cell death than control cells (Fig. 7a). This suggests that at early time points after the induction of apoptosis, loss of AIF from the mitochondria contributes to cell death. At 36 hours, however, wildtype cells with anchored AIF constructs exhibited similar rates of apoptosis as cells expressing GFP (Fig. 7a). This suggests that at later time points, the apoptotic function of endogenous AIF in the wildtype cells is the major contributor to cell death. At 12 hours and 24 hours, the presence of anchored AIF in the wildtype cells can also retain cytochrome C in the mitochondria as revealed by immunocytochemistry and subcelluar fractionation followed by western blot analysis (Fig. 7c and Fig. 7d). The mitochondrial membrane potential (Fig. 7b), ATP production (Fig. 7e) and oxygen consumption (Fig. 7f) were also maintained transiently. These suggest that during apoptosis, the presence of AIF in the wildtype mitochondria is able to transiently reduce the release of cytochrome C and maintain membrane potential. Caspase activation was also transiently delayed in wildtype cells expressing anchored AIF mutants (supp. fig. 6c), possibly due to the transient retention of cytochrome c in the mitochondria (Fig. 7c and d). To further establish the apoptotic function of endogenous AIF, we expressed anchored AIF mutants in control, $Apaf1^{-/-}$, tel. Aif^{Δ}, tel. $Aif^{\Delta}Apaf1^{-/-}$ neurons and induced apoptosis by the addition of camptothecin. Expression of anchored AIF mutants, N-AIF and D-AIF, can provide protection in tel. $Aif^{\Delta}Apaf1^{-/-}$ double null neurons since endogenous AIF is absent. In contrast, $Apaf1^{-/-}$ neurons exhibited significant apoptosis (Fig 8a and b), suggesting that the endogenous AIF existing in $Apaf1^{-/-}$ single knockout plays a prominent role in the execution of cell death. Together, these studies demonstrate that during cell death, the release and loss of AIF from mitochondria may contribute to the early phase of apoptosis,

Figure 3-8: Endogenous AIF can still execute cell death in the presence of anchored AIF mutants in the mitochondria. Cortical neurons cultured (in conventional media) from E14.5 wild type, tel. *Aif* $^{\Delta}$, *Apaf1*$^{-/-}$, and tel. *Aif* $^{\Delta}$ *Apaf1*$^{-/-}$ double mutant embryos were infected at time of plating with the anchored D-AIF and N-AIF constructs. These cells were then treated with camptothecin and cell death was assessed at the indicated time points. **a**, N-AIF (n=3). **b**, D-AIF (n=3).

a



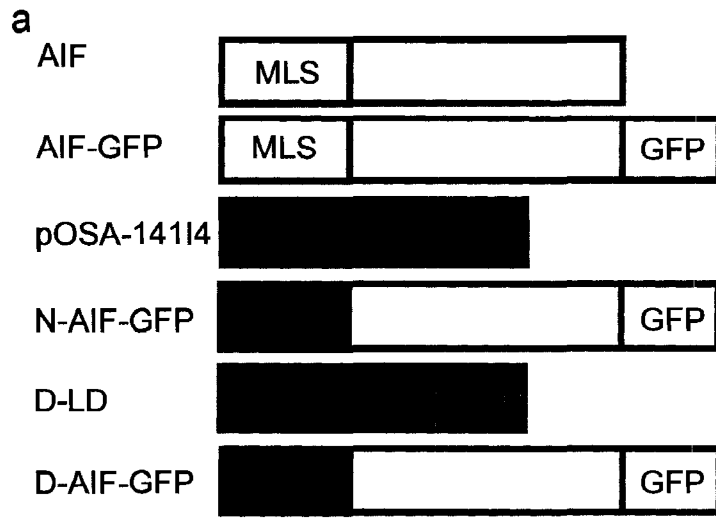
b

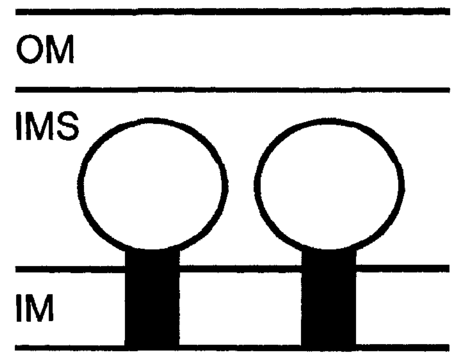


however, the pro-apoptotic function of AIF in the nucleus is a major contributor to apoptosis signaling. This is demonstrated by wild type and *Apaf1*$^{-/-}$ cells which still succumb to death even in the presence of anchored AIF in the mitochondria (Fig. 7a, 8a and 8b).

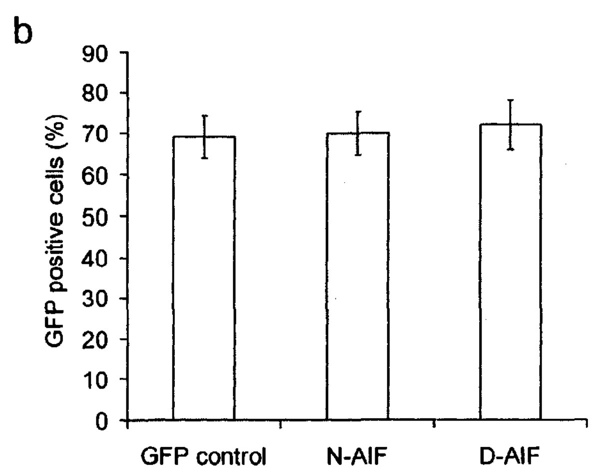
Supp. Figure 1: **Mitochondrially anchored AIF mutants.** **a**, Illustration of the anchored AIF constructs and their location in mitochondria. IM = inner membrane, OM = outer membrane, IMS = intermembrane space. **b**, Quantitative analysis of GFP fluorescence of the anchored AIF constructs 24 hours post infection. Cortical neurons from tel. Aif^{Δ} mice and wild type littermates were infected at 50 MOI with the indicated anchored AIF constructs and a control GFP only construct. **c**, Western blot analysis on the expression level of the anchored AIF constructs. **d**, Western analysis of digitonization followed by trypsin digestion of mitochondria isolated from cells expressing wt-AIF and N-AIF (both tagged with GFP). The experiment was repeated at least three times with similar results. **e**, Immunocytochemical analysis after digitonin treatment of neurons expressing wt-AIF, N-AIF and D-AIF. The experiment was repeated at least three times with similar results.

a

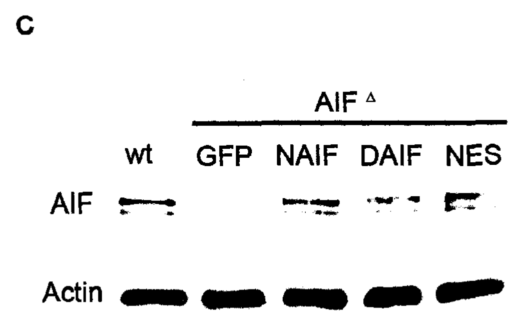




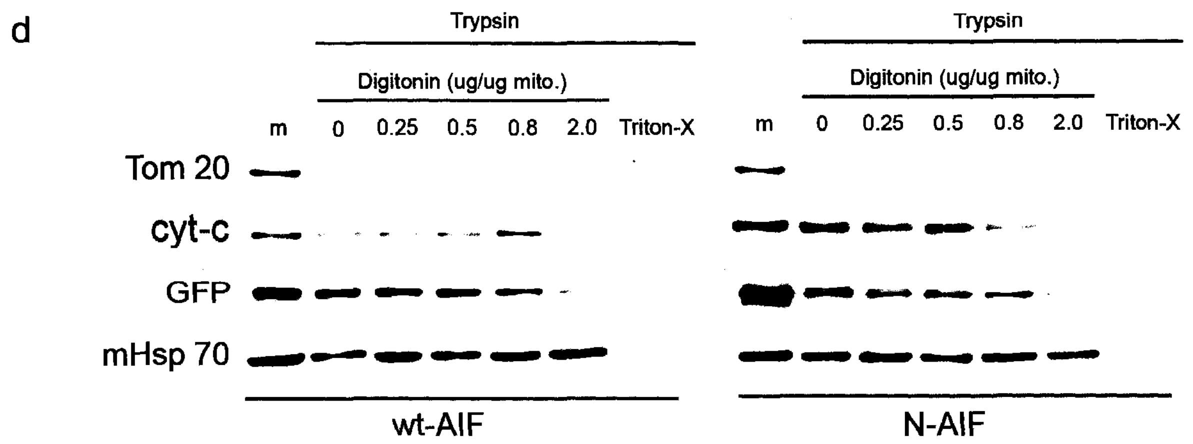
b

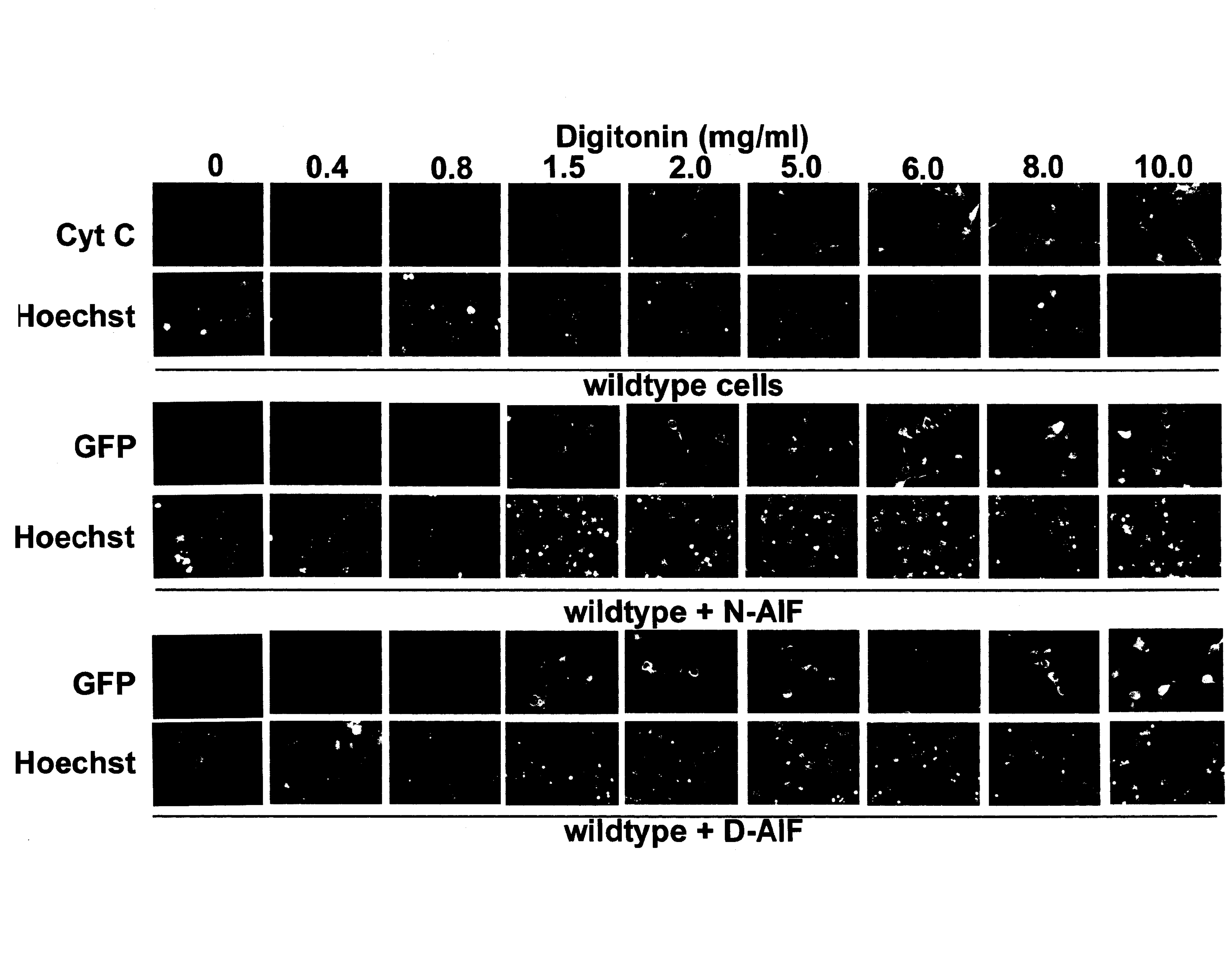


c



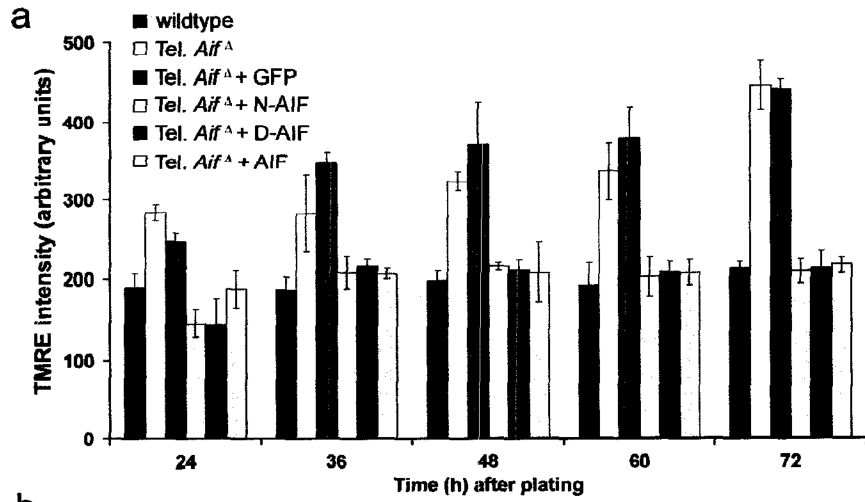
d



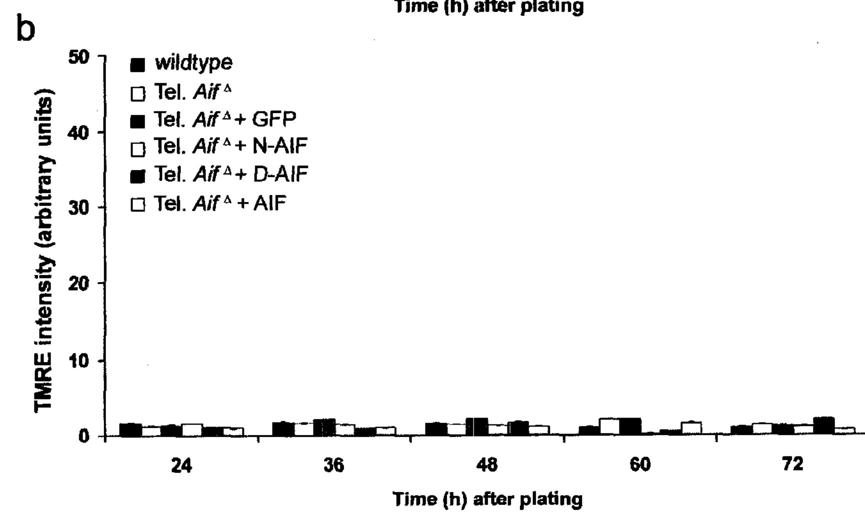


Digitonin (mg/ml)
0
0.4
0.8
1.5
2.0
5.0
6.0
8.0
10.0
Cyt C
Hoechst
wildtype cells
GFP
Hoechst
wildtype + N-AIF
GFP
Hoechst
wildtype + D-AIF

Supp. Figure 2: AIF deficient neurons exhibited abnormal mitochondrial membrane potential which can be corrected by expression of anchored AIF. Cortical neurons from E14.5 tel. *Aif* $^{\Delta}$ and wild type littermates were infected at time of plating with the anchored constructs N-AIF and D-AIF, wildtype AIF (wt AIF), or a GFP expressing control virus at 50 MOI in enriched PU media. TMRE (an indicator for mitochondrial membrane potential) was added to the cultures and the fluorescence intensity was measured. **a**, TMRE only (n=3), **b** FCCP added after TMRE application (n=3).



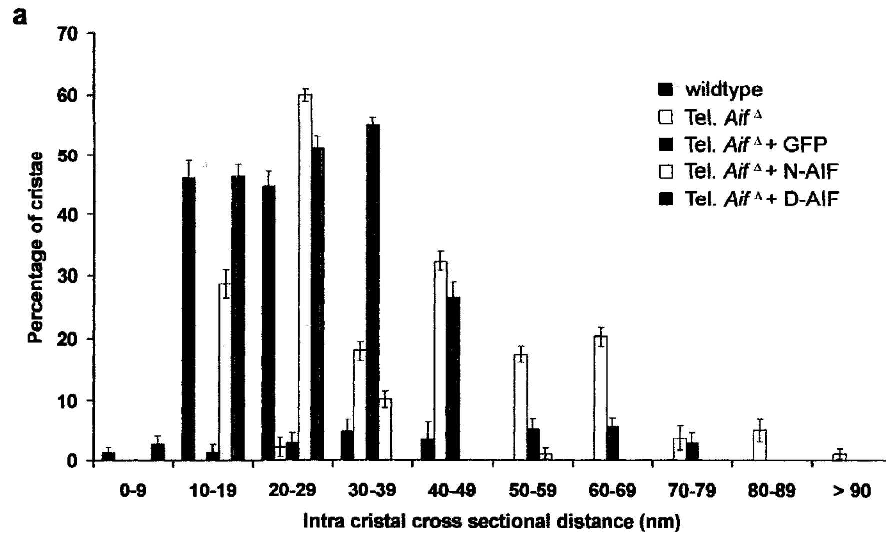
a
wildtype
Tel. Aif^Δ
Tel. Aif^Δ + GFP
Tel. Aif^Δ + N-AIF
Tel. Aif^Δ + D-AIF
Tel. Aif^Δ + AIF
TMRE intensity (arbitrary units)
500
400
300
200
100
0
24
36
48
60
72
Time (h) after plating



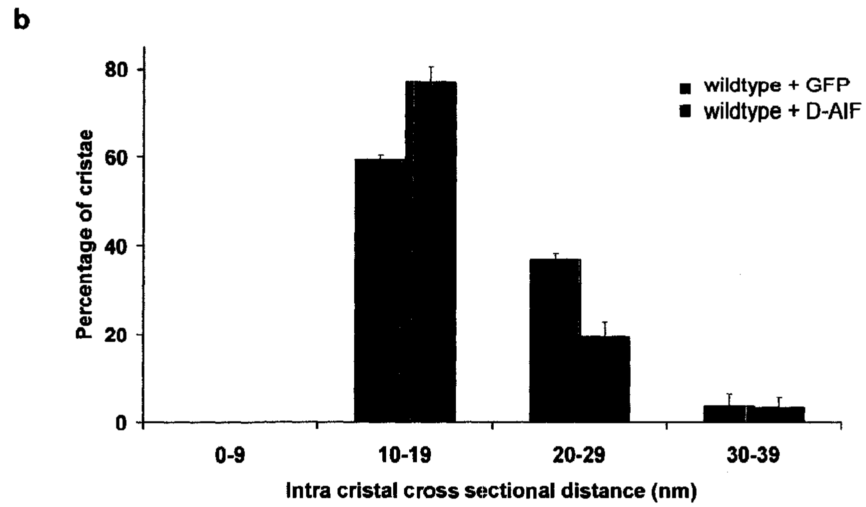
b
wildtype
Tel. Aif^Δ
Tel. Aif^Δ + GFP
Tel. Aif^Δ + N-AIF
Tel. Aif^Δ + D-AIF
Tel. Aif^Δ + AIF
TMRE intensity (arbitrary units)
50
40
30
20
10
0
24
36
48
60
72
Time (h) after plating

Supp. Figure 3: Representation of the intracristal cross-sectional distances by classifying into categories. Transmission electron microscopy of mitochondria from **a,** wildtype, tel. *Aif* $^{\Delta}$ *Apaf1*$^{-/-}$ neurons with N-AIF, D-AIF and GFP as control (n=3). **b,** wildtype with D-AIF and GFP as control (n=3).

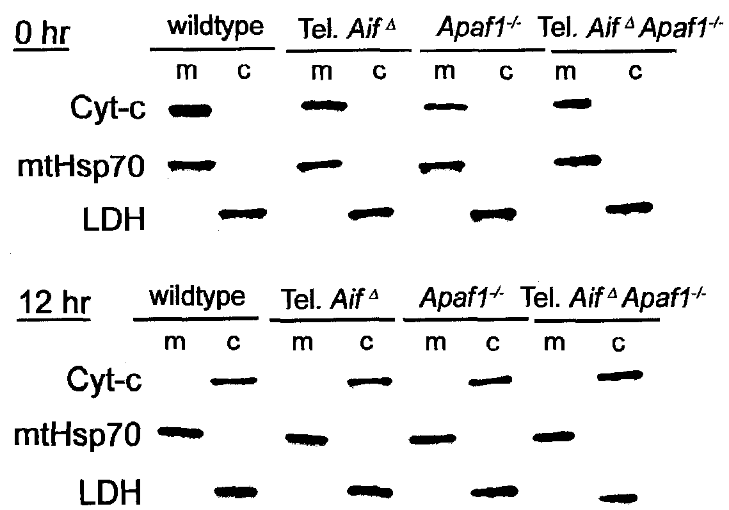
a



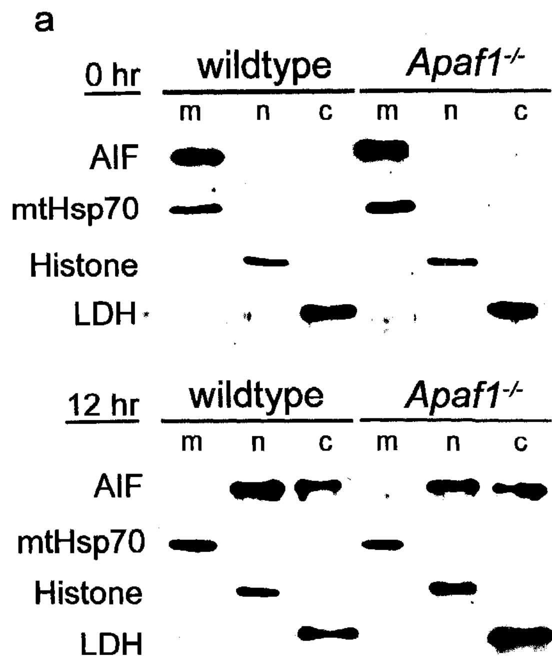
b

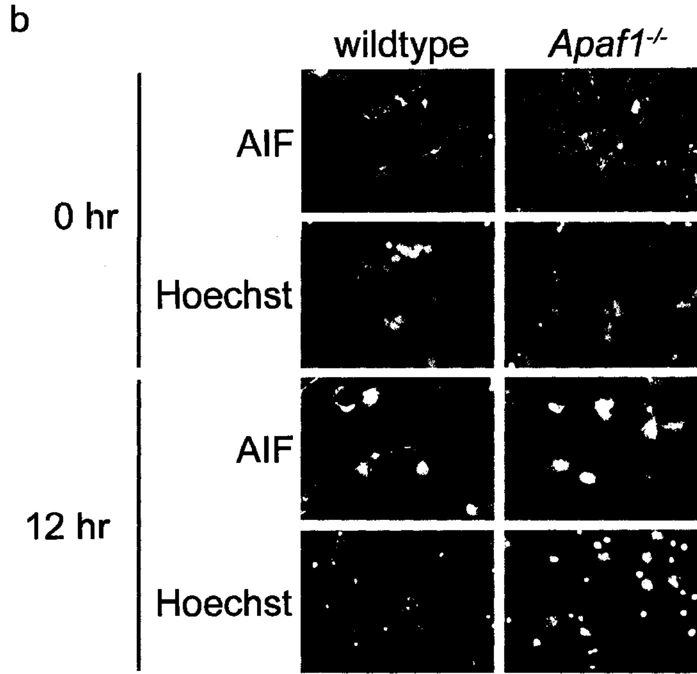


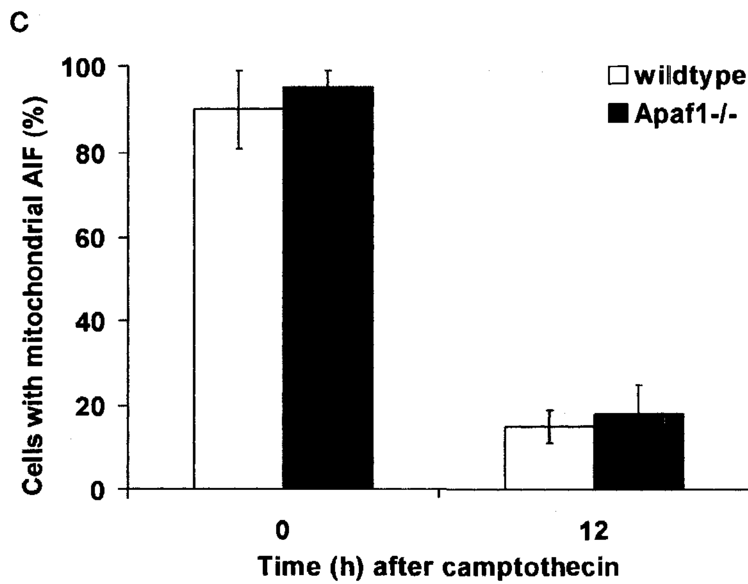
Supp. Figure 4: Cytochrome C was released after camptothecin treatment in the absence of AIF, Apafl or both. Western blot analysis of subcellular fractionation of wild type, tel. *Aif* $^{\Delta}$, *Apafl*$^{-/-}$, and tel. *Aif* $^{\Delta}$ *Apafl*$^{-/-}$ neurons after induction of apoptosis by camptothecin. Upper panel: no camptothecin, Lower panel: 12 hours after camptothecin treatment. m=mitochondrial fraction, c=cytoplasmic fraction. The experiment was repeated at least three times with similar results.


0 hr
wildtype
Tel. Aif Δ
Apaf1-/-
Tel. Aif Δ Apaf1-/-
m c m c m c m c
Cyt-c
mtHsp70
LDH
12 hr
wildtype
Tel. Aif Δ
Apaf1-/-
Tel. Aif Δ Apaf1-/-
m c m c m c m c
Cyt-c
mtHsp70
LDH

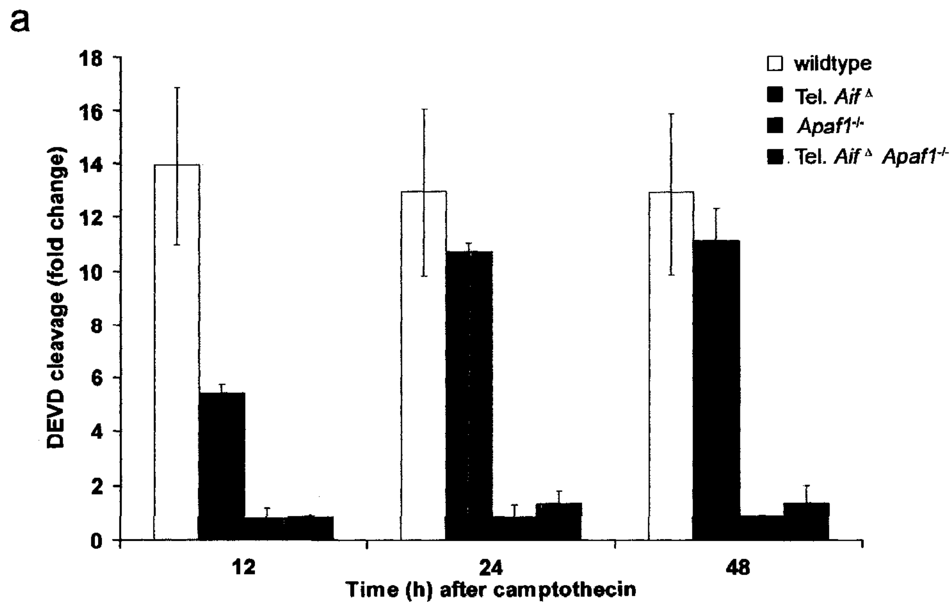
Supp. Figure 5: AIF was released after camptothecin treatment in the absence of Apaf1. **a**, Western blot analysis of subcellular fractionation of wildtype and *Apaf1*$^{-/-}$ neurons after induction of apoptosis by camptothecin. Upper panel: no camptothecin, Lower panel: 12 hours after treatment. m=mitochondrial fraction, n=nuclear fraction, c=cytoplasmic fraction. The experiment was repeated at least three times with similar results. **b**, Immunocytochemistry of wildtype and *Apaf1*$^{-/-}$ neurons showing AIF release after camptothecin treatment. Bar=20μm. **c**, Quantitative analysis of AIF release in wildtype and *Apaf1*$^{-/-}$ neurons by AIF immunostaining (n=3).

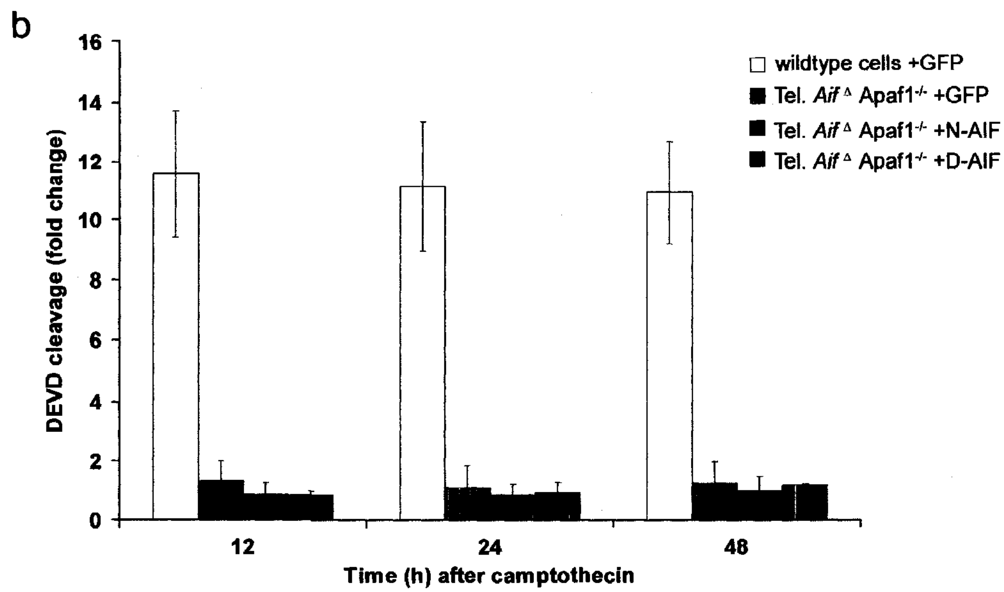

a
0 hr
wildtype
Apaf1-/-
m n c m n c
AIF
mtHsp70
Histone
LDH
12 hr
wildtype
Apaf1-/-
m n c m n c
AIF
mtHsp70
Histone
LDH

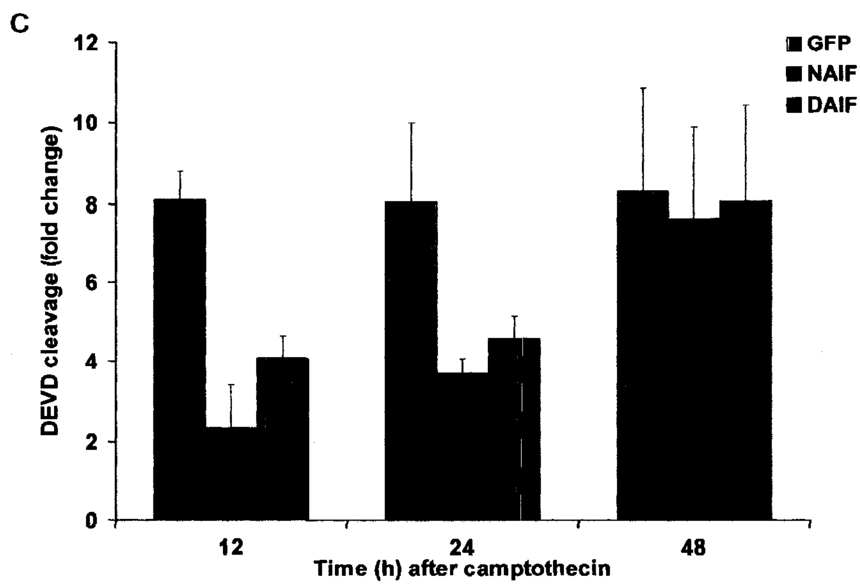

b
wildtype
Apaf1-/-
0 hr
AIF
Hoechst
12 hr
AIF
Hoechst


c
wildtype
Apaf1-/-
Cells with mitochondrial AIF (%)
100
80
60
40
20
0
0
12
Time (h) after camptothecin

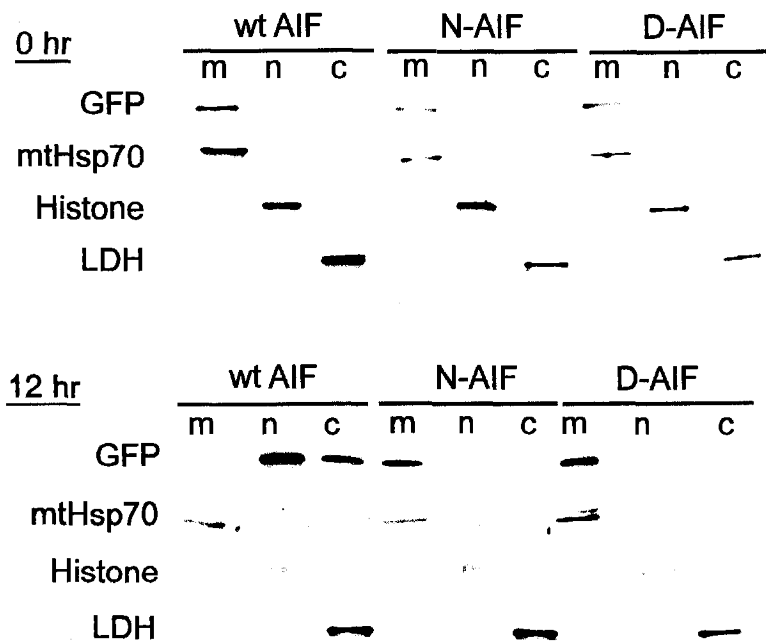
Supp. Figure 6: Caspase activity assay after camptothecin induced apoptosis. Caspase activity was measured by DEVD-AFC (*N*-acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl-coumarin)) cleavage. Caspase fold changes was calculated as fold increase over untreated cell. **a**, cortical neurons cultured from E14.5 wild type, tel. Aif^{Δ}, $Apaf1^{-/-}$, and tel. Aif^{Δ} $Apaf1^{-/-}$ double mutant embryos were infected at time of plating with the anchored D-AIF and N-AIF constructs and treated with camptothecin (n=3). **b**, cortical neurons cultured from E14.5 tel. Aif^{Δ} $Apaf1^{-/-}$ mutant neurons were infected with anchored D-AIF and N-AIF constructs and treated with camptothecin (n=3). **c**, wildtype cortical neurons were infected with anchored D-AIF and N-AIF constructs and treated with camptothecin (n=3).


a
DEVD cleavage (fold change)
0
2
4
6
8
10
12
14
16
18
wildtype
Tel. Aif$^{\Delta}$
Apaf1$^{-/-}$
Tel. Aif$^{\Delta}$ Apaf1$^{-/-}$
12
24
48
Time (h) after camptothecin


b
DEVD cleavage (fold change)
0
2
4
6
8
10
12
14
16
wildtype cells +GFP
Tel. Aif$^{\Delta}$ Apaf1$^{-/-}$ +GFP
Tel. Aif$^{\Delta}$ Apaf1$^{-/-}$ +N-AIF
Tel. Aif$^{\Delta}$ Apaf1$^{-/-}$ +D-AIF
12
24
48
Time (h) after camptothecin


c
DEVD cleavage (fold change)
0
2
4
6
8
10
12
GFP
NAIF
DAIF
12
24
48
Time (h) after camptothecin

Supp. Figure 7: Anchored AIF mutants were not released after camptothecin treatment in tel. *Aif* $^{\Delta}$ *Apaf1* $^{-/-}$ neurons. Western blot analysis of subcellular fractionation of tel. *Aif* $^{\Delta}$ *Apaf1* $^{-/-}$ neurons after induction of apoptosis by camptothecin. Upper panel: no camptothecin, Lower panel: 12 hours after camptothecin treatment. m=mitochondrial fraction, n=nuclear fraction, c=cytoplasmic fraction. The experiment was repeated at least three times with similar results.



0 hr
wt AIF
N-AIF
D-AIF
m n c m n c m n c
GFP
mtHsp70
Histone
LDH
12 hr
wt AIF
N-AIF
D-AIF
m n c m n c m n c
GFP
mtHsp70
Histone
LDH

Discussion

The anchored AIF constructs which are retained in the mitochondria during cell death provide us the means to dissociate the dual roles of AIF in cell life and death. The results of this study support a number of conclusions. First, we show that AIF is required for neuronal survival because *Aif* $^{\Delta}$ neurons exhibit fragmented mitochondria and abnormal cristae structure and undergo apoptosis during development. Second, anchored AIF expressed in tel. *Aif*$^{\Delta}$*Apaf1*$^{-/-}$ mice can protect against DNA damage induced cell death over than seen in control tel. *Aif*$^{\Delta}$*Apaf1*$^{-/-}$ neurons. Third, expression of anchored AIF in wild type cells, however, can only provide transient protection, indicating that loss of AIF from the mitochondria is not a major event in apoptosis signaling. The finding that these cells eventually die indicates that the pro-apoptotic function of AIF in the nucleus is necessary to execute cell death. The fact that AIF mutants with nuclear export signals failed to induce cell death in tel. *Aif*$^{\Delta}$*Apaf1*$^{-/-}$ cells supports this conclusion. These studies demonstrate that loss of AIF from the mitochondria is not a key apoptotic stimulus, but rather, that AIF plays an important proapoptotic function following nuclear translocation which is sufficient to induce neuronal cell death.

Previous studies have shown that apart from its apoptotic role, AIF also has an important physiological role in mitochondria. Studies in *Hq* mice, which have only 20% AIF expression, indicate that AIF may act as an ROS scavenger (Klein et al, 2002). Cells with depleted AIF have reduced electron transport chain complex I expression in the mitochondria and as a result oxidative phosphorylation is compromised (Vahsen et al, 2004). The interaction of AIF with complex I, however, has not been found, and its redox partner

remains unknown. As such, the exact role of AIF in mitochondria remains elusive and controversial.

In this report, we have identified a novel role of AIF in maintaining mitochondrial structure. Mitochondria in AIF deficient neurons are fragmented and often clustered around the nucleus, and have abnormally dilated cristae. These defects are not due to a secondary effect from reduced survival because they were cultured in enriched media to ensure survival. The abnormal cristae morphology may explain the reduced survival and the respiratory defect in *Aif*$^{-/-}$ cells (Vahsen et al, 2004), since mitochondrial cristae structure is important in regulating the respiratory processes (Frey & Mannella, 2000; Mannella, 2005). As AIF may be responsible for maintaining mitochondrial cristae, the loss of proper cristae formation due to AIF deficiency may subsequently induce mitochondrial fragmentation and bioenergetic failure. Following injury, expression of mitochondrially anchored AIF may provide enhanced protection by maintaining mitochondrial integrity. This is supported by our EM studies which reveal a tighter intra cristae cross sectional distance (Fig.4) that may account for the delay in cytochrome c release in cells expressing anchored AIF mutants (Fig 7c,d). This interpretation is consistent with previous studies which have demonstrated that cristae hold the greatest proportion of cytochrome C (Bernardi and Azzone, 1981) and cristae remodeling is required for its release (Germain et al., 2005; Scorrano et al., 2002).

The importance of AIF's physiological role is further shown in the telencephalon conditional AIF mutant, which displayed abnormal cortical development and premature death by E17. The absence of AIF during neuronal development results in mitochondrial dysfunction, which in turn may trigger multiple apoptotic pathways that may involve the activation of caspases (Narasimhaiah et al, 2005), as the AIF deficient telencephalon showed a higher number of activated caspase 3 *in vivo* during development (Fig. 1d), and lack of

AIF triggers mitochondrial dysfunction *in vitro* (Fig. 2). The enhanced cell death is mainly seen in maturing neurons but not in progenitor cells, suggesting that there is a difference in the sensitivity to the loss of AIF. Previous studies have shown that progenitors have a lower level of reactive oxygen species (ROS) compared to mature neurons in the cortex (Tsatmali et al, 2005), as well, progenitors have a higher level of telomerase than matured neurons (Mattson & Klapper, 2001), which may provide further protection against cellular stress. It has also been shown that mature neurons may recruit different apoptotic pathways compared to progenitors (D'Sa-Eipper et al, 2001). For example, neurons become more sensitive to excitotoxicity as they mature while progenitors are relatively resistant (Fannjiang et al, 2003).

That AIF deficient mitochondria exhibit hyperpolarization in the presence of a defect in oxidative phosphorylation is somewhat unexpected, however, hyperpolarization has been previously observed in situations where electron transport is defective(Reviewed in Skulachev, 2006; DiLisa and Bernardi, 1998). For example, after inhibition of the electron transport chain by NO (inhibitor of complex IV), cells respond by a defense mechanism that results in the reversal of ATP synthase to increase mitochondrial membrane potential to protect cells from death (Beltrán et al., 2000; Beltrán et al., 2002). Moreover, in a number of different cell types, including neural cells, where ATP production via oxidative phosphorylation is defective, mitochondrial membrane potential is generated by the reversal of ATP synthase using ATP produced by glycolysis (Parchman et al., 2001 PNAS; Rego et al., 2001). Thus it is possible that AIF mutant mitochondria, which do not generate ATP by oxidative phosphorylation, are using a similar mechanism to generate membrane potential. Future studies, however, are required to fully resolve this issue.

Since AIF has an important physiological role in mitochondria, one may argue that the loss of AIF from the mitochondria is the cause of cell death and the pro-apoptotic function of AIF in the nucleus is of minor consequence. To clarify this controversy, we constructed AIF anchored mutants which cannot be released from the mitochondria, to dissociate the apoptotic role and physiological role of AIF. Overexpressing AIF anchored mutants in wildtype and *Apaf1*$^{-/-}$ cells could transiently protect against cell death, however, on longer time courses these cells still died. This indicates that although loss of AIF during the initial phase of cell death can partially contribute to the cell's demise, it is the pro-apoptotic function of the endogenous wildtype AIF in nucleus that ultimately kills the cell. The pro-apoptotic role of AIF in nucleus is further supported by the use of an AIF mutant construct (AIF-NES) that fails to translocate into the nucleus during cell death. Tel. *Aif*$^{\Delta}$ *Apaf1*$^{-/-}$ cells expressing these AIF mutants had the same death rate than the cells with control construct under apoptotic induction, indicating that the nuclear translocation ability of AIF is required for its apoptotic function. Importantly, expression of anchored AIF in tel. *Aif*$^{\Delta}$ *Apaf1*$^{-/-}$ cells exhibits even greater survival after apoptosis induction than tel. *Aif*$^{\Delta}$ *Apaf1*$^{-/-}$ cells. This indicates apoptosis can be halted in two ways: (1) most importantly by preventing its pro-apoptotic action in the nucleus, and, (2) to a lesser extent by maintaining the physiological role of AIF in mitochondria.

In conclusion, this study demonstrates that AIF has a novel function in maintaining the cristae structure of mitochondria in neurons. Neurons with depleted AIF have reduced viability and defective mitochondrial cristae structure. During the initial state of apoptosis, the release of AIF from the mitochondria can partly contribute to cell death, however, it is the pro-apoptotic role of AIF in the nucleus that seals the apoptotic fate of the cell. These

studies clarify the pro-apoptotic function of AIF in signaling cell death in the nucleus, and indicate a novel role of AIF in maintenance of mitochondrial structure.

Chapter IV

Cheung et al. (2007) AIF interacts with Opa1 to maintain mitochondrial structure and function.

This manuscript further explores the mechanism of AIF in controlling mitochondrial structure and function. Here, we identified the mitochondrial fusion protein Opa1 to be the downstream target of AIF to maintain mitochondrial structure and function.

E.C. Cheung designed and conducted the experiments, with the exception of the co-immunoprecipitation experiments which were designed and performed by K. Pilon-Larose with assistance from J.G. MacLaurin. The protein cross-linking and co-immunoprecipitation results are presented in Appendix A. P. Rippstein from the laboratory of Dr. H.M. McBride performed the TEM. Dr. H.M. McBride and Dr. D.S. Park provided feedback. E.C. Cheung wrote the manuscript with the guidance and editorial assistance from Dr. R.S. Slack.

AIF INTERACTS WITH OPA1 TO MAINTAIN MITOCHONDRIAL STRUCTURE AND FUNCTION

E.C. Cheung[1], K. Pilon-Larose[1], J.G. Maclaurin[1], P. Rippstein[2], H.M. McBride[2], D.S. Park[1], and R.S. Slack[1*].
1. University of Ottawa, Dept. of Cellular and Molecular Medicine, Ottawa Health Research Institute, 451 Smyth Road, Ottawa, Canada K1H 8M5
2. University of Ottawa Heart Institute, Dept. of Pathology and Laboratory Medicine, 40 Ruskin Street, Ottawa, ON, Canada K1Y 4W7

***Correspondence:**
Ruth S. Slack, Ph.D.
Ottawa Health Research Institute,
University of Ottawa,
451 Smyth Road,
Ottawa, Ontario,
Canada K1H 8M5
Email: rslack@uottawa.ca

Keywords
AIF, Opa1, mitochondrial structure, mitochondrial cristae, neuron.

Running title
AIF regulates mitochondrial function via Opa1

Number of words: 5168
Numbe of words in abstract: 143

Acknowledgements
We thank Kelly McClellan for comments on the manuscript. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to RSS. The viral vector facility is supported by a grant from Canadian Stroke Network (RSS and DSP). ECC is supported by a CIHR studentship.

Abstract

The mitochondrial protein Apoptosis-inducing factor (AIF) has dual roles in cell survival and cell death. Recently we have suggested that AIF maintains mitochondrial structure in neurons. Here we show that AIF interacts with the mitochondrial fusion protein Opa1 to maintain mitochondrial structure and function for cell survival. Aif^{Δ} neurons have no Opa1 oligomers which is essential for mitochondrial cristae formation. Only Opa1 expression can rescue Aif^{Δ} neurons: manipulating other mitochondrial fission/fusion proteins, or the addition of ROS scavenger, cannot rescue the defects. These underscores the specificity of AIF-Opa1 functional interaction in maintaining mitochondrial integrity. AIF works upstream of Opa1 because mitochondrial AIF cannot rescue Opa1 deficiency. AIF physically interacts with Opa1 and is part of the Opa1 oligomer, indicating that AIF assists Opa1 oligomerization for cristae formation. These results revealed the mechanism of AIF in maintaining mitochondrial function and cell survival via Opa1.

Introduction

Mitochondria mediate multiple functions in a cell such as cellular respiration, metabolism and cell death (Green & Kroemer, 2004; McBride et al, 2006). Some of the mitochondrial intermembrane proteins, such as cytochrome c and AIF, have dual functions in mitochondrial respiration as well as initiating cell death (Li et al, 2000; Modjtahedi et al, 2006; Vahsen et al, 2004). AIF has been shown to have a key role in executing caspase independent apoptosis following release from the mitochondria and translocation to the nucleus (Cheung et al, 2005; Cregan et al, 2002; Polster et al, 2005; Susin et al, 1999b; Wang et al, 2004). Using mitochondrially anchored AIF that cannot be released during cell death, we have definitively shown that AIF is indeed a cell death factor, since the presence of mitochondrial AIF cannot negate the pro-apoptotic effect of AIF in the nucleus (Cheung et al, 2006). Once AIF is in the nucleus, it induces chromatin condensation and large-scale DNA fragmentation, which ultimately lead to caspase independent cell death (Cregan et al, 2004).

AIF also has an important physiological role in the mitochondria to maintain cell survival. *Hq* mice, which have only 20% AIF expression, have degenerated cerebella and retina. The brain and heart of these mice have increased ROS levels and are more vulnerable to H_2O_2 damage (Klein et al, 2002; van Empel et al, 2005; van Empel et al, 2006). AIF germline knockout mice are embryonic lethal at E8-9, and the muscle specific AIF mutant shows extensive muscle atropy (Brown et al, 2006; Joza et al, 2005). In the neuronal system, we have previously shown that AIF is also required for the proper development of the CNS (Cheung et al, 2006). AIF deficient cells are impaired in mitochondrial respiration, and have reduced expression of mitochondrial electron transport chain complex 1 and to a lesser extent complex III (Cheung et al, 2006; Joza et al, 2005; Pospisilik et al, 2007; Vahsen et al,

2004). Hence, it has been inferred that AIF may either work as a ROS scavenger, or somehow control the stability of the complexes in the electron transport chain in the mitochondria (Bonni, 2003; Klein & Ackerman, 2003; Lipton & Bossy-Wetzel, 2002; Porter & Urbano, 2006; Urbano et al, 2005). A recent study, however, has questioned the role of AIF as ROS scavenger since it is not able to transfer electrons from NADH to superoxide anion, hydrogen peroxide, and other free radicals (Churbanova & Sevrioukova, 2007). The mechanism by which AIF affects mitochondrial function therefore remains unknown. Recently, using AIF deficient neurons we have identified a novel role of AIF in controlling mitochondrial structure. The mitochondria of AIF deficient neurons are fragmented, and the mitochondrial cristae are dilated and disorganized. This was observed even when the cells were cultured in enriched media to support survival and energy metabolism. Conversly, cells expressing elevated levels of AIF exhibit elongated mitochondria and tighter cristae (Cheung et al, 2006). Taken together these results suggested that AIF may also have a role in maintaining mitochondrial structure, which is essential for mitochondrial function and cell survival.

The abnormal mitochondrial morphology that we have previously observed in AIF deficient cells leads us to ask whether AIF may have a role in regulating the mitochondrial fission and fusion proteins which controls mitochondrial shape. In the mammalian system, mitochondrial fission and fusion is controlled by the fusion proteins Mfn1/2 and Opa1, and the fission proteins Drp1 and hFis1 (Chan, 2006; Meeusen & Nunnari, 2005; Okamoto & Shaw, 2005). The importance of these proteins in neuronal survival is highlighted by the fact that mutations in Opa1 and Mfn2 cause autosomal dominant optical atrophy and Charcot-Marie-Tooth type 2A neuropathy, respectively (Alexander et al, 2000a; Chan, 2006; Delettre et al, 2000a; Detmer et al, 2007; Kijima et al, 2005; Meeusen et al, 2006). Opa1 is a

dynamin related GTPase located in the intermembrane space of the mitochondria, where it forms high molecular weight oligomers for proper mitochondrial cristae formation. The loss of Opa1 oligomers during cell death results in cristae remodeling and complete release of cyt c (Frezza et al, 2006; Gottlieb, 2006; Olichon et al, 2002a). The formation of oligomers depends on the correct processing of Opa1 into the long and short form by mitochondrial proteases such as PARL, Yme1L, and paraplegin (Cipolat et al, 2006; Griparic et al, 2007; Herlan et al, 2003; Ishihara et al, 2006; Jeyaraju et al, 2006; Song et al, 2007). Interestingly, Opa1 deficient cells have striking similarities to AIF deficient cells. Cells lacking Opa1 have fragmented mitochondria, dilated mitochondrial cristae, reduced activity of complex 1 and IV in the electron transport chain, oxidative damage, and defective mitochondrial respiration which leads to cell death (Chen et al, 2005; Griparic et al, 2004; Herlan et al, 2003). Mfn1/2 are located on the outer mitochondrial membrane which form hetero/homodimer for mitochondrial fusion (Chen et al, 2003; Koshiba et al, 2004; Rojo et al, 2002; Santel & Fuller, 2001). Recently, a cerebellar specific Mfn2 mutant was shown to result in progressive degeneration of the Purkinje cells, indicating an essential role of mitochondrial fusion in maintaining neuronal survival (Chen et al, 2007). Apart from the physiological role, the fission/fusion machinery has also been implicated in cell death control (for review see (Cheung et al, 2007; Youle & Karbowski, 2005)). For example, by inhibiting fission using dominant negative Drp1 (dnDrp1), cytochrome C release and cell death can be blocked (Frank et al, 2001). By enhancing the fusion machinery via Mfn1/2, neurons are more resistant to cell death by camptothecin and ROS (Bossy-Wetzel et al, 2003; Jahani-Asl et al, 2007). These studies indicate that the mitochondrial fission/fusion machinery are important for proper mitochondrial function and cellular survival, and in the regulation of cell death after various cytotoxic insults.

As our previous studies suggest that AIF may have a role in controlling mitochondrial morphology (Cheung et al, 2006), we asked whether AIF may interact with the fission/fusion machinery to maintain proper mitochondrial architecture and cell survival. Since other studies suggested that AIF acts as a ROS scavenger, we first asked if the addition of ROS scavenger NAC (N-acetylcysteine) can rescue *Aif* $^{\Delta}$ neurons. Our result indicate that NAC alone cannot rescue the mitochondrial defects in *Aif* $^{\Delta}$ neurons, suggesting that AIF function goes beyond the role of a ROS scavenger. We next explored the possibility that AIF may modulate the function of the mitochondrial fission/fusion machinery and we showed that only Opa1 expression, but not Mfn2 or dominant negative Drp1 (dnDrp1), can rescue the defects in *Aif* $^{\Delta}$ neurons. In contrast, the anchored form of AIF cannot rescue the defects in shOpa1 neurons, indicating that AIF works upstream of Opa1 to control mitochondrial shape. *Aif* $^{\Delta}$ neurons have no Opa1 oligomers and importantly, AIF can interact physically with Opa1 and is part of the Opa1 oligomer complex. Our results demonstrate that under physiological conditions, AIF interacts with Opa1 in the mitochondria to facilitate the formation of Opa1 oligomers to maintain cristae structure, which are essential to maintain the mitochondrial metabolism and structure for cell survival. AIF therefore has an essential role to connect mitochondrial metabolism to regulation of mitochondrial structure.

Materials and methods

Mice and cortical neuronal cultures

Telencephalon specific AIF conditional mutants (*$Aif^{flox/flox}$;$cre^{+/-}$*) are generated from floxed AIF mice and Foxg1-cre mice as described previously (Cheung et al, 2006). *$Aif^{flox/x}$;$cre^{+/-}$* were generated as control. Cortical neurons from these AIF conditional mutants (*Aif^{Δ}* neurons) and control littermates (control neurons) were cultured as described previously (Fortin et al, 2001, Cheung et al, 2005). Enriched meida was prepared as described previously (Cheung et al, 2006; Li et al, 2000). Recombinant adenoviral vectors containing N-AIF, GFP, Mfn2, Opa1, and dnDrp1 were constructed and used as described previously (Cheung et al, 2006; Cregan et al, 2000; Jahani-Asl et al, 2007) at 100 MOI (multiplicity of infection).

ATP production and O_2 consumption assays

ATP levels were measured using a luciferase-based CellTitre-Glo assay kit (Promega) as described previously (Barsoum et al, 2006). O_2 consumption levels were measured using fluorescent dye embedded 96 well microplate BD oxygen biosensor system (BD Biosciences) as described previously (Cheung et al, 2006).

Cell viability and mitochondrial superoxide measurement

Cell death was measured by the characteristic nuclear morphology of chromatin condensation revealed by Hoechst staining as described previously (Cregan et al. 1999). Mitochondrial superoxide ($O_2^{\cdot-}$) was measured by MitoSOX^{TM} Red, a mitochondrial

superoxide fluorogenic indicator (Molecular Probles, M36008) for highly selective detection of mitochondrial superoxide of live cells. Five μM of MitoSOX™ Red in culture media was applied to neurons growing on coverslips. After 10 mins incubation in 37 °C, cells were washed three times in warm media, and the coverslips were mounted for fluorescence microscopy (510/580nm) and the fluorescence intensity was measued per cell.

Mitochondrial isolation, co-immunoprecipitation, protein cross-linking and western blot analysis

Mitochondrial isolation was performed as described previously (Cheung et al, 2006; Yu et al, 2002). Protein crosslinking was performed as described previously (Frezza et al, 2006; Meeusen et al, 2006).

Transmission electron microscopy (TEM)

TEM was performed as described before (Neuspiel et al, 2005). Briefly, after two days of culture, neurons were isolated, washed with PBS, fixed in 1.6% glutaraldehyde and embedded in SPURR resin (Mariva, Quebec). Thin section were cut with a Leica Ultracut E ultramicrotome and counterstained with lead citrate and uranyl acetate. Digital images were taken using a JEOL 1230 TEM at 60kV adapted with a 2K x 2K bottom mount CCD digital camera (Hamamatsu, Japan) and AMT software.

Quantifications and statistical analysis

For cell death analysis, a minimum of 500 cells for each independent experiment was scored at the indicated time points. For mitochondrial analysis, a minimum 1000 cells for

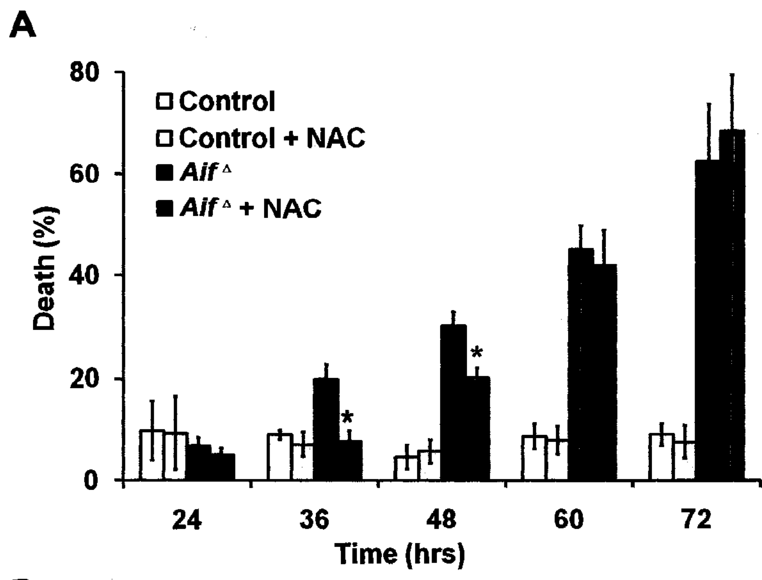
each independent experiment was classified with the indicated mitochondrial shape (normal, fragmented, clump, and intermediate, see Figure 2a-d) and expressed as percentage of the total number of cells. Only cells infected with the adenovirus containing the constructs were counted in the cell death analysis and the mitochondrial measurement. For the Mfn2, dnDrp1, and Opa1 experiments (Figure 3-6), the experiments were performed at the same time. We seperated Opa1 data from Mfn2 and dnDrp1 data, and used the same control (control cells with GFP, and Aif^{Δ} with GFP) for clarity. The data represent mean values +/- s.d. from three independent experiments (n=3) unless otherwise noted. P-values were obtained using two-way ANOVA and Fisher's post hoc tests.

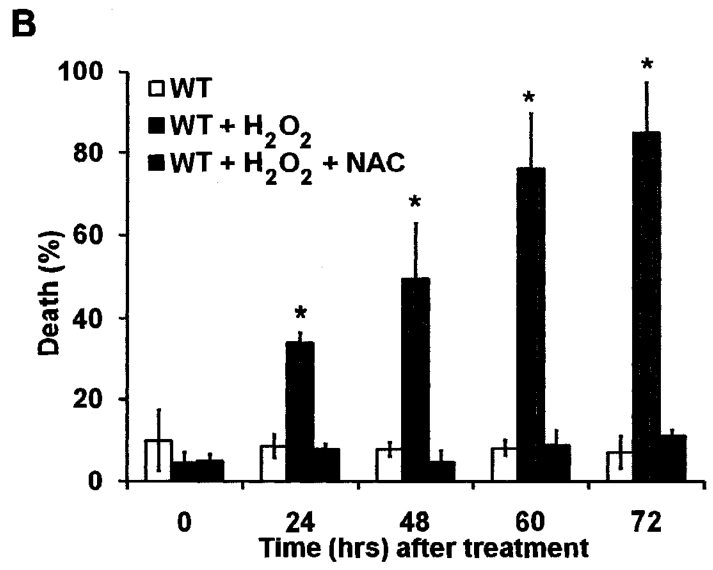
Results

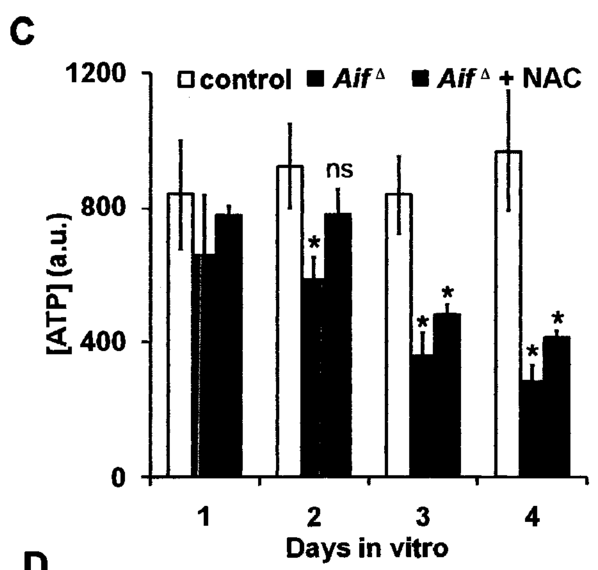
AIF has functions beyond scavenging ROS

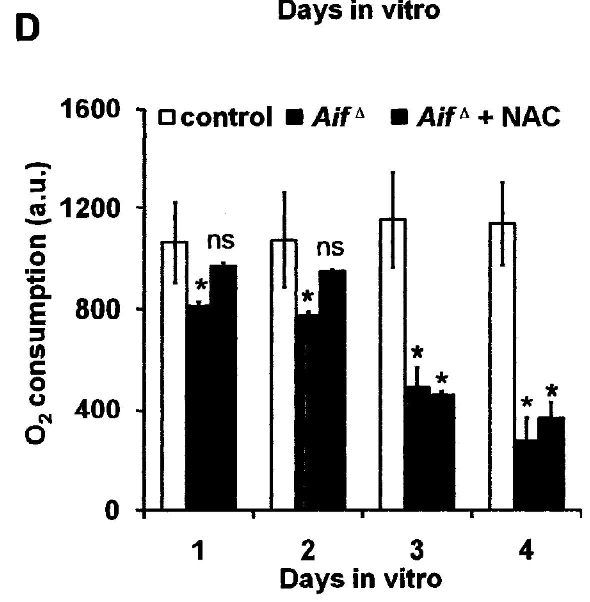
We have previously shown that *Aif* $^{\Delta}$ neurons have fragmented mitochondria and dilated mitochondrial cristae, implicating a vital role of AIF in maintaining mitochondrial structure (Cheung et al, 2006). Other studies have suggested that AIF acts as a ROS scavenger (Klein et al, 2002; Urbano et al, 2005; van Empel et al, 2005; van Empel et al, 2006), since the loss of AIF in neurons and in heart muscles induces sensitivity to ROS. If AIF indeed acts as a ROS scavenger, application of ROS scavenger such as NAC (N-acetyl cysteine) should be able to maintain survival of *Aif* $^{\Delta}$ neurons. NAC has been used extensively to protect neurons against oxidative damage (Colton et al, 1995; Ferrari et al, 1995; Henderson et al, 1996). We first asked if ROS scavenger can rescue the cell death in *Aif* $^{\Delta}$ neurons. In the presence of 1mM NAC, the survival of *Aif* $^{\Delta}$ neurons was only slightly rescued at early time points (36 and 48 hours) compared to *Aif* $^{\Delta}$ neurons without NAC. After 48 hours the *Aif* $^{\Delta}$ neuronal culture with NAC had a similar percentage of cell death compared to the cultures without NAC (Figure 1a). In order to show that NAC did not lose its activity after 24 hours, the same protocol was used to rescue neurons challenged with 10mM H_2O_2 to induce oxidative stress. At all time points 1mM of NAC is able to maintain survival of cells challenged with H_2O_2, indicating that the ROS scavenging activity of NAC was indeed functional at later time points (Figure 1b). Since *Aif* $^{\Delta}$ cells have defective mitochondrial respiration (Cheung et al, 2006; Joza et al, 2005; Vahsen et al, 2004), it is possible that AIF may act as a ROS scavenger to clean up the ROS produced by complex 1 and III in the electron transport chain when some electrons are leaked and oxidize O_2 during

Figure 4-1: ROS scavenger NAC cannot rescue survival and metabolic defects of *Aif*$^{\Delta}$ neurons. **(A)** Cortical neurons from E15.5 control and *Aif*$^{\Delta}$ littermates were cultured with or without 1mM N-acetylcholine (NAC), a ROS scavenger. Cell death is quantified by nuclear morphology revealed by Hoechst staining (n=3). **(B)** Wildtype cortical neurons were treated with or without H_2O_2 in the absence or presence of 1mM NAC. Cell death is quantified by nuclear morphology revealed by Hoechst (n=3). **(C)** ATP production of the control and *Aif*$^{\Delta}$ neurons with or without NAC (n=5). **(D)** O_2 consumption of the control and *Aif*$^{\Delta}$ neurons with or without NAC (n=5). * $p<0.05$ compared to control, n.s. : not significant compared to control.


A
Control
Control + NAC
Aif^Δ
Aif^Δ + NAC
Death (%)
Time (hrs)


B
WT
WT + H2O2
WT + H2O2 + NAC
Death (%)
Time (hrs) after treatment

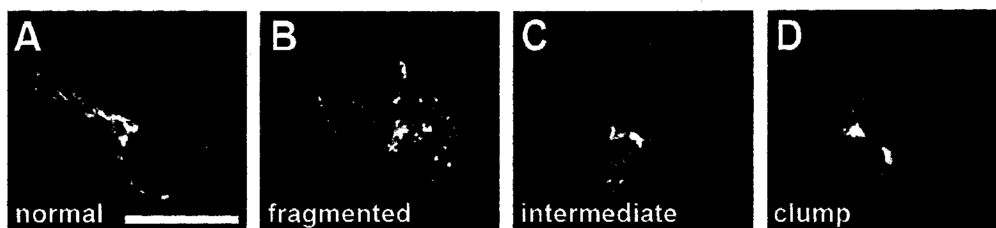

C
control
Aif^Δ
Aif^Δ + NAC
[ATP] (a.u.)
Days in vitro
ns

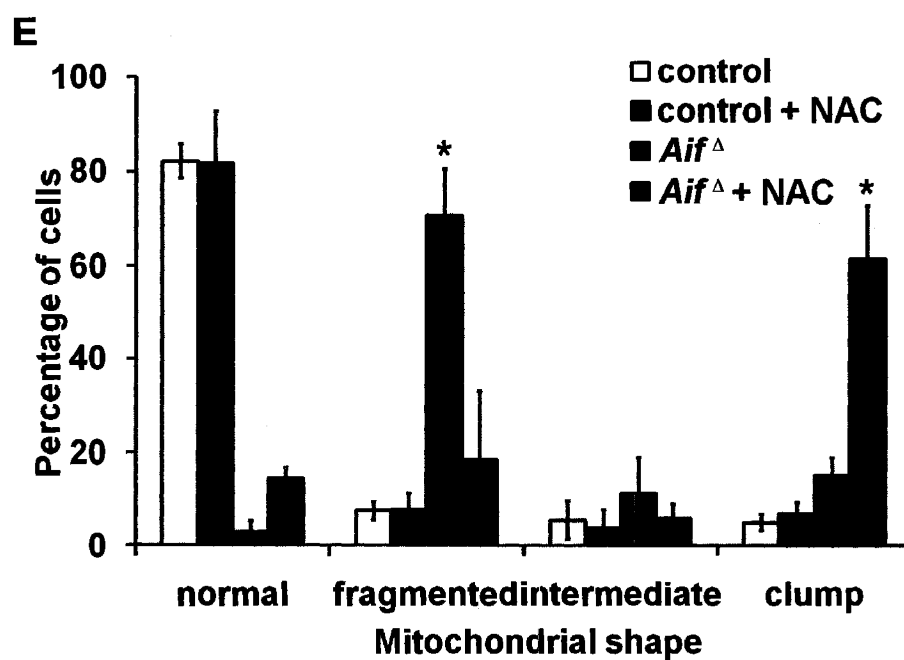

D
control
Aif^Δ
Aif^Δ + NAC
O2 consumption (a.u.)
Days in vitro
ns

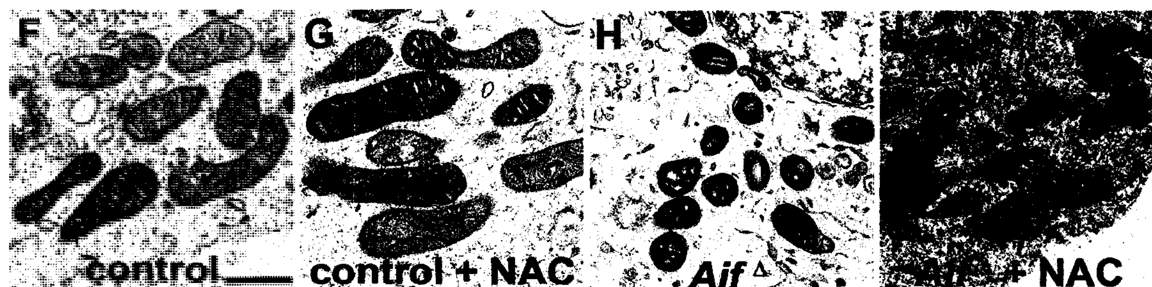
normal respiration. This will ensure that the ROS produced by complex I and III during physiological situations will not attack the proteins in the electron transport chain and undermine cellular respiration. Hence, we asked whether inhibiting ROS can rescue the mitochondrial respiration defects in the *Aif* $^{\Delta}$ neurons. We examined the mitochondrial function as indicated by ATP production and O_2 consumption level. Similar to the survival experiment above, the presence of the ROS scavenger NAC can only rescue the ATP production and O_2 consumption of the *Aif* $^{\Delta}$ neurons to the control level after 1 and 2 days in vitro. At longer time points, both ATP production and O_2 consumption are decreased to the same level as *Aif* $^{\Delta}$ neurons without NAC (Figure 1c and d). This indicates that ROS scavenger cannot rescue the defects of the mitochondrial function of the *Aif* $^{\Delta}$ neurons. All of the above experiments show that the ROS scavenger NAC cannot rescue any of the defects in the *Aif* $^{\Delta}$ neurons; hence AIF must have functions beyond scavenging ROS to maintain mitochondrial function and cell survival. This is in agreement with a recent report indicating that AIF does not have ROS scavenging activity in cell free systems (Churbanova & Sevrioukova, 2007).

As there was a slight rescue at early timepoints with NAC, we then asked if NAC could rescue mitochondrial morphology in *Aif* $^{\Delta}$ neurons. These results indicated that ROS scavenger cannot rescue the survival of *Aif* $^{\Delta}$ neurons. AIF therefore does not simply act as a ROS scavenger to maintain neuronal survival. Consistent to what we have shown previously (Cheung et al, 2006), *Aif* $^{\Delta}$ neurons, even in the presence of an alternative energy source, exhibit fragmented mitochondria compared to the control neurons (Figure 2a-e). In the presence of NAC, the mitochondria of these *Aif* $^{\Delta}$ neurons are not rescued to the wildtype morphology. Most of NAC treated *Aif* $^{\Delta}$ neurons have clump-like mitochondria or

Figure 4-2: ROS scavenger cannot rescue structural defects in *Aif* $^{\Delta}$ mitochondria. Cortical neurons from E15.5 control and *Aif* $^{\Delta}$ littermates were cultured with or without 1mM NAC. **(A-D)** Representative images of different types of mitochondria revealed by Tom20 immunostaining. Bar, 10μm. **(E)** Quantitative analysis of neurons with the indicated mitochondrial shape in control and *Aif* $^{\Delta}$ neurons treated with or without NAC (n=5). * $p<0.05$ compared to *Aif* $^{\Delta}$.**(F-I)** Representative transmission electron microscopy (TEM) images of mitochondria of control and $AIF^{-/-}$ neurons treated with or without NAC. Bar, 500nm.


A
normal
B
fragmented
C
intermediate
D
clump


E
100
80
60
40
20
0
Percentage of cells
control
control + NAC
Aif Δ
Aif Δ + NAC
*
*
normal
fragmented
intermediate
clump
Mitochondrial shape

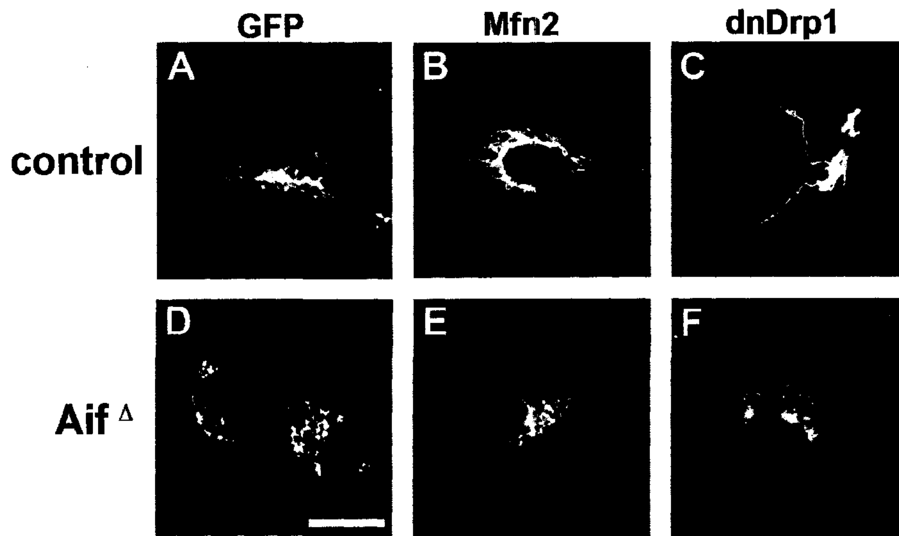

F
control
G
control + NAC
H
Aif Δ
+ NAC

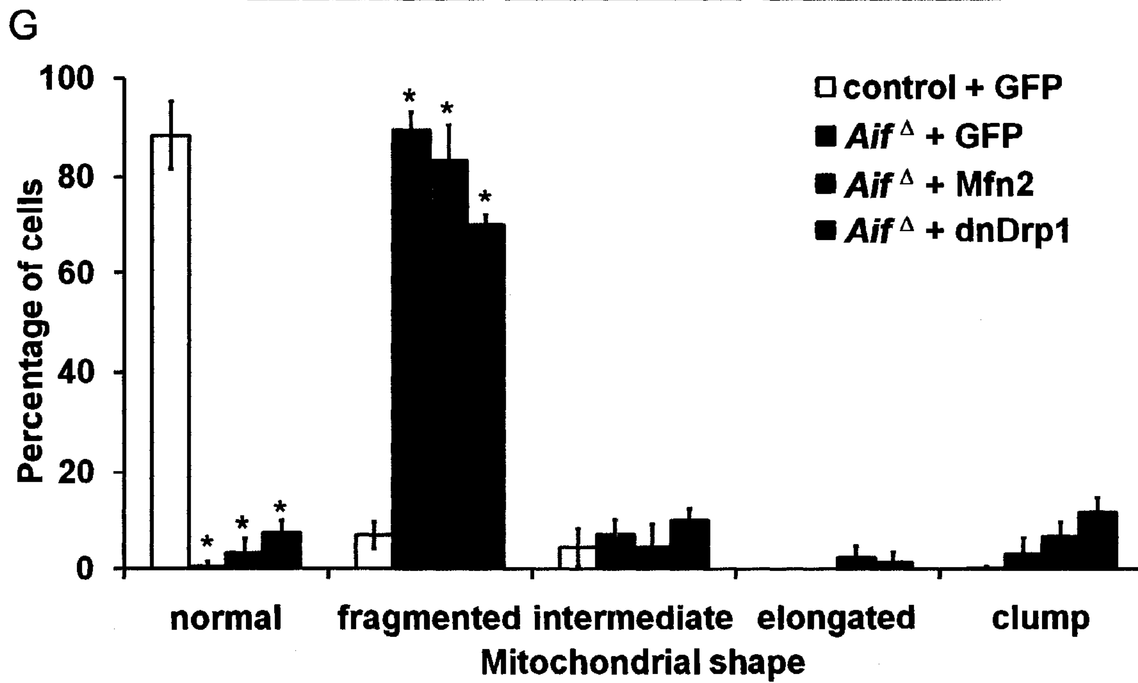
fragmented mitochondria (~80%), and only a minor fraction (~20%) exhibited normal morphology. This is in stark contrast to wildtype neurons in which ~80% of the cells have normal mitochondria (Figure 2a-d,e). Next we looked at the ultra-structure of mitochondria by TEM. Similar to our previous study (Cheung et al, 2006), the mitochondria in the *Aif* $^{\Delta}$ neurons have dilated and unorganized cristae (Figure 2h), compared to the control mitochondrial cristae which are more organized and compact (Figure 2f). Consistent with the mitochondrial morphology revealed by TMRE, the TEM study show that the mitochondria of NAC treated *Aif* $^{\Delta}$ neurons are usually in close proximity to each other (Figure 2i), and are generally larger than those without NAC (Figure 2h), which correlates to the clump-like structure revealed by TMRE (Figure 2f-2i). Most importantly, the presence of NAC in the *Aif* $^{\Delta}$ neurons was unable to rescue the defective cristae structure back to control levels (Figure 2f-2i), indicating that the cristae defect in *Aif* $^{\Delta}$ neurons is not simply due to an increased level of ROS. The gross and ultrastructural studies of mitochondrial morphology show that the ROS scavenger NAC cannot rescue the mitochondrial defects in *Aif* $^{\Delta}$ neurons, and thus indicate that the defects seen in *Aif* $^{\Delta}$ neurons are not simply due to the increased level of ROS in the *Aif* $^{\Delta}$ neurons.

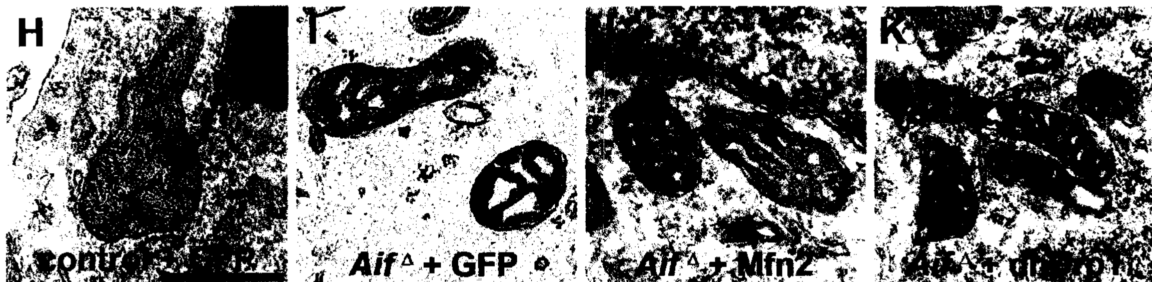
The role of AIF in regulating mitochondrial structure

Previously we have shown that *Aif* $^{\Delta}$ neurons have fragmented mitochondria with dilated and unorganized cristae, therefore, AIF may have a role in maintaining mitochondrial structure ((Cheung et al, 2006) and also Figure 2). To ask if AIF modulates mitochondrial fission and fusion, we manipulated the mitochondrial fission/fusion machinery in the *Aif* $^{\Delta}$ neurons. We increased mitochondrial fusion by increasing the expression of mitochondrial

Figure 4-3: Mfn2 or dnDrp1 cannot rescue the mitochondria structural defects in *Aif*$^{\Delta}$ neurons. (A-F) Representative images of mitochondria revealed by Tom20 immunostaining from control and *Aif*$^{\Delta}$ neurons expressing Mfn2, dnDrp1 and GFP control (n=3). Bar, 10μm. **(G)** Quantitative analysis of control and *Aif*$^{\Delta}$ neurons expressing Mfn2, dnDrp1, and GFP control with the indicated mitochondrial shape (n=3). * $p<0.05$ compared to control + GFP. **(H-K)** Representative TEM images of control neurons expressing GFP control, and *Aif*$^{\Delta}$ neurons expressing Mfn2, dnDrp1, and GFP control (n=3). Bar, 500nm.

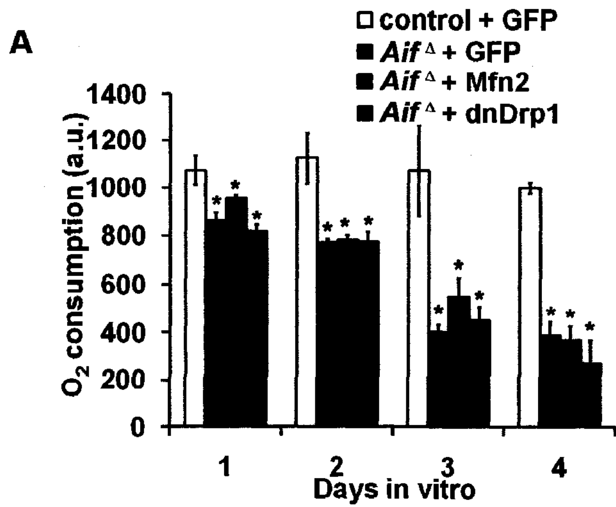

GFP
Mfn2
dnDrp1
A
B
C
control
D
E
F
Aif Δ

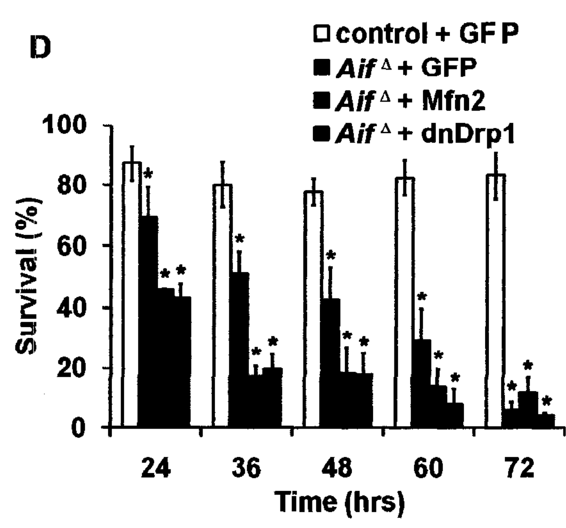

G
100
80
60
40
20
0
Percentage of cells
control + GFP
Aif Δ + GFP
Aif Δ + Mfn2
Aif Δ + dnDrp1
normal
fragmented
intermediate
elongated
clump
Mitochondrial shape

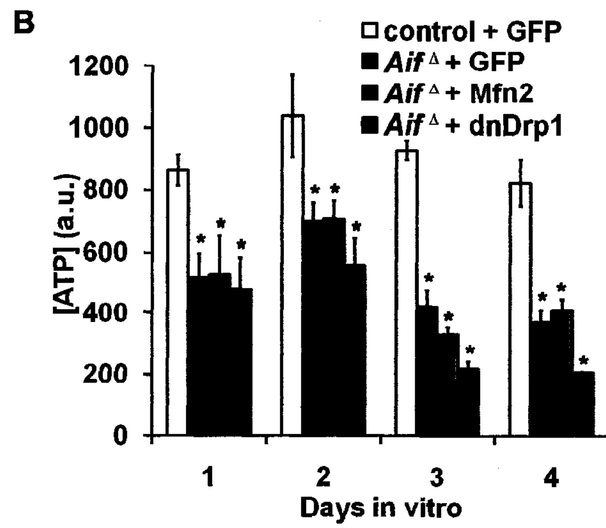

H
Aif Δ + GFP

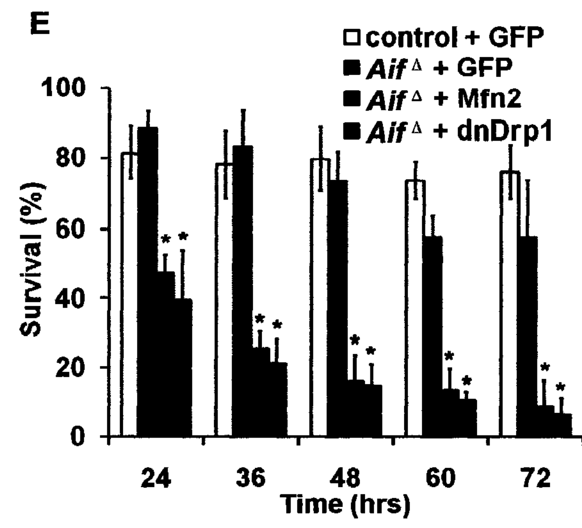
fusion protein Mfn2, and we inhibited mitochondrial fission by expressing the dominant negative form of the fission protein Drp1 (dnDrp1) (Frank et al, 2001). In control cortical neurons, the expression of Mfn2 and dnDrp1 can increase mitochondrial length compared to the GFP control (Figure 3a-c), confirming that these proteins function as expected in primary cortical neurons. We first looked at whether increasing fusion or inhibiting fission can rescue the fragmented mitochondria in *Aif* $^{\Delta}$ neurons. *Aif* $^{\Delta}$ neurons with either Mfn2 or dnDrp1 have similar level of mitochondrial fragmentation compared to *Aif* $^{\Delta}$ neurons with GFP control, indicating that increasing mitochondrial fusion or inhibiting fission cannot rescue mitochondrial fragmentation in *Aif* $^{\Delta}$ neurons (Figure 3d-f,g). Ultrastructure of the mitochondria revealed by TEM shows that expression of dnDrp1 or Mfn2 cannot rescue the dilated and disorganized mitochondrial crisate in *Aif* $^{\Delta}$ neurons (Figure 3h-k), indicating that promoting mitochondrial fusion or inhibiting fission is not able to rescue the mitochondrial cristae defect of *Aif* $^{\Delta}$. All of these studies indicate that manipulating the mitochondrial fission and fusion machinery is not able to rescue the mitochondrial structural defects in *Aif* $^{\Delta}$ neurons. Next we asked whether Mfn2 or dnDrp1 can rescue the metabolic defects in the *Aif* $^{\Delta}$ neurons. ATP production or O_2 consumption of *Aif* $^{\Delta}$ neurons cannot be rescued by Mfn2 and dnDrp1 compared to the control neurons with GFP (Figure 4a-b). We examined the mitochondrial superoxide ($O_2^{-\cdot}$) level and asked whether the expression of Mfn2 or dnDrp1 is able to inhibit the high level of mitochondrial $O_2^{-\cdot}$ in the *Aif* $^{\Delta}$ neurons. *Aif* $^{\Delta}$ neurons have ~5 times higher mitochondrial $O_2^{-\cdot}$ level than control cells (Figure 4c, ~5 a.u. control vs ~25 a.u. *Aif* $^{\Delta}$). The expression of either Mfn2 or dnDrp1 cannot reduce the $O_2^{-\cdot}$ level of *Aif* $^{\Delta}$ back to the level in control neurons with GFP (~5 a.u.). The levels are, however, somewhat reduced compared to *Aif* $^{\Delta}$ with GFP control (Figure 4c, ~25 a.u. in GFP

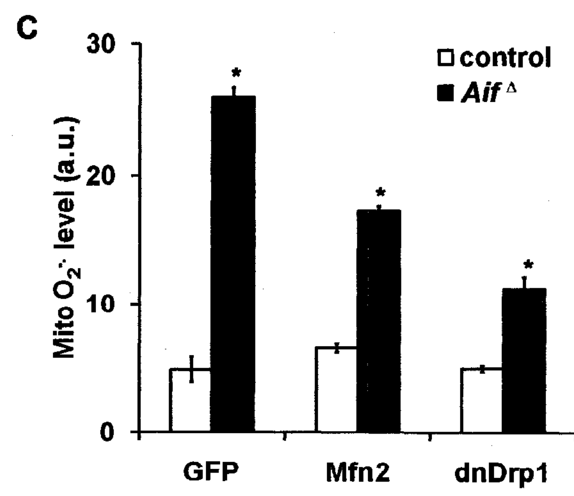
Figure 4-4: Mfn2 or dominant negative Drp1 (dnDrp1) cannot rescue metablic, survival and ROS defects of *Aif*$^{\Delta}$ neurons. **(A)** O_2 consumption of the control neurons expressing GFP, and *Aif*$^{\Delta}$ neurons expressing Mfn2, dnDrp1, and GFP control (n=5). **(B)** ATP production of the control neurons expressing GFP, and *Aif*$^{\Delta}$ neurons expressing Mfn2, dnDrp1, and GFP control (n=5). **(C)** Mitochondrial superoxide ($O_2^{\cdot-}$) level of the control and *Aif*$^{\Delta}$ neurons expressing Mfn2, dnDrp1, and GFP control after 3 DIV (n=3). **(D)** Quantitative analysis of cell death of control and *Aif*$^{\Delta}$ neurons in normal media expressing Mfn2, dnDrp1, and GFP as control (n=3). **(E)** Quantitative analysis of cell death of control and *Aif*$^{\Delta}$ neurons in enriched media expressing Mfn2, dnDrp1, and GFP as control (n=3). * $p<0.05$ compared to control + GFP.


A
control + GFP
Aif$^{\Delta}$ + GFP
Aif$^{\Delta}$ + Mfn2
Aif$^{\Delta}$ + dnDrp1
O_2 consumption (a.u.)
Days in vitro


D
control + GFP
Aif$^{\Delta}$ + GFP
Aif$^{\Delta}$ + Mfn2
Aif$^{\Delta}$ + dnDrp1
Survival (%)
Time (hrs)


B
control + GFP
Aif$^{\Delta}$ + GFP
Aif$^{\Delta}$ + Mfn2
Aif$^{\Delta}$ + dnDrp1
[ATP] (a.u.)
Days in vitro


E
control + GFP
Aif$^{\Delta}$ + GFP
Aif$^{\Delta}$ + Mfn2
Aif$^{\Delta}$ + dnDrp1
Survival (%)
Time (hrs)

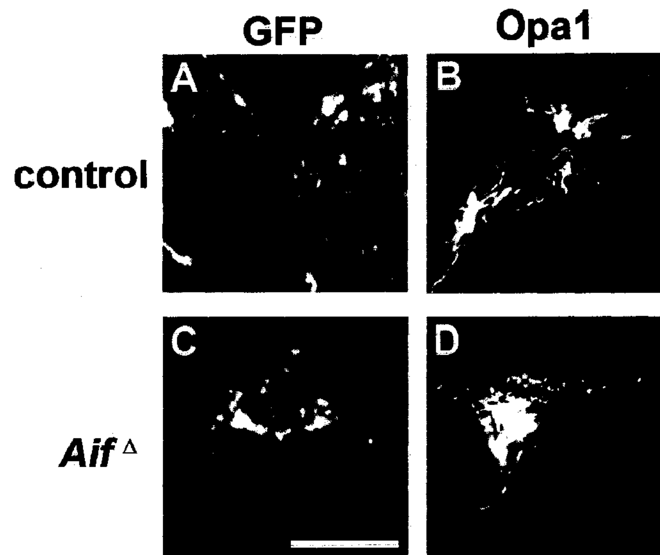

C
control
Aif$^{\Delta}$
Mito $O_2^{\cdot-}$ level (a.u.)
GFP
Mfn2
dnDrp1

vs. ~17 a.u. in Mfn2 and ~12 a.u. in dnDrp1). This could be due to the fusion independent role of Mfn2 in controlling metabolism (Bach et al, 2003; Pich et al, 2005), and inhibiting fisssion may have a similar effect. Finally, we asked if inhibition of fission or enhancement of fusion can increase survival of Aif^{Δ} neurons. The presence of either Mfn2 or dnDrp1 was not able to increase survival of Aif^{Δ} neurons to the control level, indicating that increasing mitochondrial fusion or inhibiting fission is not able to rescue the survival of Aif^{Δ} neurons (Figure 4d). Interestingly, even in the presence of an enriched media, which can rescue Aif^{Δ} neurons by itself via promoting althernative energy production (Cheung et al, 2006; Li et al, 2000), in these neurons the presence of Mfn2 and dnDrp1 actually induces cell death in Aif^{Δ} neurons compared to Aif^{Δ} neurons with GFP control (Figure 4e). This further indicates that the defects of Aif^{Δ} neurons are not simply due to a lack of mitochondrial fusion or abnormal increase of fission, since surviving Aif^{Δ} neurons in the presence of enriched media were actually killed by increasing mitochondria fusion or by inhibiting fission. Taken together, these results indicate that the defects in Aif^{Δ} neurons are not due to disregulation of fission and fusion as the primary cause. AIF therefore does not have a direct role in modulating mitochondrial fusion and fission via Mfn2 or Drp1 to maintain mitochondrial structure and cell survival.

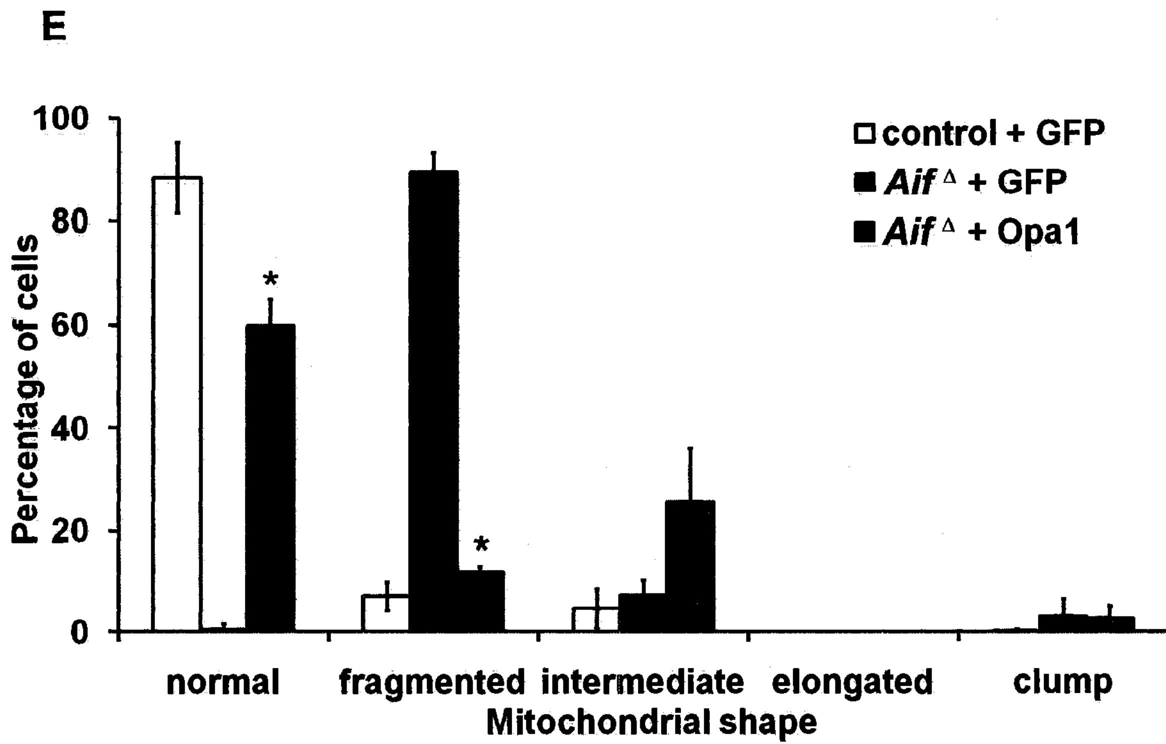
Opa1 functions downstream of AIF to maintain mitochondrial structure and function

We next turned our attention to another mitochondrial protein involved in maintaining proper structure: Opa1. Opa1 is a mitochondrial fusion protein responsible for maintaining inner membrane structure and also for mitochondrial fusion by interacting with Mfn1 (Cipolat et al, 2004; Meeusen et al, 2006). Opa1 shares the same location as AIF in the

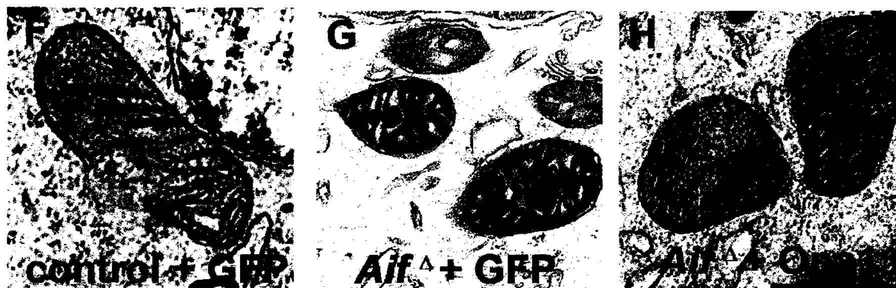
Figure 4-5: Opa1 can rescue the mitochondrial structural defects in *Aif*$^{\Delta}$ neurons. (A-D) Representative images of mitochondria revealed by Tom20 immunostaining from control and *Aif*$^{\Delta}$ neurons expressing Opa1 and GFP control (n=3). Bar, 10 μm. **(E)** Quantitative analysis of control and *Aif*$^{\Delta}$ neurons expressing Opa1 and GFP control with the indicated mitochondrial shape (n=3). * $p<0.05$ compared to *Aif*$^{\Delta}$ + GFP. **(F-H)** Representative TEM images of control neurons expressing GFP control, and *Aif*$^{\Delta}$ neurons expressing Opa1 and GFP control (n=3). Bar, 500 nm.



GFP
Opa1
control
Aif Δ
A
B
C
D



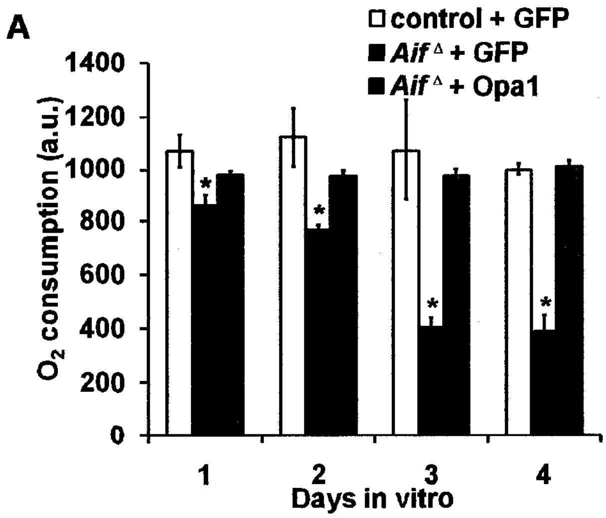
E
Percentage of cells
100
80
60
40
20
0
control + GFP
Aif Δ + GFP
Aif Δ + Opa1
*
*
normal
fragmented
intermediate
elongated
clump
Mitochondrial shape

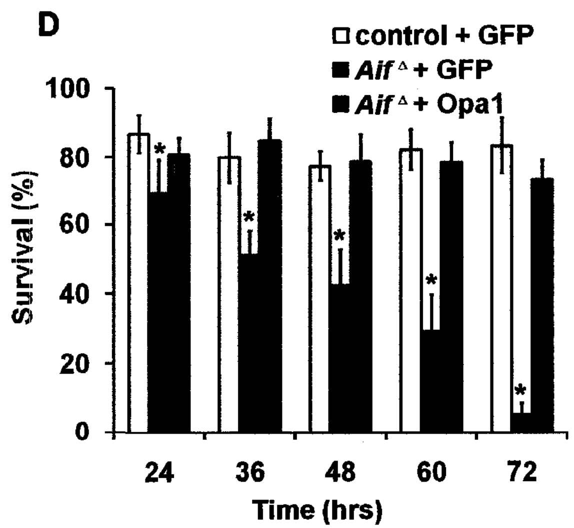


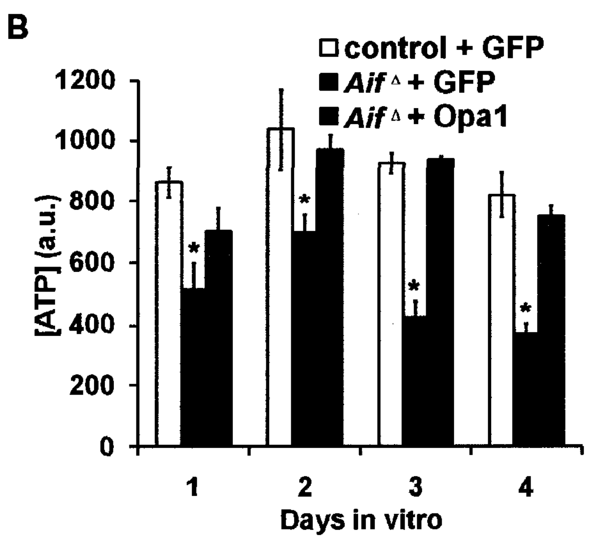
G
H
control + GFP
Aif Δ + GFP

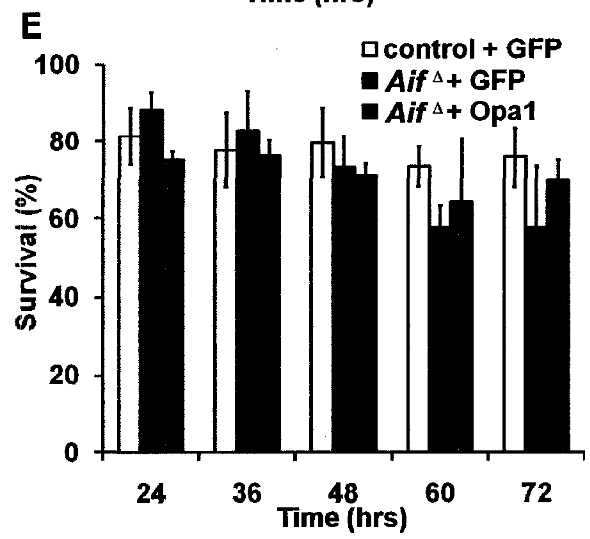
intermembrane space of the mitochondria associated with the inner membrane (Ishihara et al, 2006; Olichon et al, 2002a; Otera et al, 2005). Similar to AIF deficiency, loss of Opa1 also induces mitochondrial fragmentation and cristae dilation which culminates in a defect in respiration and cell death (Chen et al, 2005; Griparic et al, 2004; Herlan et al, 2003). These similarities between Opa1 and AIF suggest that they may work in the same pathway in regulating mitochondrial function. First we asked whether the expression of Opa1 can rescue the mitochondrial architecture defects in *Aif* $^{\Delta}$ neurons. Opa1 expression in control neurons can enhance mitochondrial fusion, confirming Opa1 works as a mitochondrial fusion protein in neurons (Figure 5a-b). Unlike Mfn2 and dnDrp1, expression of Opa1 is able to rescue the gross mitochondrial structural defect of *Aif* $^{\Delta}$ neurons (Figure 5c-d compared to Figure 3e-f). Quantitative analysis showed that Opa1 treated *Aif* $^{\Delta}$ neurons have signficantly fewer fragmented mitochondria compared to the *Aif* $^{\Delta}$ neurons with GFP. In fact, the level of the fragmented mitochondria is rescued to the control neurons with GFP (Figure 5e). Similarly, there are significantly more neurons with normal mitochondria in Opa1 expressing *Aif* $^{\Delta}$ neurons compared to those with GFP as control, indicating that Opa1 is able to rescue mitochondrial fragmentation in *Aif* $^{\Delta}$ neurons (Figure 5e). We next looked at the mitochondrial structure more closely by TEM. As we have seen previously ((Cheung et al, 2006) and figure 2f-i), the mitochondrial cristae of *Aif* $^{\Delta}$ neurons are more disorganzied and dilated compared to those from control neurons (Figure 5f,g). Again, in contrast to Mfn2 and dnDrp1 (Figure 3j,k), compared to the *Aif* $^{\Delta}$ with control GFP (Figure 5g), the mitocondrial cristae in Opa1 expressing *Aif* $^{\Delta}$ neurons are tighter and more organized (Figure 5h), similar to those in the wildtype control mitochondria (Figure 5f). All of these results indicate that Opa1 is also able to rescue the mitochondrial structural defect in the *Aif* $^{\Delta}$ neurons. We next

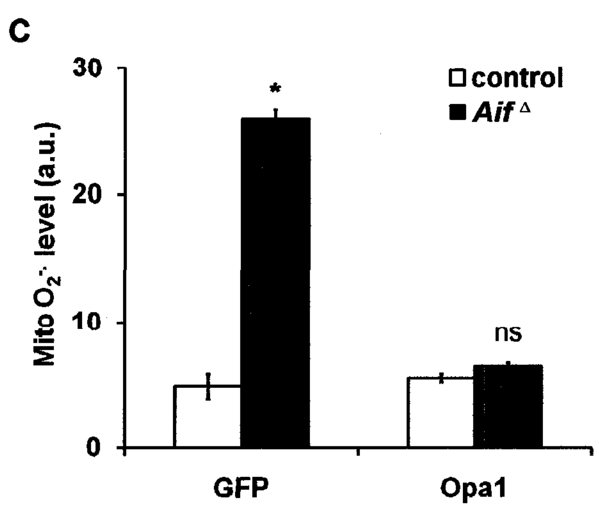
Figure 4-6: Opa1 can rescue metabolic, survival and ROS defects of *Aif*$^{\Delta}$ neurons. (A) O_2 consumption of the control neurons expressing GFP, and *Aif*$^{\Delta}$ neurons expressing Opa1 and GFP control (n=5). **(B)** ATP production of the control neurons expressing GFP, and *Aif*$^{\Delta}$ neurons expressing Opa1 and GFP control (n=5). **(C)** Mitochondrial superoxide ($O_2^{\cdot-}$) level of the control and *Aif*$^{\Delta}$ neurons expressing Opa1 and GFP control after 3 DIV (n=3). **(D)** Quantitative analysis of cell death of control and *Aif*$^{\Delta}$ neurons in normal media expressing Opa1 and GFP as control (n=3). **(E)** Quantitative analysis of cell death of control and *Aif*$^{\Delta}$ neurons in enriched media expressing Opa1 and GFP as control (n=3). * $p<0.05$ compared to control + GFP.


A
control + GFP
Aif^Δ + GFP
Aif^Δ + Opa1
O2 consumption (a.u.)
1400
1200
1000
800
600
400
200
0
1
2
3
4
Days in vitro


D
control + GFP
Aif^Δ + GFP
Aif^Δ + Opa1
Survival (%)
100
80
60
40
20
0
24
36
48
60
72
Time (hrs)


B
control + GFP
Aif^Δ + GFP
Aif^Δ + Opa1
[ATP] (a.u.)
1200
1000
800
600
400
200
0
1
2
3
4
Days in vitro


E
control + GFP
Aif^Δ + GFP
Aif^Δ + Opa1
Survival (%)
100
80
60
40
20
0
24
36
48
60
72
Time (hrs)


C
control
Aif^Δ
Mito O2^·- level (a.u.)
30
20
10
0
ns
GFP
Opa1

asked whether Opa1 can also rescue the metabolic defects of the *Aif* $^{\Delta}$ neurons. By expressing Opa1 in *Aif* $^{\Delta}$ neurons, we were able to rescue both O_2 consumption and ATP production in $AIF^{-/-}$ neurons back to the control level (Figure 6a and b). Indeed, the mitochondrial superoxide ($O_2^{\cdot-}$) level in *Aif* $^{\Delta}$ neurons is significantly reduced after Opa1 expression compared to GFP expressing *Aif* $^{\Delta}$ neurons. In contrast to Mfn2 or dnDrp1 that only partially rescue *Aif* $^{\Delta}$ neurons (Figure 4c), the presence of Opa1 is able to rescue mitochondrial $O_2^{\cdot-}$ level back to control level (both around ~5 a.u., Figure 6c). This indicates that the increase of ROS level in *Aif* $^{\Delta}$ neurons is due to the lack of proper Opa1 activity in these cells. Finally, we asked whether the expression of Opa1 may affect the survival of *Aif* $^{\Delta}$ neurons. Remarkably, in stark contrast to other mitochondrial proteins (Figure 4d) and to the ROS scavenger NAC (Figure 1a), *Aif* $^{\Delta}$ neurons with Opa1 have significantly higher survival than *Aif* $^{\Delta}$ neurons with GFP control, indicating that Opa1 is able to rescue the survival of *Aif* $^{\Delta}$ neurons (Figure 6d). Also, the presence of Opa1, unlike Mfn2 or dnDrp1, does not kill the *Aif* $^{\Delta}$ neurons in enriched media (Figure 6e), again underscore the specificity of Opa1. Taken together, these results show that the expression of Opa1, but not Mfn2 or dnDrp1 can rescue a variety of mitochondrial defects in *Aif* $^{\Delta}$ neurons. AIF therefore controls the mitochondrial structure and function, and subsequently cellular survival, specifically via Opa1.

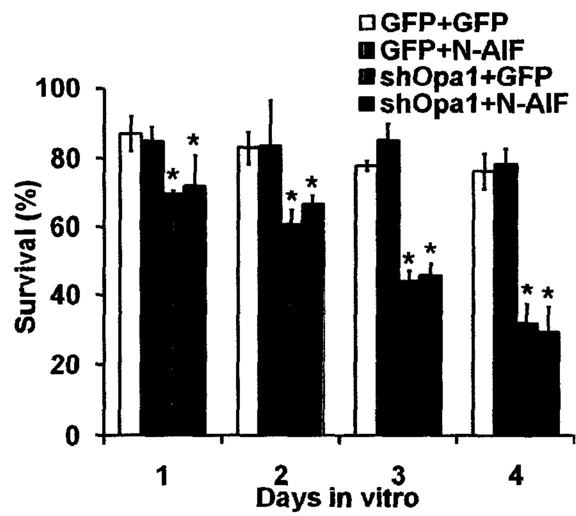
Since Opa1 expression rescues the defects of AIF deficient neurons, this suggests that Opa1 works downstream of AIF to maintain cell survival. If Opa1 does indeed function downstream of AIF then AIF would not be functional in Opa1 deficient neurons. To test this we asked whether AIF can rescue neurons depleted in Opa1 using shOpa1 to knockdown Opa1 expression. Similar to other cell types with reduced Opa1 (Chen et al, 2005; Griparic

et al, 2004; Herlan et al, 2003), neurons treated with shOpa1 also have reduced survival compared to GFP control. Opa1 knockdown neurons expressing mitochondrially anchored AIF (Cheung et al, 2006) have the same death rate compared to those expressing GFP control, indicating that mitochondrial AIF cannot rescue the survival of shOpa1 treated neurons (Figure 7). Our results demonstrating that Opa1 can rescue AIF deficiency (Figure 4d) but mitochondrial AIF cannot rescue Opa1 deficiency (Figure 7), place AIF upstream of Opa1 in the regulation of mitochondrial function.

AIF binds with Opa1 to form Opa1 oligomers

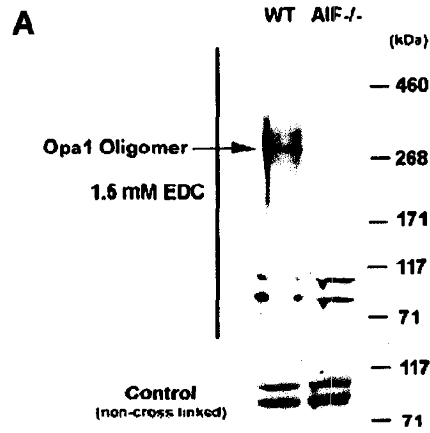
Next we questioned how AIF may control Opa1 activity in maintaining mitochondrial structure. Previous studies have indicated that Opa1 forms oligomers for the formation of cristae junction, and the absence of Opa1 causes mitochondrial cristae malformation and defective oxidative phosphorylation (Frezza et al, 2006; Griparic et al, 2004; Herlan et al, 2003). Since AIF works upstream of Opa1, we first determined whether AIF is essential for the oligomerization of Opa1. Western blot of mitochondria from $AIF^{-/-}$ neurons after protein cross-linking showed that Aif^{Δ} mitochondria have no high molecular weight Opa1 complex compared to the control animals (Supplementary figure 8a). This is not due to the loss of Opa1 monomers as western blot analysis without cross-linking showed similar level of Opa1 monomers between the Aif^{Δ} neurons and the control (Supplementary figure 8a). This indicates that AIF is required for oligomerization of Opa1. Next we asked whether AIF modulates Opa1 oligomerization by a direct physical interaction. Co-immunoprecipitation using AIF antibody to pull down Opa1 suggest that AIF and Opa1 may interact physically (Supplementary figure 8b).

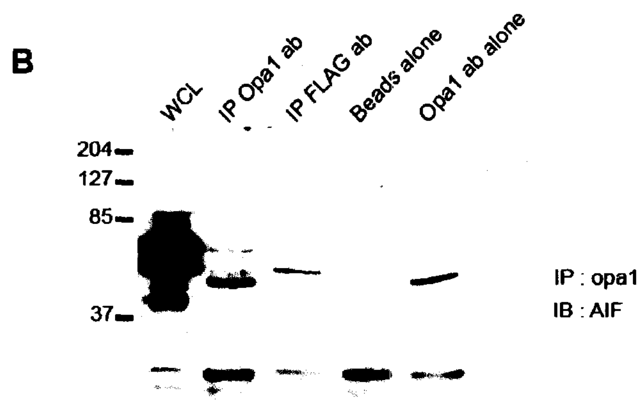
Figure 4-7: AIF works upstream of Opa1 to maintain survival. Quantitative analysis of cell death of wildtype cortical neurons expressing GFP+N-AIF, shOpa1+GFP, N-AIF+shOpa1 and GFP+GFP (each treatment: 100 MOI in total) as control (n=5). * $p<0.05$ compared GFP+GFP and GFP+N-AIF.



GFP+GFP
GFP+N-AIF
shOpa1+GFP
shOpa1+N-AIF
Survival (%)
100
80
60
40
20
0
*
1
2
3
4
Days in vitro

Supp. Figure 8: AIF interacts with Opa1 and control Opa1 oligomerization. (A) Western blot analysis of isolated proteins of mitochondria from *Aif* $^{\Delta}$ and control neurons that were crosslinked with EDC. Non crosslinked proteins show the presence of Opa1 monomers in both *Aif* $^{\Delta}$ and control neurons. (B) Co-immunoprecipitation of Opa1 and AIF. Mitochondria (200ug) isolated from 293T cells were resuspended in 5 ml of IP Lysis Buffer. Mitochondria were lysed for 2 hours at 4°C and then centrifuged 15 min at 12 000 rpm. The supernatant was incubated overnight with α-opa1 or α-FLAG. Protein G conjugated agarose beads were blocked with BSA and added to the samples. After 2 hours, the beads were collected and washed 5 times with TBS. Samples were analyzed by SDS-PAGE.


A
WT AIF-/-
(kDa)
460
Opa1 Oligomer
268
1.5 mM EDC
171
117
71
Control
(non-cross linked)
117
71


B
WCL
IP Opa1 ab
IP FLAG ab
Beads alone
Opa1 ab alone
204
127
85
37
IP : opa1
IB : AIF

To further confirm that AIF is present in a complex with Opa1 we asked if AIF is associated with Opa1 oligomers by probing a western blot with Opa1 oligomers using AIF antibody. A band detected by antibodies against AIF colocalizes with the Opa1 oliogmers (Cheung, Pilon-Larose, and Slack unpublished observation). Taken together, these results suggest that AIF physically interacts with Opa1 in the complexes to maintain Opa1 oligomerization. Loss of AIF causes disintegration of the oligomers, and subsequently the breakdown of the mitochondrial cristae architecture which results in impaired mitochondrial metabolism and cell death. Our functional as well as biochemical analysis, therefore, indicate a novel role of AIF in maintaining mitochondrial integrity and cell survival through the mitochondrial inner membrane fusion protein Opa1.

Discussion

Previous studies have shown that AIF has a physiological role in the mitochondria, however, the exact mechanism is not yet clear. This study identified Opa1 as a target of AIF to maintain mitochondrial function through regulating the structure. We first demonstrated that AIF does not simply act as a ROS scavenger, as the ROS scavenger NAC cannot rescue any defects of the *Aif*$^{\Delta}$ neurons. Instead, *Aif*$^{\Delta}$ neurons can be rescued by expressing Opa1, but not by promoting fusion using Mfn2 or by inhibiting fission through dominant negative Drp1. This indicates that Opa1 maintains the survival of AIF mutant due to its specific activity in controlling cristae structure, not the fusion promoting activity of Opa1 *per se*. This is further supported by the lack of high molecular weight Opa1 oligomer complex in the *Aif* $^{\Delta}$ neurons. Opa1 complex has been shown to be important for maintaining mitochondrial cristae structure (Frezza et al, 2006). Finally, we showed that AIF interacts physically with Opa1 and is also part of the Opa1 complex, further strengthening the role of AIF in regulating mitochondrial function via Opa1.

Different views on how AIF maintains cell survival, including AIF as a ROS scavenger, as part of the electron transport chain, or as a stabilizer of mitochondrial complex 1 and IV, have been proposed previously (Klein et al, 2002; Urbano et al, 2005; van Empel et al, 2005; van Empel et al, 2006). Since no genes that can rescue the defects in cells lacking AIF has been identified in these studies, it is difficult to pinpoint the exact mechanism of AIF in maintaining cell survival. A recent report has indicated that AIF is part of the complex I in heart mitochondria using MS analysis (Palmisano et al, 2007). It is, however, difficult to explain why the stability of other complexes such as complex IV is also affected if the role of AIF is to stabilize complex I by direct interaction. Here, we first show

that AIF has function beyond being a ROS scavenger, since the ROS scavenger NAC can only marginally rescue the defects in *Aif*$^{\Delta}$ neurons. AIF deficient mice such as *Hq* mice have increased level and increased sensitivity to ROS are possibly a result of secondary effects due to malfunctioned mitochondria resulted from the defective cristae structure and deregulated fission/fusion events caused by the lack of proper Opa1 activity. In fact, a recent report has shown that AIF cannot transfer electrons from NADH to scavenge ROS such as superoxide and hydrogen peroxide, indicating that AIF does not act merely as a ROS scavenger (Churbanova & Sevrioukova, 2007). On the other hand, similar to AIF deficient cells, Opa1 knockdown cells also have increased level of ROS, reduced mitochondrial function which leads to spontaneous cell death (Chen et al, 2005; Griparic et al, 2004; Herlan et al, 2003). The exact relationship between mitochondrial cristae structure and the stability of mitochondrial respiration complexes is not known. It has been suggested that a defective membrane structure may destabilize the mitochondrial respiration complexes that are embedded in the membrane, or the unstable membrane structure may cause electron leakage from the electron transport chain which increase the production of ROS (Benard et al, 2007; Vercesi et al, 1997). In addition, the lack of fusion in the *Aif*$^{\Delta}$ cells may hinder the mixing of mitochondria, which is essential for the maintanence of mitocondrial DNA. As some of the components of the mitochondrial complex I and IV are encoded by mtNDA, a defect in fusion may cause a decrease of complex I and IV expression (Chen et al, 2007; Detmer & Chan, 2007b). Our model, therefore, is able to explain the observations that other studies has indicated in AIF deficient cells and animals, and has resolved the physiological role of AIF in the mitochondria, that is, to control mitochondrial structure through its interaction with Opa1.

Apart from the physiological role, the interaction between AIF and Opa1 may also unravel a novel connection between mitochondrial metabolism and the cell death pathway during cellular stress. AIF may act as a sensor of changes in metabolic rate or ROS level in the mitochondria which indicates how healthy the cell is. Under adverse conditions such as decreased level of energy production, or increased level of mitochondrial ROS production due to pathological Ca^{2+} influx such as excitotoxicity, AIF may relay these signals to Opa1, which may result in the breakdown of the Opa1 oligomers. Subsequently, this will induce the unravelling of the mitochondrial cristae which facilitate quick and complete cytochrome c release (Frezza et al, 2006; Germain et al, 2005; Scorrano et al, 2002). If the physiological role of AIF is retained in the mitochondrial after cell death induction, the cells will be able to survive longer as the function of Opal is maintained and the cristae do not undergo remodelling for complete cytochrome C release. This is supported by our previous studies in which by expressing mitochondrially anchored AIF that cannot be released during cell death in *Apafl/Aif*$^{\Delta}$ double knockout neurons, the cells exhibit higher survival and maintain proper mitochondrial function after cell death induction. As well, overexpression of mitochondrially anchored AIF in wildtype neurons is able to retain cyt c in the mitochondrial during cell death longer than the control cells (Cheung et al, 2006). Hence, in addition to the importance of the AIF-Opa1 pathway in maintaining mitochondrial structure and function, the regulation of this pathway may also play a critical role in the progression of apoptosis after cytotoxic insults, especially those that can also directly disrupt mitocondria such as Ca^{2+} and ROS.

Chapter V: Conclusion

AIF in the regulation of neuronal cell death

Previously, the role of AIF has been implicated in studies where the translocation of AIF has been observed in neurons undergoing cell death *in vitro* and *in vivo* (see table 5-1). Overexpression of AIF alone can induce neuronal cell death, and the inhibition of AIF translocation using neutralizing AIF antibodies can increase cell survival during DNA damage induced cell death when caspases are also inhibited (Cregan et al, 2002; Wang et al, 2004). While these studies suggested a role for AIF in neuronal cell death, more direct studies using AIF deficient animals were lacking. The experiments in Chapter two provide direct evidence using *Hq* mice which have 20% AIF expression. *Hq* neurons are more resistant to excitotoxicity *in vitro*, which is largely Bax and caspase independent. In particular, *Hq* neurons are more resistant to NMDA and KA induced cell death, but not AMPA, indicating that AIF may be selectively activated after NMDA and KA induced cell death. As AIF is a downstream target of PARP-1 (Yu et al, 2002), this result is in accordance with PARP-1 induced cell death, which is also dependent on NMDA and KA but not AMPA. When caspases are inhibited using *Apaf1*$^{-/-}$ mice, the inhibition of AIF can confer long term protection against DNA damage compared to *Apaf1*$^{-/-}$ neurons alone (Cheung et al, 2005). This indicates that AIF is the caspase independent cell death factor in DNA damage induced cell death, which is Bax dependent (Cregan et al, 1999). The critical role for AIF in caspase independent cell death is further demonstrated in an *in vivo* excitotoxic model using adult mice injected with KA to induce seizure and hippocampal damage. *Hq* adult mice are more resistant to KA induced cell death in the hippocampus *in vivo*, indicating that AIF is a

key factor in excitotoxicity (Cheung et al, 2005). Shortly after our paper was published, another report also indicated a critical role of AIF in neuronal cell death using a stroke model (middle cerebral artery occlusion) (Culmsee et al, 2005). Taken together, my study in chapter three is the first to directly show a critical role of AIF in mediating cell death during acute neuronal injury.

AIF in the regulation of neuronal survival

The discovery of the *Hq* mice showed a surprising role of AIF in maintaining neuronal survival, as these animals exhibited neurodegeneration at an older age (Klein et al, 2002). Studies on AIF null cells also indicate an important role of AIF in maintaining mitochondrial health, such that the complete absence of AIF leads to the loss of mitochondrial function (Joza et al, 2005; Vahsen et al, 2004). As described in chapter three, a conditional mutant of AIF in the forebrain has reduced neuronal survival due to mitochondria dysfunction. The role of AIF in cell death is thus called into question as it seems like the main function of AIF is to maintain survival in the mitochondria. It is possible that after cell death induction, the cells are dying due to the loss of mitochondrial AIF and the subsequent mitochondrial malfunction: the "apoptotic" role of AIF in the nucleus may not be required. In order to solve this controversy, we designed an AIF construct that is anchored into the mitochondria and cannot be released during cell death. The presence of mitochondrially-anchored AIF in the *Apaf1*$^{-/-}$*Aif*$^{-/-}$ neurons can provide long term survival after DNA damaged induced cell death. In contrast, *Apaf1*$^{-/-}$ cells (containing endogenous wildtype AIF) with the mitochondrially-anchored AIF still go on to die, indicating that even

in the presence of mitochondrial AIF, the wildtype AIF that is released and translocated into the nucleus can still kill the cell. The pro-apoptotic role of AIF in the nucleus is further demonstrated using AIF mutant that cannot be translocated into the nucleus, which cannot induce cell death when it is expressed in *Aif*$^{-/-}$ neurons (Cheung et al, 2006). Taken together, these studies provided the first definitive evidence that the pro-apoptotic activity of AIF in the nucleus is a key factor in inducing cell death, in addition to its role in survival.

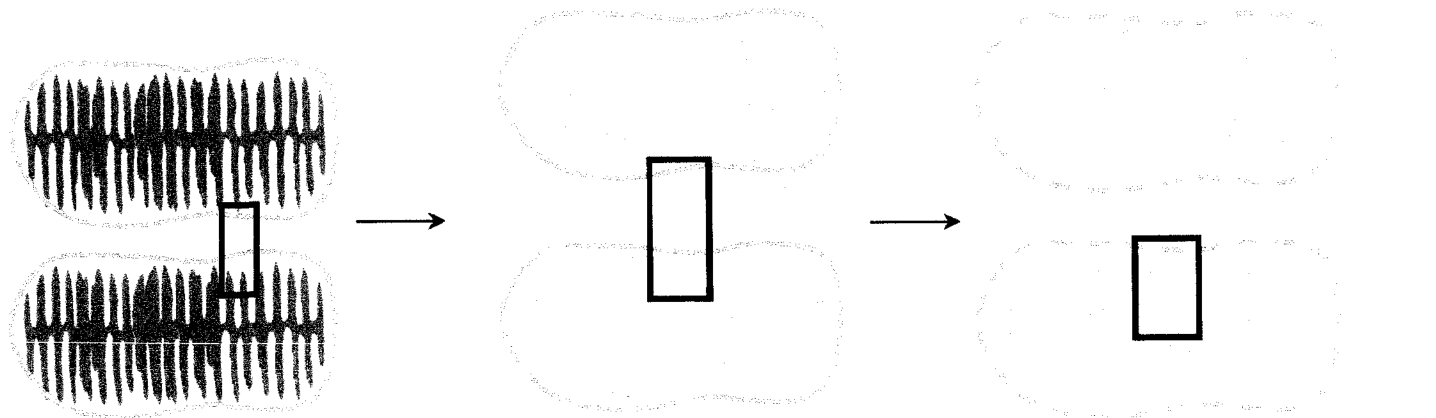
There are two proposed mechanisms on how AIF maintains cell survival: acting as a ROS scavenger, or stabilizing complex I of the mitochondrial electron transport chain. Since binding partners of AIF has not been identified in the mitochondria, it is difficult to demonstrate the role of AIF in the mitochondria. As described in chapter three, the mitochondria in the *Aif*$^{-/-}$ neurons are fragmented with dilated cristae. Addition of AIF in these mutant cells can rescue the phenotypes back to wildtype level, and more importantly, wildtype cells that are expressing the mitochondrially anchored AIF have longer mitochondria with more compact cristae. All of these indicate a possible function of AIF in controlling mitochondrial structure. The addition of ROS scavenger can only partially rescue the defects in the AIF mutant cells, thus the primary activity of AIF is probably not in ROS scavenging. We further explore this possibly by rescuing the AIF mutant phenotype using the mitochondrial fission/fusion machinery. Only the expression of the mitochondrial fusion protein Opa1 is able to rescue the mitochondrial defects and maintain the survival of the AIF mutant cells. This indicates that AIF may have a similar function as Opa1, or Opa1 is acting downstream of AIF. Expression of AIF in Opa1 deficient cells cannot rescue the phenotype, suggesting that Opa1 is upstream of AIF for the maintenance of mitochondrial structure. In combination of a recent study that shows AIF and Opa1 can interact with each other

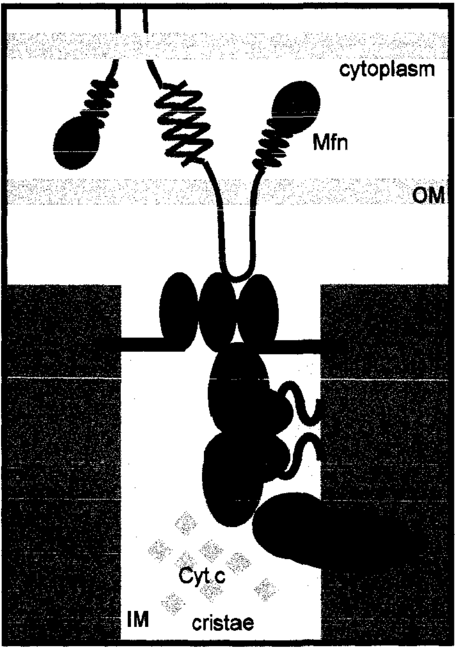
physically (Zanna et al, 2008), the results in chapter 3 and 4 indicate, for the very first time, that the role of AIF is to maintain mitochondrial shape through Opa1, and may provide a link between mitochonrial metabolism and the control of mitochondrial architecture.

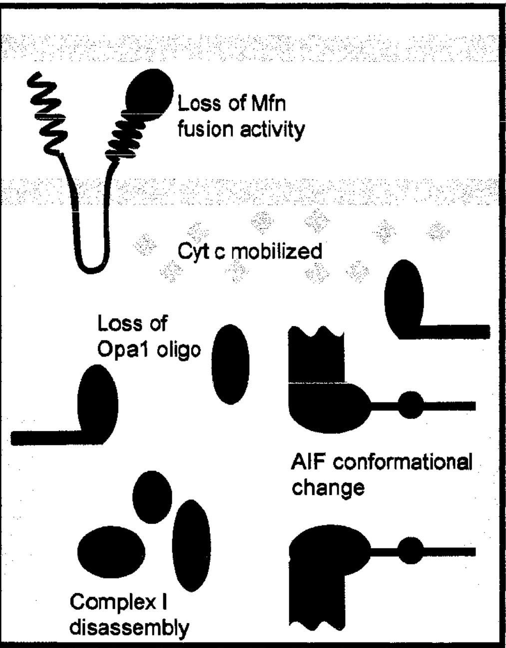
A working model of how AIF works in the mitochondria (Figure 5-1)

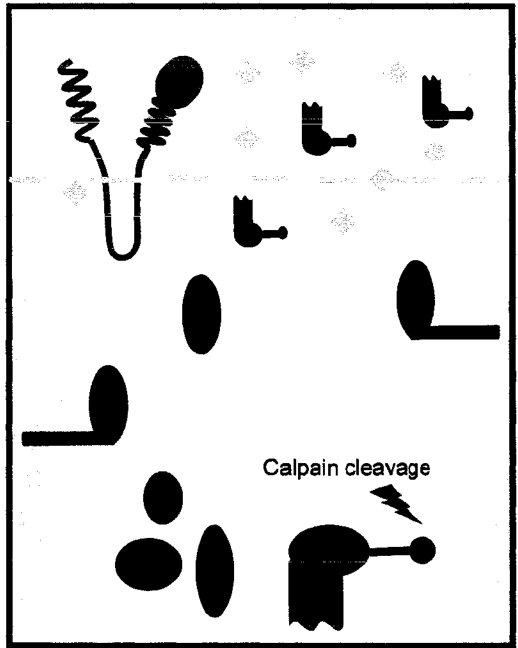
Using the results presented in chapter 2-4 in combination with the recent literature on AIF, we can now propose a model of how AIF works in the mitochondria. In healthy mitochondria under a normal level of NAD(P)H, AIF exists as NAD(P)H-bound dimers (Churbanova & Sevrioukova, 2007). This dimer may bind to Opa1 through the C terminal of AIF, and this interaction is essential for the formation of the Opa1 oligomers between the long and short Opa1 isoforms. The Opa1 oligomers, as discussed in section 6, are essential for the formation of tight mitochondrial cristae. During cell death, the loss of Opa1 oligomers can induce cristae remodelling/dilation that mobilizes cyt c for its complete release (Frezza et al, 2006). In mammalian system, Opa1 requires Mfn1 for mitochonrial fusion (Cipolat et al, 2004), therefore, the interaction between AIF and Opa1 may also regulate the fusogenic activity of Mfn. AIF dimers may also interact with complex I for stability (Palmisano et al, 2007; Zanna et al, 2008). When the cells are under metabolic stress (e.g. when the energy production is low), the interactions between AIF/Opa1/Mfn1/complex I are disrupted due to the conformational change at the C terminal of AIF. The conformational change is the result of the lack of NAD(P)H binding at the NADH binding domain of AIF. In addition to the changes at the C terminal, the dimerization of AIF is also disrupted, and the calpain I cleavage site at the N terminal is also exposed

Figure 5-1: A model of how AIF works in the mitochondria.




Healthy cells
cytoplasm
Mfn
OM
Cyt c
IM
cristae


Metabolic stress, low NAD(P)H
Loss of Mfn
fusion activity
Cyt c mobilized
Loss of
Opa1 oligo
AIF conformational
change
Complex I
disassembly


MOMP by Bax/Bak
Calpain cleavage

(Churbanova & Sevrioukova, 2007). As a result, mitochondrial cristae are dilated due to the lack of Opa1 oligomers, fusion activity of Mfn1 is abrogated, and the cells are thus sensitized to cell death. Cyt c (and other pro-apoptotic proteins in the mitochondria) in the cristae are mobilized and are ready for a complete release once MOMP or MTP is initiated after a cytotoxic insult.

Future directions

The above proposed model based on the results in chapter 2-4 can be used as a starting point to further delineate the mechanism of AIF in regulating mitochondrial structure and function. As described in chapter 3 and 4, AIF may bind to Opa1 for the maintenance of the mitochondrial structure. Other studies also suggest a possible role of AIF in complex I stability (Palmisano et al, 2007; Vahsen et al, 2004; Zanna et al, 2008). Hence, two hypotheses can be proposed for the role of AIF in maintaining mitochondrial function (Figure 5-2). The first hypothesis is that AIF binding to Opa1 is the first critical step for the maintenance of mitochondrial structure and function. The second one is that AIF binding to complex I is the critical step. It is worth noting that the two scenarios may not be mutually exclusive, as AIF binding to both proteins could be required to keep the mitochondria healthy.

The role of AIF in mitochondrial fusion can be confirmed by using the photoactivation assay. This is performed by expressing a mitochondrially targeted photoactivable GFP in the AIF deficient neurons, followed by photoactivating the GFP in a small area of the mitochondrial network. The rate of fusion is then assessed by the time it

Figure 5-2: Two hypothesis of how AIF regulates mitochondrial structure.

Hypothesis 1 (Opa1 binding is critical):

Cristae formation: AIF binds to Opa1 → Opal oligomerize → cristae formation → binding of AIF to complex I → CI stability

Mitochondrial fusion: AIF binds to Opa1 → Opa1 binds to Mfn1 → mitochondrial fusion → complex I stable

Hypothesis 2 (Complex I binding is critical):

Cristae formation: AIF binds to CI → CI is stable → Opa1 oligomerize → binding of AIF to Opa1 → cristae formation

Mitochondrial fusion: AIF binds to CI → CI is stable → Opa1 binds to Mfn1 → mitochondrial fusion

takes for the GFP signal to spread across the cell due to the fusion of mitochondria. If AIF is indeed required for fusion, the rate of speading in AIF deficient cells should be signficantly less than wildtype cells. Since the fusion between two membranes often requires the presence of the fusion protein at both membranes (i.e. in trans) (Chen et al, 2003; Hoppins et al, 2007; Meeusen & Nunnari, 2005), similarly AIF mediated cristae formation/mitochondrial fusion may also require AIF to act in trans. This can be assessed by PEG mediated fusion assay between two cells. This allows the mixing of two different populations of mitochondria between two cells, one with AIF and labelled with mitochondrial-CFP (blue), the other without AIF and labelled with mitochondrial-YFP (yellow). If AIF works in trans for mitochondrial fusion, then there will be no fusion of mitochondrial between these two populations and the mitochodria in the fused cell remain separate (the mitochondria will either be blue or yellow). If AIF does not have to work in trans, then the presence of AIF in one population of mitochondria should be enough to fuse with those without AIF. All the mitochondria should be green due to the fusion of the blue and yellow mitochondria. These experiments will definitely indicate the role of AIF in mitochondrial fusion, which is shown in chapter 3 and 4.

First hypothesis: AIF binding to Opa1 is the critical event for maintaining mitochondrial architecture

While chapter 4 shows that AIF may interact with Opa1 for maintaining the mitochondrial structure, whether AIF binds to the long or the short form of Opa1 remains unknown. This can be revealed by expressing uncleavable l-Opa1 or s-Opa1 in Opa1

deficient cells, and then perform co-immunoprecipitation using AIF antibody. If AIF interacts only with either one of the isoforms, then we will see co-immunoprecipitation between AIF and the specific long or short isoforms. If AIF interacts with both isoforms, then expression of both isoforms in Opa1 deficient cells are required. Also suggested in chapter 4 is that binding of AIF to Opa1 may be necessary for the formation of Opa1 oligomers, which in turn is essential for mitochondrial cristae formation and the stability of Complex I. The critical role of binding betwen AIF and Opa1 can be further demonstrated by disrupting the interaction between AIF and Opa1 and then assess Complex I stability and Opa1 oligomer formation. To acheive this, the domain on Opa1 that binds to AIF has to be identified by mutational studies on Opa1 followed by co-immunoprecipitation. After the binding domain is identified on Opa1, we can express the AIF binding mutant of Opa1 in Opa1 defiient cells and see if this can rescue the Opa1 oligomerization, cristae malformation, and the loss of complex I activity compared to the wildtype Opa1 construct. If the AIF-Opa1 interaction is indeed required for the Opa1 activity and complex I stability, then the AIF binding mutant of Opa1 will not be able to rescue the mitochondrial cristae defects and complex I defects in Opa1 deficient cells. In addition to chapter 3 and 4, these studies will further demonstrate the mechanism of AIF-Opa1 interaction in maintaining proper mitochondrial function.

Chapters 3 and 4 indicate that the mitochondria in AIF knockout neurons are also fragmented, which point to a possible role of AIF in regulating mitochondrial fusion through Opa1. As Mfn1 requires Opa1 for mitochondrial fusion (Cipolat et al, 2004), AIF may regulate the interaction between Mfn1 and Opa1. To see whether AIF mediates Mfn1-Opa1 interaction, the presence or absence of Mfn1-Opa1 interaction can be determined in AIF

deficient neurons by co-immunoprecipitation between Mfn1 and Opa1. The interaction between Mfn1 and Opa1 should be absent in AIF deficient neurons, compared to wildtype cells. To test whether AIF-Opa1 interaction is required for mitocondria fusion via Mfn1-Opa1 interaction, the AIF binding mutant of Opa1 can be used. This mutant construct should be unable to rescue the fragmented mitochondria in the Opa1 deficient cells, and there will be no interaction between Opa1 and Mfn1. After the importance of AIF-Opa1 interaction in mitochondrial fusion through Mfn1 is shown, then the mechanism of AIF in Mfn1-Opa1 interaction can be determined. AIF may directly interact with Mfn1 and Opa1, thus linking Mfn1 and Opa1 in a similar fashion as Ugo1 in yeast that links Mgm1p and Fzo1p (Sesaki & Jensen, 2004). This can be shown by co-immunoprecipitation studies using AIF antibody to pull down Mfn1 or Opa1, and vice versa, and see whether AIF interacts directly with only Opa1 or both Opa1 and Mfn1. Alternatively, if AIF does not bind to either one of those proteins, it may still indirectly regulate Mfn1-Opa1 interaction via the NAD(P)H redox activity. This can be tested by expressing AIF with mutated NADH domain in AIF deficient cells, and compare the mitochondria fusion activity and Mfn1-Opa1 interaction with those that are expressing the wildtype AIF. If the NADH domain is required, then there will be no interaction between Mfn1 and Opa1 when the NADH domain mutant is expressed in AIF deficient cells. Taken together, these experiments will define the mechanism of AIF in controlling Opa1 activity for mitochondrial fusion and cristae formation.

Second hypothesis: AIF binding to complex I is the critical event for maintaining mitochondrial architecture

While the interaction between AIF and Complex I has been suggested (Palmisano et al, 2007; Zanna et al, 2008), the exact mechanism of this interaction, as well as whether this interaction is required for Complex I stability, are not yet clear and has not been fully addressed in chapter 4. It is possible that the interaction between AIF and complex I regulates the activity and oligomerization of Opa1, which in turn controls mitochondrial shape. We have previously identified the complex I subunit NDUFA8 by yeast two hybrid assay as a potential binding partner of AIF (McLaurin, Cheung, and Slack unpublished). Rather than directly interacting with Opa1 as suggested in chapter 4, AIF may instead interact with complex I through NDUFA8, and the loss of this interaction causes a decrease in Opa1 oligomerization which leads to cristae malformation and loss of mitochondrial fusion. In order to test this hypothesis, first the interaction between AIF and NDUFA8 has to be confirmed by co-immunoprecipitation. Then we can ask if NDUFA8 is required for the physiological role of AIF in the mitochondria by using siRNA of NDUFA8. First, we can assess whether AIF can still bind to Complex I in cells without NDUFA8 by performing coimmunoprecipitation studies in NDUFA8 deficient cells. Next, the stability and activity of complex I in cells lacking NDUFA8 can be determined by performing blue native PAGE to seperate the mitochondrial complexes in their native form and activity. If AIF interacts with NDUFA8 for stabilizing complex I, then the lack of NDUFA8 will lead to lack of AIF interaction, and the destabilization of complex I. While it is obvious that hypothesis can be rejected if AIF-NDUFA8 interaction is not required for complex I stability/activity, the interaction between AIF-NDUFA8 (or any other complex I subunits) itself may still affect Opa1 activity without the involvement of complex I activity (for example, NDUFA8 acts as a structural protein for Opa1-AIF interaction). The above studies will provide the important

first step to further explore the role of AIF-complex I interaction in maintaining mitochondrial structure and function.

If the lack of NDUFA8 does indeed inhibit AIF binding to complex I and induce complex I instability, we can then ask whether AIF binds to NDUFA8 to regulate Opa1 activity and oligomerization to maintain mitochondrial cristae structure and function. If this is true, then cells with reduced NDUFA8 will have disrupted Opa1 oligomers and dilated mitochondrial cristae. We can then ask if AIF-Complex I interaction through NDUFA8 is required for proper mitochondrial fusion via Mfn1 and Opa1. We can determine whether Mfn1 and Opa1 can still interact with each other in the absence of NDUFA8. In addition, we can assess whether fusion is disrupted by the photoactive mitochondrial-GFP fusion assay. If NDUFA8 is indeed required for Mfn1-Opa1 interaction, in the abscence of NDUFA8, Mfn1 and Opa1 will not be able to interact and subsequently mitochondrial fusion will be reduced. If changes in the mitochondrial fusion ability in the absence of NDUFA8 is subtle due to other compensatory mechanisms, the sensitivity of the mitochondrial fusion assay can be amplified in cells lacking NDUFA8 by increasing the rate of mitochondrial fusion using Mfn1 overexpression or dominant negative Drp1. If indeed NDUFA8 has a regulatory role in fusion (but is not absolutely required for fusion), then in the presence of an increased rate of fusion (or decrease rate of fission), cells lacking NDUFA8 should have less increase in fusion compared to wildtype. Another possible caveat is that AIF may not bind to complex I through NDUFA8, or the binding does not affect the complex I stability. In this case, we can disrupt the stability of the whole protein by using siRNA of complex I subunits that are responsible for the assembly (Brandt, 2006; Ogilvie et al, 2005; Schulte et al, 1998), and then use these to perform the above experiments to see if complex I stability regulates Opa1

oligomerization and activity. If on the other hand the depletion of NDUFA8 is too detrimental to complex I stability and causes rapid mitochondrial failure and cell death, then the domain that is responsible for AIF binding can be determined, and then the binding mutant can be used instead. Taken together, these future studies will address the possibility that instead of interacting with Opa1 directly as suggested in chapter 4, AIF may rather interact with complex I directly through NDUFA8 and this interaction is critical for Opa1 to function properly.

Therapeutic implications: targeting mitochondria for the treatment of neuronal injury

The ultimate goal of this study is to discover novel therapeutic targets for the treatment of stroke. Presently, the only available treatment for stroke at this moment is tPA (tissue plasminogen activator), which only serves as a clot diffusing agent and does not stop the cell death process once it has started. The window of treatment for tPA is thus three hours, and after that even though the blood flow is re-established, the cell death process is already in progress. Various pharmacological molecules that target the apoptotic pathway have been developed in animal stroke models, but unfortunately most of these treatments failed in clinical settings (Ikonomidou & Turski, 2002; Kemp & McKernan, 2002). These stroke treatments failed not only because of the recruitment of multiple cell death pathways during neuornal cell death, but more importantly, the design of these drugs overlooked the vital and physiological roles of these "pro-apoptotic" drug targets. For example, MK801 is a very effective NMDA receptor blocker that can inhibit excitotoxicity in animal models, but at the same time the pro-survival role of NMDA is also blocked, which has detrimental

consequences to neuronal survival (Ikonomidou & Turski, 2002; Lo et al, 2003). Similarly, as indicated in chapter 3 and 4, AIF, once thought to be purely apoptotic, also has an important role in cell survival. One can imagine the devastating outcomes if drugs are designed to inhibit AIF activity completely as a treatment for maintaining neuronal survival. In fact, similar to the absence of AIF during embryonic brain development as indicating in chapter 3, the absence of AIF in post-mitototic hippocampal neurons can also induce neurodegeneration (Kelly, Cheung, and Slack unpublished), further supporting the danger of inhibiting AIF indiscriminately as a strategy for treating neuronal injury. The studies in this thesis highlight the emerging theme in cell death study: the "day jobs" of pro-apoptotic proteins that are critical for cell survival in healthy cells (**Table 5-1**). These essential vital functions of the so-called "death proteins" cannot be ignored when designing therapeutic treatments against the apoptotic activity of these proteins. A more feasible way to design therapeutic strategy is to dissociate the vital and lethal functions of these proteins such that only the apoptotic activity is inhibited while the vital role of these proteins in cell survival is maintained or even augmented.

In chapter three, I have provided a method to dissociate the cell death role of AIF from the cell survival role by using a mitochondrially anchored AIF that cannot be released during cell death. By using this construct, the cell death role of AIF is eliminated while preserving its vital role in the mitochondria. The expression of this construct in *Aif/Apaf1*$^{-/-}$ double knockout can provide long term protection against DNA damage compared to the double knockout neurons alone, indicating that the preservation of mitochondrial function during cell death can provide further protection against cell death. In this study, however, the impact of cyt c release to cell survival has not been addressed. Since the loss of cyt c from

Protein	Cell death activity	"Day job"	Reference
Cyt c	Activate caspases	Electron transport in mitochondria	(Hao et al, 2005; Li et al, 2000)
Caspases	Cell death protease	Development, inflammation, proliferation	(Algeciras-Schimnich et al, 2002; Lamkanfi et al, 2007; Launay et al, 2005; Schwerk & Schulze-Osthoff, 2003)
Calpain	Cell death protease	Neuronal remodelling, cell spreading and retraction	(Chakrabarti et al, 1993; Faddis et al, 1997; Flevaris et al, 2007)
AIF	Caspase independent cell death	Mitochondrial function: architecture, metabolism	(Cregan et al, 2004; Krantic et al, 2007; Modjtahedi et al, 2006)
Omi/HtrA2	Caspase dependent cell death, cell death protease	Protein quality control, chaperone	(Jones et al, 2003; Plun-Favreau et al, 2007; Suzuki et al, 2001; van Loo et al, 2002)
Bax/Bak	MOMP	Mitochondrial fusion	(Karbowski et al, 2006)
NMDA receptors	Excitotoxicity	Signal transduction, neuronal survival	(Hardingham & Bading, 2003; Ikonomidou & Turski, 2002; Kemp & McKernan, 2002)

Table 5.1: The "day jobs" of the cell death proteins. Some of the cell death proteins, when not activated during cell death, have important physiological functions in healthy cells.

mitochondrial during cell death can be detrimental to mitochondrial function and cell survival (Li et al, 2000), the retention of both cyt c and AIF in the mitochondria during cell death may provide even stronger protection against cell death, especially during ischemia in which the energy level of the neuron is severely compromised. While there are no studies using mitochonrdially anchored cyt c in cell death, there is one similar study using cyt c knock-in mutant mouse in which the amino acid responsible for caspase activation is mutated (Hao et al, 2005). Surprisingly, cell death progession after DNA damage is not reduced, suggesting that the release of cyt c itself may cause cell death even though caspases are not activated. It will be interesting to anchor cyt c in the mitochondria in a similar fashion as depicted in chapter 3 to assess the impact of cyt c release on mitochondrial function during cell death. If the release of cyt c itself indeed causes mitochondrial dysfunction in addition to caspase activation during cell death, one can envisage that anchoring both AIF and cyt c in the mitochondria can provide a powerful protection during neuronal cell death.

A proposal of using AIF in the treatment of neuronal injury

While the introduction of mitochondrially anchored AIF in the absence of endogenous AIF is not feasible in clinical settings, our studies nevertheless serve as a proof-of-principle strategy in which retaining AIF activity in the mitochondria in the absence of its pro-apoptotic activity can be used for inhibiting neuronal cell death in combination with other treatments such as caspase inhibitors. There are other ways to retain AIF's physiological activity in the mitochondria during cell death while selectively inhibit the pro-

apoptotic activity. When the cell is metabolically stressed, AIF undergoes conformational changes at the N terminal which exposures the calpain cleavage site for the release during cell death, and also at the C terminal that are responsible for interacting the proteins that are essential for its phyiological function (Churbanova & Sevrioukova, 2007). To retain AIF's activity in mitochondria, we need to stop its release from the mitochondria, as well as maintaining its activity in the mitochondria. To stop the release, we can use calpain inhibitors in combination with MOMP inhibitors such as pro-survival Bcl-2 peptides and MPT inhibitors such as cyclosporine A that inhibits CypD (Kroemer et al, 2007; Rasola & Bernardi, 2007; Zamzami et al, 2005). To retain the function of AIF, we can introduce the C terminal peptide into the mitochondria such that all the physiological interactions between AIF and the essential mitochondrial proteins are preserved. Since the redox domain is responsible for the conformational change at the C terminal, AIF C terminal on its own should be able to resist the conformational change, provided that the peptide retains the physiological conformation. Drugs that act as NAD(P)H analogs specific to AIF can also be used to "lock" the AIF conformation in the physiological state even if the endogenous NAD(P)H level is low during cell death. The combination of retaining AIF activity in mitochondrial with caspase inhibition (and cyt c retention in mitochondria) could stop the cell death machinery as well as preserving the mitochondrial function during neuronal cell death.

This study also underscore the importance of mitochondria as targets for the treatment of neuronal injury, since not only they are the hubs for different cell death pathways (Figure 1-1), but they are also essential for the well-being of a neuron. While the inhibition of cell death pathways can stop the neurons from dying, the preservation of

healthy mitochondria is more critical for the neurons to recover and regain their physiological function. Drugs that target the mitochondria to prevent their damage during cell death can be used in combination with various inhibitors of pro-apoptotic pathways during neuronal injury. This will ensure not only the survival of the neurons, but also the recovery and proper functioning of the surviving neurons when the cell death stimuli are removed. In this regard, more studies should be focused on the fate of the mitochondria during acute neuronal injury such as stroke, and the pathways that lead to the mitochondrial demise should be identified and used as a pharmacological target for the treatment of neuronal injury.

Chapter VI: Reference

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Many thanks,

Eric Cheung.

Eric Cheung

PhD candidate

Department of Cellular and Molecular Medicine

Appendix B Curriculum Vitae

Eric C. Cheung

EDUCATION

2002-present	Ph D candidate, Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada
1999-2001	B. Sc. Biology, University of British Columbia, Vancouver, BC, Canada
1995-1999	B. Sc. Biochemistry, University of British Columbia, Vancouver, BC, Canada

APPOINTMENT

2005-2007	VP Administration, Graduate Student Association of Cellular and Molecular Medicine and Neuroscience (CMM/NSC), University of Ottawa, ON, Canada

TEACHING EXPERIENCE

2002	Teaching assistant, Department of Chemistry, University of Ottawa, ON, Canada

RESEARCH KEYWORDS

Apoptosis, Excitotoxicity, acute neuronal injury, Caspase independent cell death, Mitochondrial dynamics, oxidative phosphorylation, Apoptosis Inducing Factor (AIF).

AWARDS AND HONOURS

2007	Brain Star Award, Canadian Institutes of Health Research (CIHR), Institute of Neurosciences, Mental Health and Addiction $1000
2006	University of Ottawa, Award of Excellence in Graduate Studies, Faculty of Medicine $500
2005-2008	CIHR Doctoral Research Award $21000/yr

2005-2009	University of Ottawa Excellence Scholarship
2005-2006	Ontario Graduate Scholarship (declined)
2005-2008	Heart and Stroke Foundation of Canada Doctoral Research Award (declined)
2006	The Fifth European Workshop on Cell Death Travel Award
2005	Gerry Taichman Award for Research Excellence, University of Ottawa $250
2005	Brain Star Award, CIHR Institute of Neurosciences, Mental Health and Addiction $1000
2005	National Research Forum for Young Investigators in Circulatory and Respiratory Health Travel Award
1995	British Columbia Provincial Scholarship for Undergraduates $1000

PUBLICATIONS

1. **Eric C. Cheung**, Nicholas Joza, Nancy A.E. Steenaart, Kelly A. McClellan, Margaret Neuspiel, Stephen McNamara, Jason G. MacLaurin, Peter Rippstein, David S. Park, Gordon C. Shore, Heidi M. McBride, Josef M. Penninger, and Ruth S. Slack (2006) Dissociating the dual roles of AIF in apoptosis and mitochondrial structure. **The EMBO Journal** 25, 4061–4073

2. **Eric C. Cheung**, Lysanne Melanson-Drapeau, Sean P. Cregan, Jacqueline L. Vanderluit, Kerry L. Ferguson, William C. McIntosh, David S. Park, Steffany A. L. Bennett, and Ruth S. Slack (2005) AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. **Journal of Neuroscience** 25(6):1324-34.

3. **Eric C. Cheung**, Heidi M McBride, and Ruth S. Slack (2007) Mitochondrial dynamics and the regulation of neuronal cell death. **Apoptosis** 12(5):979-92.

4. **Eric C. Cheung** and Ruth S. Slack (2004) Emerging role for ERK as a key regulator of neuronal apoptosis. **Science's STKE** Sep 14;2004(251):PE45.

5. Arezu Jahani-Asl, **Eric C. Cheung**, Margaret Neuspiel, Jason G. MacLaurin, Andre Fortin, David S Park, Heidi McBride, Ruth S. Slack (2007) Mitofusin 2 protects cerebellar granule neurons against injury induced cell death. **Journal of Biological Chemistry** 282(33):23788-98

6. Nicole A. Arbour, Grace O. Iyirhiaro, **Eric C. Cheung**, Jason G. MacLaurin, Steven M. Callaghan, David S. Park, and Ruth S. Slack (2007) Mcl-1 protects against neuronal cell death through interactions with BH3-only proteins. *Submitted to* **Journal of Neuroscience**.

7. Sean P. Cregan, Nicole A. Arbour, Jason G. MacLaurin, Steven M. Callaghan, Andre Fortin, **Eric C. Cheung**, Daniel S. Guberman, David S. Park and Ruth S. Slack (2004) P53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. **Journal of Neuroscience** 24(44):10003-12.

INVITED PLATFORM PRESENTATIONS

2007 "The role of apoptosis-inducing factor (AIF) in regulating mitochondrial function in neurons."
September 2007, Cold Spring Harbour Cell Death Meeting, Cold Spring Harbour, NY, USA

2007 "The vital and lethal roles of the mitochondrial protein apoptosis-inducing factor (AIF) in neurons."
March 6, 2007, University of Ottawa, Ottawa, ON, Canada.

2006 "The dual roles of apoptosis inducing factor (AIF): vital and lethal."
Nov 30, 2006, The 6th Annual OHRI Research Day, Ottawa, ON, Canada

2006 "Dissociating the dual roles of AIF in maintaining mitochondrial structure and apoptosis in neurons"
Oct 18, 2006, Society for Neuroscience meeting, Atlanta, Georgia, USA.

2006 "Dssociating the dual roles of AIF in apoptosis and mitochondrial structure"
May 31, 2006, The Fifth European Workshop on Cell Death, Rolduc, Krekrade, the Netherlands.

2005 "Cell death in nervous system and its implication in stroke therapy"
Feb 12, 2005, Continuing Education Seminar Series, Animal Care and Veterinary Service, University of Ottawa, Ottawa, ON, Canada.

ABSTRACTS

1. **Eric C. C. Cheung**, Nicholas Joza, Margaret Neuspiel, Nancy Steenaart, Peter Rippstein, Jason G MacLaurin, David S. Park, Gordon C. Shore, Heidi M. McBride, Joseph Penninger, and Ruth S. Slack A novel role of AIF in maintaining mitochondrial cristae morphology and neuronal survival. Gordon Research Conference: Cell Death. Sept. 10-15, 2006, Big Sky, Montana, USA.

2. **Eric C. C. Cheung**, Nicholas Joza, Margaret Neuspiel, Nancy Steenaart, Peter Rippstein, Jason G MacLaurin, David S. Park, Gordon C. Shore, Heidi M. McBride, Joseph Penninger, and Ruth S. Slack A novel role of AIF in maintaining mitochondrial cristae morphology and neuronal survival. FEBS-IUBMB Workshop: Mitochondrial Dynamics in Cell Life and Death. August 27-30, 2005, Venetian Institute of Molecular Medicine, Padova, Italy.

3. **Eric C. C. Cheung**, Nicholas Joza, Margaret Neuspiel, Nancy Steenaart, Peter Rippstein, Jason G MacLaurin, David S. Park, Gordon C. Shore, Heidi M. McBride, Joseph Penninger, and Ruth S. Slack A novel role of AIF in maintaining mitochondrial cristae morphology and neuronal survival. OHRI Research Day, 2005, Ottawa, ON, Canada

4. **Eric C. C. Cheung**, Lysanne Melanson-Drapeau, Sean P. Cregan, Jacqueline L. Vanderluit, Kerry L. Ferguson, William C. McIntosh, David S. Park, Steffany A. L. Bennett, and Ruth S. Slack AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. National Research Forum for Young Investigators in Circulatory and Respiratory Health. Apr. 28-May 1, 2005, Winnipeg, MB, Canada.

5. **Eric C. C. Cheung**, Lysanne Melanson-Drapeau, Sean P. Cregan, Jacqueline L. Vanderluit, Kerry L. Ferguson, William C. McIntosh, David S. Park, Steffany A. L. Bennett, and Ruth S. Slack AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. Keystone Symposia, Cellular Senescence and Cell Death. Mar. 3-9, 2005, Keystone, CO, U.S.A.

6. **Eric C. C. Cheung**, Lysanne Melanson-Drapeau, Sean P. Cregan, Jacqueline L. Vanderluit, Kerry L. Ferguson, William C. McIntosh, David S. Park, Steffany A. L. Bennett, and Ruth S. Slack AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. Society for Neuroscience meeting, Oct. 23-27, 2004, San Diego, CA, U.S.A.

7. **Eric. C. C. Cheung**, Lysanne Melanson-Drapeau, Sean P. Cregan, Jacqueline L. Vanderluit, Kerry L. Ferguson, William C. McIntosh, David S. Park, Steffany A. L. Bennett, and Ruth S. Slack AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. OHRI Research Day, 2004, Ottawa, ON, Canada

8. **Eric. C. C. Cheung**, Lysanne Melanson-Drapeau, Sean P. Cregan, Jacqueline L. Vanderluit, Kerry L. Ferguson, William C. McIntosh, David S. Park, Steffany A. L. Bennett, and Ruth S. Slack AIF is a key factor in neuronal cell death propagated by Bax-dependent and Bax-independent mechanisms. Cold Spring Harbour Laboratory, Mouse Molecular Genetics Meeting. Sept. 1-5, 2004, Cold Spring Harbour, NY, U.S.A.

9. **Eric C.C. Cheung**, Sean P. Cregan, Carl W. McIntosh, Steven M. Callaghan, David S. Park, and Ruth S. Slack The role of apoptosis inducing factor (AIF) in neuronal cell death. OHRI Research Day, 2003, Ottawa, ON, Canada

10. **Eric C.C. Cheung**, Sean P. Cregan, Carl W. McIntosh, Steven M. Callaghan, David S. Park, and Ruth S. Slack The role of apoptosis inducing factor (AIF) in neuronal cell death. Society for Neuroscience meeting. Nov. 8-12, 2003, New Orleans, LA, U.S.A.