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Apoptosis-Inducing Factor (AIF) Forms a Complex with Optic Atrophy 1 (Opa1) to Maintain Mitochondrial Structure and Function

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Apoptosis-Inducing Factor (AIF) forms a complex with Optic Atrophy 1 (Opa1) to maintain mitochondrial structure and function

By

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STATEMENT OF CONTRIBUTION

A manuscript titled "AIF interacts with mitochondrial GTPase Opa1 to link mitochondrial structure to apoptosis signaling", of which I am co-first author and that includes data reported here, is in preparation for re-submission. Also, data presented here appear in the manuscript "Regulation of mitochondrial dynamics following deregulation of calcium through NMDA receptors", in preparation for submission, of which I am second author. Experiment in Figure 12 A was performed in collaboration with E. Cheung.

ABSTRACT

The mitochondrial protein Apoptosis-inducing factor (AIF) is a redox active flavoprotein that has a dual role in the regulation of cell death and survival. We have previously identified a novel role for AIF in mitochondrial structure. Here, we examine the mechanism by which AIF controls mitochondrial structure and metabolism and found that AIF deficiency results in mitochondrial fragmentation, cristae malformation and a defect in oxidative phosphorylation. Mitochondrial AIF is essential for organelle fusion as the fission/fusion proteins Mfn1 and dnDrp1 fail to rescue the structural defect seen in AIF deficiency. In contrast, upregulation of Opa1 in AIF deficient neurons restores mitochondrial structure, metabolism and cellular survival. We show that AIF functions upstream of Opa1 because increased mitochondrial AIF cannot rescue neuronal cell death induced by Opa1 deficiency. AIF-deficient neurons display reduced Opa1 oligomerization resulting in impaired cristae formation. Furthermore, we show that AIF interacts with Opa1 to maintain Opa1 oligomerization. This interaction is critical during apoptosis signaling, as Opa1 oligomerization can be preserved by expression of mitochondrial AIF. Apart from AIF, we also identified novel factors that affect the degree of Opa1 oligomerization. Indeed, Opa1 oligomers seem to be modulated by cell metabolism according to levels of NADH and NAD^+. These results identify a novel functional interaction between AIF, Opa1 and cell metabolism and links the control of mitochondrial structure with apoptosis signaling and the metabolic state of the cell.

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LIST OF ABBREVIATIONS

AA	Amino acids
AIF	Apoptosis Inducing Factor
ADP	Adenosine Diphosphate
Apaf-1	Apoptosis-protease activating factor 1
ATP	Adenosine Triphosphate
Ca2+	Calcium ion
CCCP	Carbonylcyanide-3-chlorophenylhydrazone
Cyt C	Cytochrome C
DNA	Deoxyribonucleic acid
dnDrp1	Dominant negative Dynamin-related protein 1
Drp1	Dynamin-related protein 1
DSS	Disuccinimidyl suberate
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
ER	Endoplasmic reticulum
ETC	Electron Transport Chain
FAD	Flavin adenine dinucleotide
GFP	Green fluorescent protein
GTP	Guanosine Triphosphate
hFis1	Fission 1 (mitochondrial outer membrane) human homolog
Hq	Harlequin
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MLS	Mitochondrial localization sequence
MOMP	Mitochondrial outer membrane permeabilization
MPP	Mitochondrial processing peptidase
MPTP	Membrane permeability transition pore
mRNA	Messenger Ribonucleic acid
mtDNA	mitochondrial Deoxyribonucleic acid
NAC	N-acetyl cysteine
NAD+	Nicotinamide adenine dinucleotide

NADH	Reduced Nicotinamide adenine dinucleotide
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
$O_2^{\cdot -}$	Superoxide
OM	Outer membrane
Opa1	Optic Atrophy 1
Parp-1	Poly-ADP ribose polymerase-1
ROS	Reactive Oxygen Species
SH3	SRC homology 3 domain
siRNA	Small interfering RNA
Sirt-3,-4,-5	Sirtuins 3, 4 and 5
WW	Simplest β-sheet structure

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First and foremost, I would like to thank Dr Ruth Slack for her intellectual and moral support throughout this project. Thank you for giving me the opportunity to join your laboratory and to go to great conferences. A special thanks to Dr Eric Cheung, former lab member, with whom it was extremely fulfilling to collaborate. He helped me a lot and trusted me to take over his project. Also, I want to thank Dr Melissa Kelly and Luis Rivero-Lopez for reviewing my thesis. It was a hard job, since I am not a good writer in English, and I am extremely grateful for that. I want to acknowledge Dr Marc Germain, our senior post-doctoral fellow, for all the scientific discussions and his help with some experiments. A special thanks to my committee members, Dr Heidi McBride and Dr Rob Screaton, for their critical opinion on my work and their helpful advice. Merci à mon amoureux Luis pour m'avoir encouragée pendant ces deux années et pour m'avoir supportée pendant les moments plus difficiles. Finally, a special thanks to Melissa and Angela for helping me with my mouse colony and to all the members of the Slack lab for moral support and great friendship throughout those years.

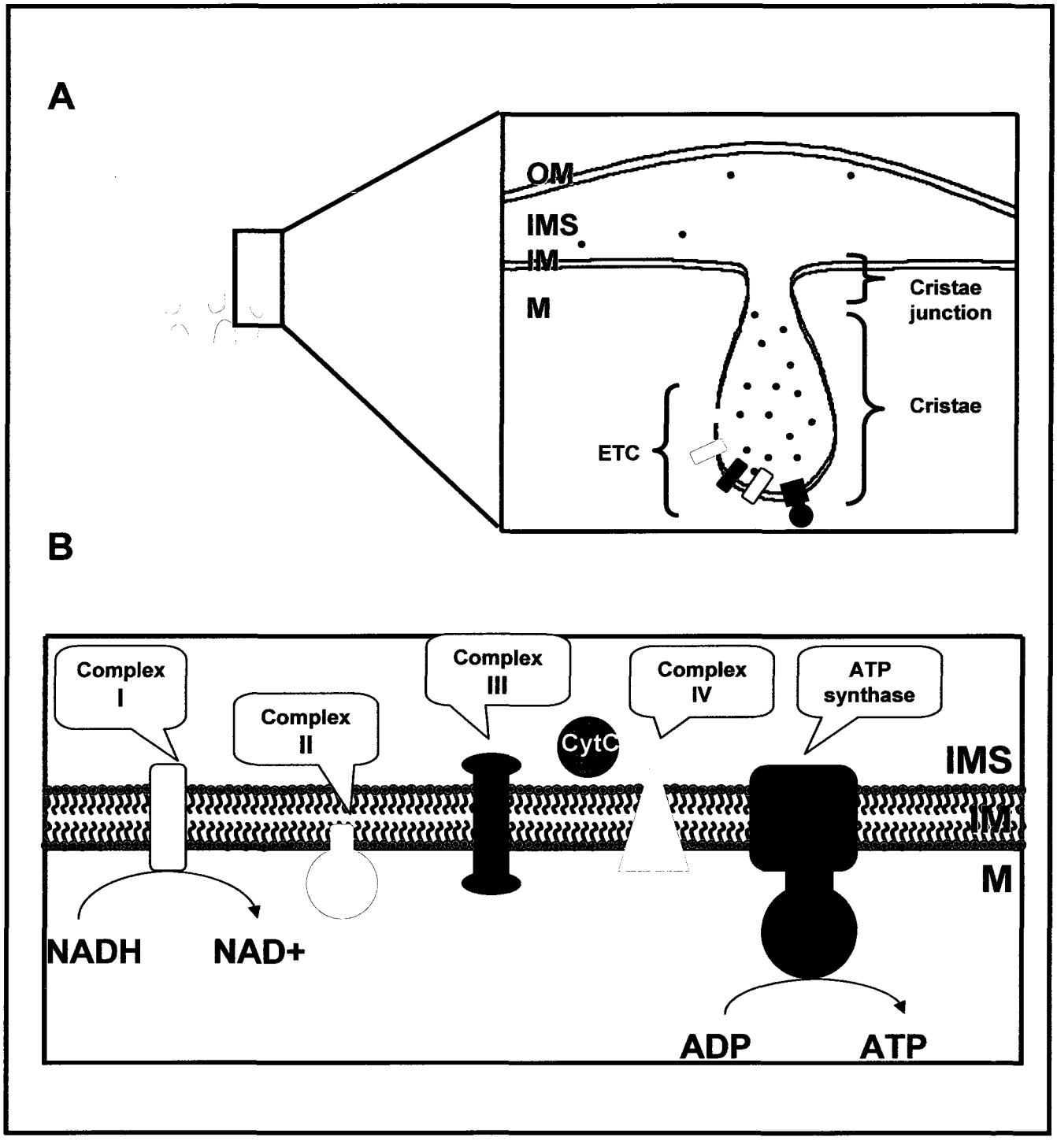
INTRODUCTION

1. The Mitochondria: at the centre life and death

Mitochondria are rod-shaped organelles common to all eukaryotes. They are composed an outer membrane and an inner membrane that separates the inter membrane space from the matrix (Figure 1A). Studies on isolated mitochondria during the mid 20th century uncovered one of its fundamental functions: the production of adenosine triphosphate (ATP), the major energy source of the cell. ATP is produced via the electron transport chain (ETC), a series of supra-molecular complexes embedded in the inner membrane (Figure 1B). Throughout the years, mitochondria were also found to be implicated in many other cellular activities such as cell growth, cell cycle regulation and cellular differentiation (Mandal et al., 2005; Owusu-Ansah et al., 2008). Among others, the discovery of its pivotal role in apoptotic signalling established the mitochondrion as an essential and bi-functional organelle that has the capacity to sustain and end life. First thought to be rather isolated organelles, mitochondria are part of a dynamic network where individual mitochondria constantly divide and fuse with other mitochondria (Chan, 2006b). The study of mitochondrial structural dynamics has recently generated heightened interest, now that we more clearly understand its impact on mitochondrial energy production, cell survival and initiation of cell death.

In this thesis, I will describe the mechanism by which the Apoptosis Inducing Factor protein (AIF) regulates mitochondrial structure, and the importance for proper mitochondrial function and cellular survival. In the following sections, I will describe the central role of the mitochondria in apoptotic signalling and how the mitochondrial protein AIF is implicated in the apoptotic cascade. I will then give further details on the dual role

Figure 1. The mitochondrion and the Electron Transport Chain. (A) Mitochondria is composed of two compartments, the matrix (M) and the intermembrane space (IMS) separated by two lipid bilayers; the outer membrane (OM) and the inner membrane (IM). Invagination of the IM forms a structure called cristae, where the complexes of the Electron Transport Chain (ETC) are localized. The cristae connect to the IM by through the cristae junction. **(B)** The ETC is composed of five complexes imbedded in the IM. Complex V, also called ATP synthase, catalyzes the production of ATP.


A
OM
IMS
IM
M
Cristae junction
Cristae
ETC
B
Complex I
Complex II
Complex III
Complex IV
ATP synthase
CytC
IMS
IM
M
NADH
NAD+
ADP
ATP

of AIF as a pro-apoptotic and pro-survival protein and explain how AIF might affect mitochondrial structure by functionally interacting with the pro-fusion protein Opa1. In order to better understand this novel role of AIF in regulating mitochondrial structure, I will also review the mitochondrial fission and fusion pathways and their implication in metabolism.

1.2. The mechanisms of mitochondrial apoptosis

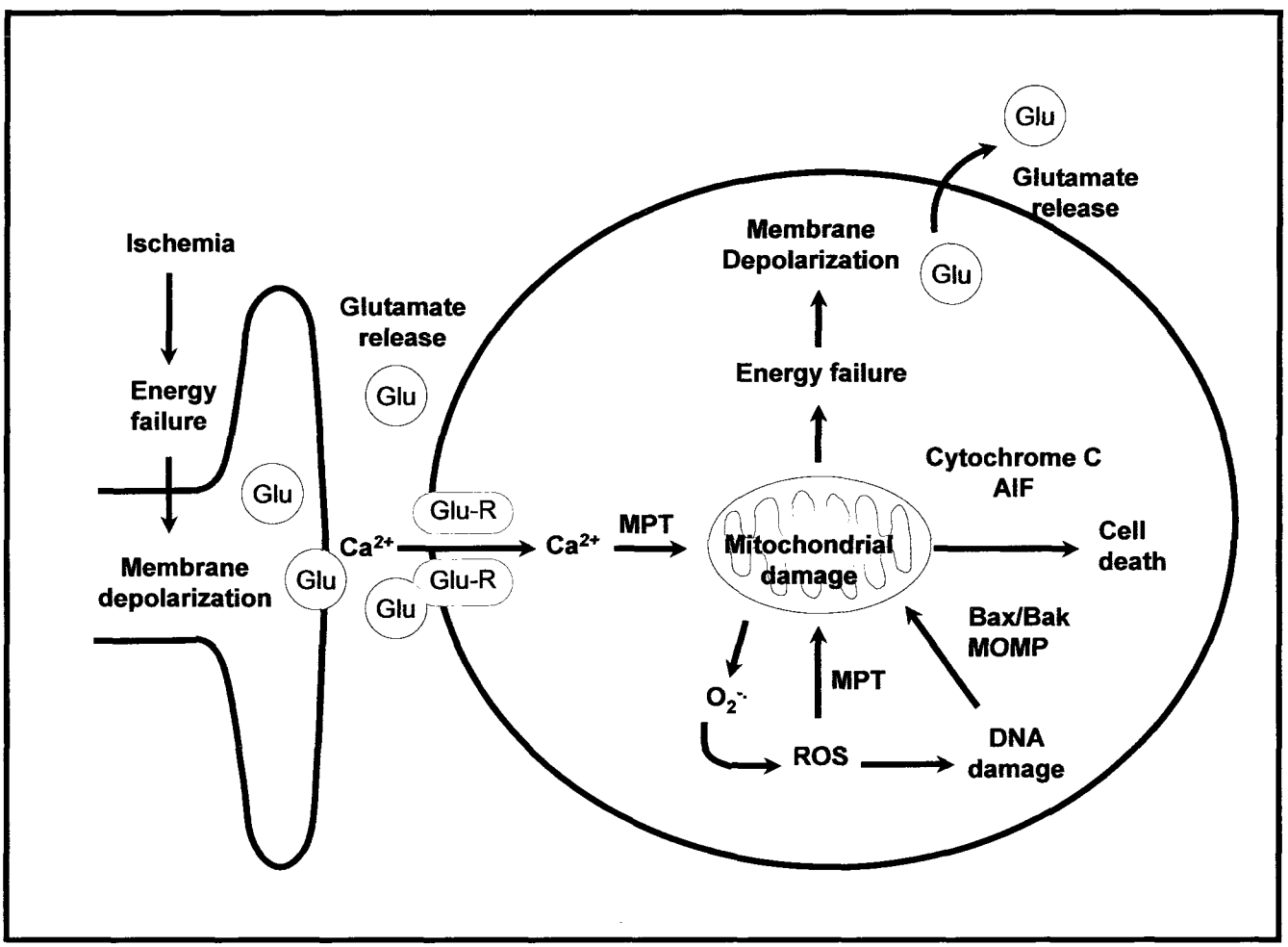
Throughout life, from embryonic development to death, a phenomenal amount of cells will die by apoptosis. Apoptosis is characterized by specific morphological features that differentiate it from other forms of cell death such as necrosis and autophagy (Kerr et al., 1972). The fundamental biochemical and morphological hallmarks of apoptosis are nuclear condensation, DNA fragmentation and activation of pro-apoptotic proteins (reviewed in Kroemer et al., 2009). Apoptotic cell death can be initiated through a wide range of signals which can originate from outside (extrinsic) or inside (intrinsic) the cell. In general, the extrinsic cell death pathway signals through death receptors, which are present at the cell surface. When those receptors are activated by binding of their respective ligand, they initiate an apoptotic cascade and terminate life of the cell (reviewed in Wallach et al., 2008). The intrinsic pathway of apoptosis is initiated after a major cellular stress that greatly damages the cell. This internal damage acts as the trigger activating the apoptotic pathway. Even though the two pathways are independent, both can use the contribution of the mitochondria to complete apoptosis and ultimately converge on caspase activation. In the next sections, I will focus on the role of the

mitochondria as central relaying stations in apoptotic signalling following an intrinsic cell death insult such as excitotoxicity.

1.2.1. Excitotoxic-induced neuronal cell death

Neuronal excitotoxicity is caused by over-stimulation of glutamate receptors in the nervous system following an acute neuronal injury (Figure 2) (Arundine and Tymianski, 2004; Nicholls and Budd, 1998). Interruption of blood flow during ischemia results in reduced ATP production, necessary for the neuron to maintain its membrane potential. Loss of membrane potential causes massive release of glutamate in the synaptic cleft, causing over-stimulation of the glutamate receptors on the post-synaptic neuron. Activation of glutamate receptors induces an influx of Ca^{2+} which will activate downstream pathways ultimately leading to cell death (Lipton, 1999). Ca^{2+} directly affects the mitochondria. After a major calcium influx, the mitochondria and endoplasmic reticulum (ER) attempt to re-equilibrate cytosolic levels of Ca^{2+} by absorbing the excess ion. Increased mitochondrial Ca^{2+} can affect the electron transport chain by disrupting membrane potential and the flow of electrons, causing increased production of reactive oxygen species (ROS) (Dirnagl et al., 1999; Sattler and Tymianski, 2000). Elevated levels of ROS can be detrimental to the mitochondria as well as the cell itself (Giulivi et al., 1995; Abramov et al., 2007). Furthermore, excessive amounts of Ca^{2+} induce formation of membrane permeability transition pore (MPTP) and mitochondrial outer-membrane permeabilization (MOMP) (Bernardi and Rasola, 2007; Green and Kroemer, 2004), leading to the release of cytochrome C and AIF, resulting in caspase-dependent and –independent apoptotic signalling respectively.

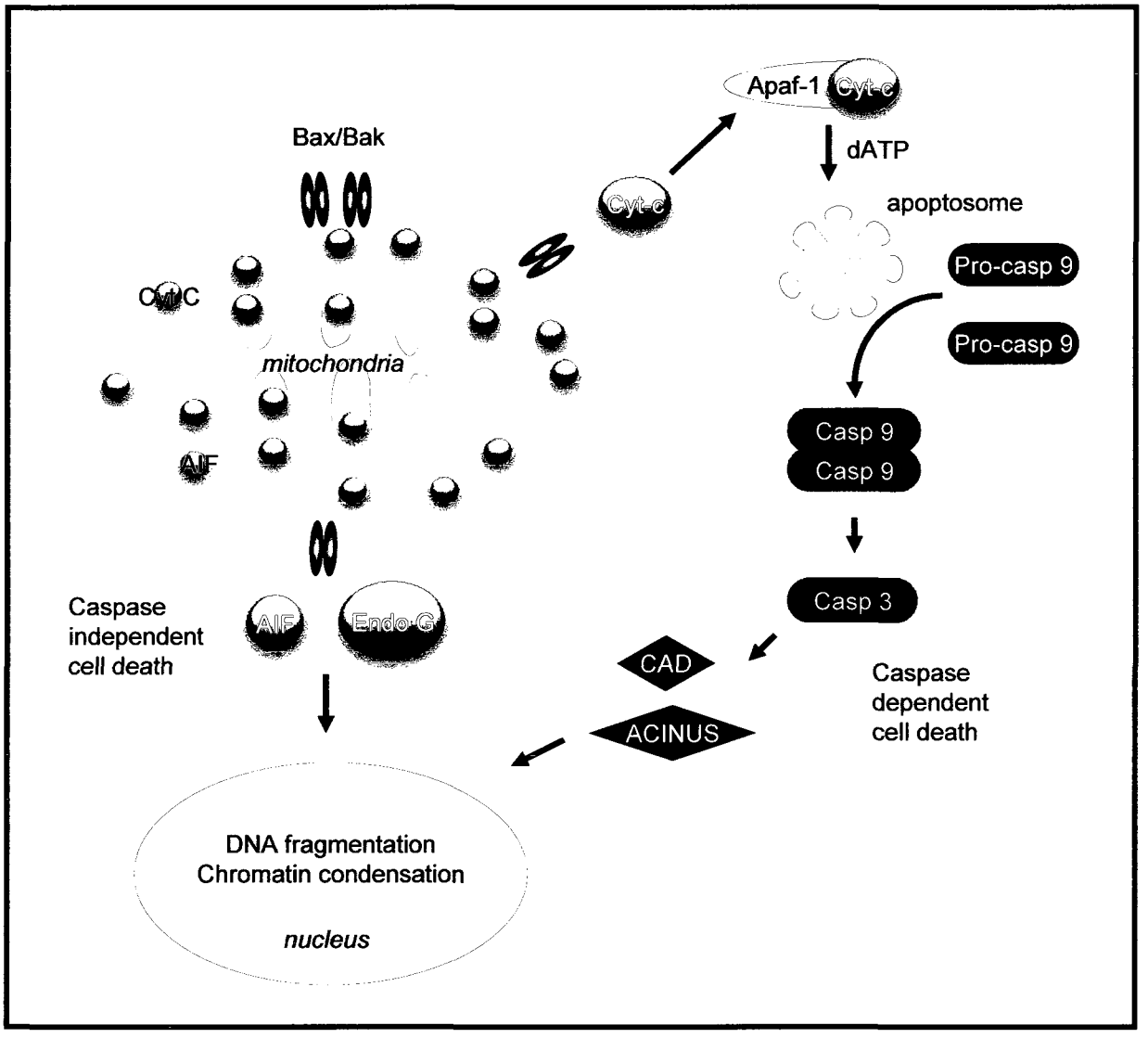
Figure 2. Excitotoxicity. After ischemia, the activation of glutamate receptors induces an influx of Ca^{2+} which will activate downstream pathway ultimately leading to cell death. Ca^{2+} overload directly affect the mitochondria, leading to AIF release and induction of caspase-independent cell death. Secondary effects of mitochondrial damage caused by the excess of Ca^{2+} include production of superoxides, membrane permeability transition (MPT) and DNA damage, further damaging the cell.


Ischemia
Energy failure
Membrane depolarization
Glu
Glutamate release
Glu
Glu-R
Glu
Glu-R
Glu
Ca2+
Ca2+
MPT
Mitochondrial damage
Membrane Depolarization
Glu
Glu
Glutamate release
Energy failure
Cytochrome C
AIF
Cell death
Bax/Bak
MOMP
MPT
O2-
ROS
DNA damage

1.2.2. Caspase-dependent mitochondrial apoptosis

Mitochondrial apoptosis is controlled by the Bcl-2 family of proteins (reviewed in Brunelle and Letai, 2009). Either pro- or anti-apoptotic, they are initiators of the mitochondrial apoptotic cascade by controlling mitochondrial outer membrane permeabilization (MOMP) (Sharpe et al., 2004). Following a death insult, the BH3 only proteins, a subset of the Bcl-2 family, become activated and bind the anti-apoptotic Bcl-2 members. Eventually, this will cause activation and oligomerization of Bax and Bak at the outer membrane of the mitochondria to create pores allowing release of mitochondrial resident proteins such as cytochrome C (Figure 3) (Newmeyer and Ferguson-Miller, 2003). Once released, cytochrome C initiates caspase-mediated apoptosis by activating the adaptor molecule apoptosis-protease activating factor 1 (Apaf-1). Together, Apaf-1 and cytochrome C form a structure called the apoptosome that recruits and activates pro-caspase 9 (Boatright et al., 2003), which in turn will cleave and activate executioner caspases such as caspase 3 and 7. Once activated, the executioner caspases can turn on an array of apoptotic proteins such as nucleases, which result in chromatin condensation, DNA degradation and culminate in cell death (Riedl and Salvesen, 2007). Although caspases are very efficient death effectors, they are not the sole pathway through which the cell can execute apoptosis. In the next section, I will describe the caspase-independent pathway of apoptosis and one of its central participants, AIF.

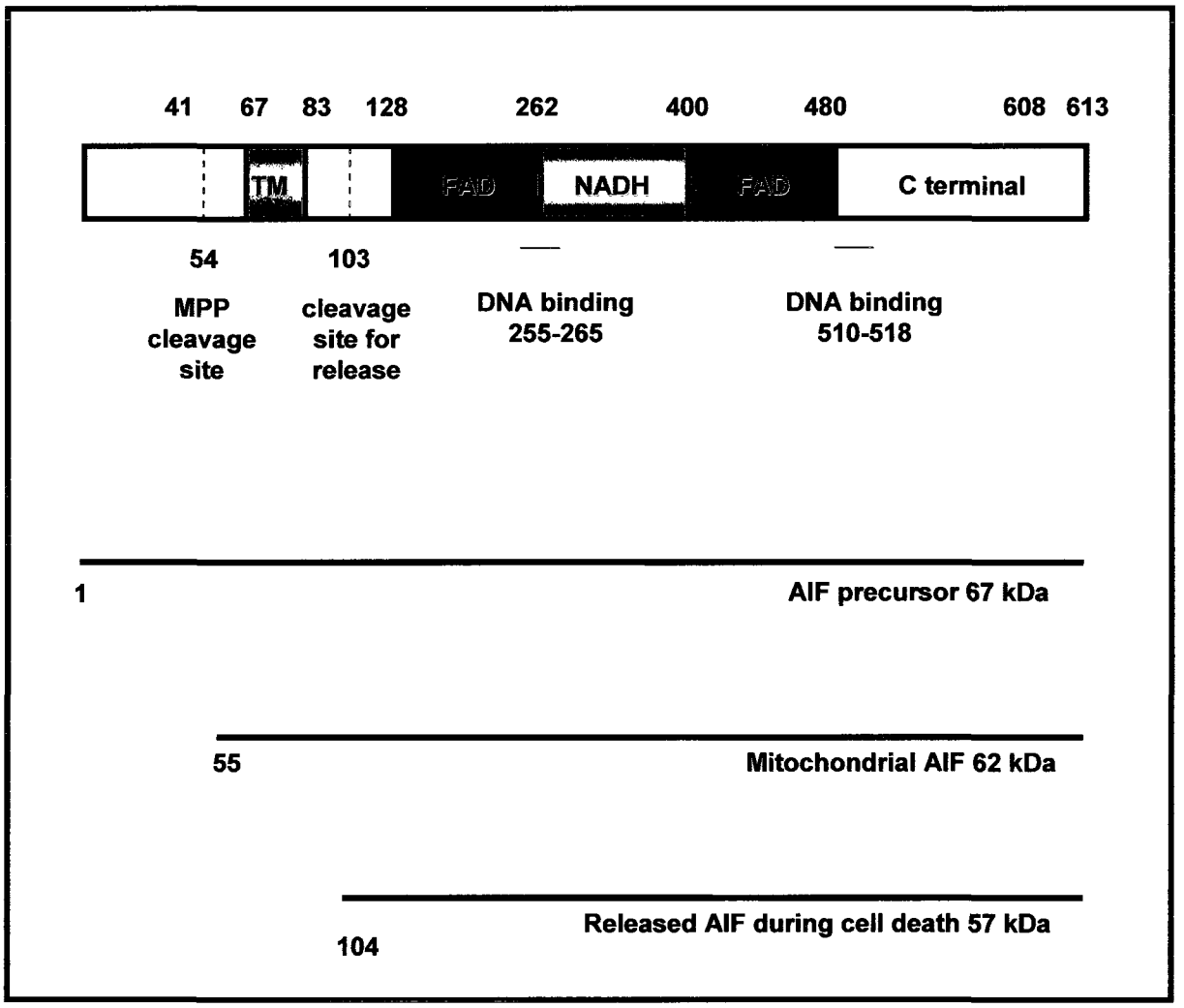
Figure 3. The release of mitochondrial pro-apoptotic proteins. After mitochondrial outer membrane permeabilization (MOMP), cyt C is released and activates the caspase pathway via Apaf-1. The activation of caspases activates proteins such as Cad and Acinus (DNases), that are responsible for DNA fragmentation and chromatin condensation. AIF and Endonuclease G (Endo G) are the caspase-independent apoptotic proteins that are also released from the mitochondria and translocate to the nucleus. They can also induce DNA fragmentation and chromatin condensation.


Apaf-1
Cyt-c
dATP
apoptosome
Pro-casp 9
Pro-casp 9
Casp 9
Casp 9
Casp 3
Bax/Bak
Cyt-c
Cyt C
mitochondria
AIF
Caspase
independent
cell death
AIF
Endo G
CAD
ACINUS
Caspase
dependent
cell death
DNA fragmentation
Chromatin condensation
nucleus

1.3. Apoptosis Inducing Factor

Following permeabilization of the mitochondrial outer-membrane, one of the proteins released from the mitochondrial inter-membrane space is Apoptosis Inducing Factor (AIF). Once in the cytosol, AIF has been found to translocate to the nucleus where it induces large scale DNA fragmentation and cell death (Cregan et al., 2004; Daugas et al., 2000a; Daugas et al., 2000b; Lorenzo et al., 1999). AIF is a nuclear encoded flavoprotein composed of a mitochondrial localization sequence (MLS), a transmembrane domain (TM) as well as two FAD- and one NADH-binding domains (Figure 4). In its C-terminal portion, AIF contains a PEST sequence, which could be responsible for protein turnover and a poly-proline-2 helix motif (PPII) (Mate et al., 2002). The latter suggests that AIF could potentially interact with proteins containing SH3 domains and WW sequences (Kay et al., 2000). AIF is translated as a 67 kDa precursor protein targeted to the mitochondria by its mitochondrial localization sequence (MLS) present at the N-terminus. After mitochondrial import, AIF is inserted in the mitochondrial inner membrane facing the inter membrane space and mitochondrial processing peptidases (MPP) cleave the MLS, forming the 62 kDa isoform. AIF has a weak NADH oxidoreductase activity, which can be separated from its apoptotic function (Daugas et al., 2000a; Loeffler et al., 2001; Miramar et al., 2001). During apoptosis, AIF is detached from the mitochondrial inner membrane by proteolytic processing (Arnoult et al., 2002; Otera et al., 2005; Munoz-Pinedo et al., 2006) and the 57 kDa form of the protein is released to the cytosol following permeabilization of the outer-membrane. Calpains have recently been implicated in the mechanism of AIF release during apoptosis.

Figure 4. AIF domains. AIF consists of MLS (mitochondrial localization signal), putative transmembrane (TM) domain, FAD binding domains and NADH binding domain. After mitochondrial import, AIF precursor (67kDa) is inserted in the inner-membrane and the mitochondrial localization sequence (MLS) is cleaved by mitochondrial membrane peptidases (MMP), forming the 62kDa isoform. During cell death, mitochondrial AIF may be cleaved at a.a. 103 and the processed form (57kDa) is then released from the mitochondria.


41
67
83
128
262
400
480
608
613
TM
FAD
NADH
FAD
C terminal
54
103
MPP cleavage site
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

This family of proteins are non-caspase cysteine proteases activated by Ca^{2+} and mostly located in the cytoplasm (Chan and Mattson, 1999; Takano et al., 2005; Lankiewicz et al., 2000). More specifically, Calpain I has been suggested to induce AIF cleavage between residues 101-102 in isolated mitochondria incubated with activated pro-apoptotic form of Bid (t-Bid) and in an injury model of oxygen and glucose deprivation (Polster et al., 2005; Cao et al., 2007). Other proteins, such as PARP-1, have been implicated in AIF release (Komjati et al., 2004; Yu et al., 2002). Although the exact mechanism of this release is not clear, it is probable that more than one protein is involved in AIF cleavage, since the kinetics of AIF release vary depending on the cell type and apoptotic stimulus (Munoz-Pinedo et al., 2006).

1.3.1. Pro-apoptotic function of AIF

Subsequent to the induction of apoptosis, mitochondria release several proteins to the cytoplasm due to disruption of its outer membrane. Upon mitochondrial release, AIF translocates to the nucleus, where it induces large scale DNA fragmentation and chromatin condensation (Cregan et al., 2004; Krantic et al., 2007). Even though AIF can bind directly to DNA in a sequence-independent manner, it alone can not induce DNA fragmentation and chromatin condensation (Mate et al., 2002; Ye et al., 2002; Vahsen et al., 2006; Susin et al., 1999). Therefore, it requires other factors to carry out his apoptogenic activity in the nucleus. CypA, a peptidyl-prolyl *cis–trans* isomerase (chaperone) localized to the cytosol and the nucleus, has been previously shown to interact with AIF in the nucleus under apoptotic conditions (Cande et al., 2004). Mutagenesis of the CypA-binding domain on AIF significantly reduces the apoptogenic

activity of the protein, demonstrating a potential cooperation between AIF and CypA to complete apoptosis once it binds to DNA. Another potential partner for AIF's apoptogenic activity in the nucleus is EndoG, a mitochondrial protein released during apoptosis that possesses caspase-independent DNase activity (Li et al., 2001; Parrish et al., 2001; Widlak et al., 2001). In *C. elegans*, WAH-1 and CSP-6 (homologues of AIF and EndoG respectively) physically interact to promote DNA fragmentation (Wang et al., 2002). Although the exact mechanism of AIF apoptogenic activity remains unclear, it is evident that AIF has a pro-apoptotic function in the nucleus. Studies in Harlequin (Hq) mice, which possess a mutation in intron 1 of AIF gene such that its expression is drastically reduced to only 20% (Klein et al., 2002), show a protection against cell death after oxygen and glucose deprivation as well as excitotoxicity *in vitro* and *in vivo* (Cheung et al., 2005; Wang et al., 2004). This supports a role for AIF as a pro-apoptotic factor. Furthermore, an *in vitro* study on cortical neurons performed by our group has shown that a major part of AIF's pro-apoptotic function is mediated in the nucleus. Expression of AIF tagged with a nuclear export sequence (NES) in AIF and Apaf-1 null neurons, inhibiting AIF from remaining inside the nucleus, fails to mediate caspase-independent cell death (Cheung et al., 2006). Hence, AIF functions as a pro-apoptotic factor mediating caspase-independent apoptosis after its translocation to the cell nucleus by causing DNA fragmentation and chromatin condensation through a potential interaction with CypA and EndoG.

1.3.2. Pro-survival function of AIF

The physiological significance of AIF is highlighted in various mouse models; whole body knockout of the AIF gene, for example, is lethal for the developing embryo, which die at E12.5 (Joza et al., 2005; Brown et al., 2006). Telencephalon-specific loss of AIF is also embryonic lethal, due to elevated apoptosis and drastic cell loss (Cheung et al., 2006). Harlequin (Hq) mice, which exhibit approximately 80% knock-down of AIF levels due to a mutation in intron 1, further validate that AIF is more than a pro-apoptotic protein. The dramatic reduction of AIF levels in Hq mice results in cerebellar and retinal degradation by three months of age (Klein et al., 2002; El Ghouzzi et al., 2007). Interestingly, Hq mice are also more sensitive to oxidative stress and have defective oxidative phosphorylation due to decreased levels of complex I of the ETC (Klein et al., 2002; Cheung et al., 2006; Vahsen et al., 2004). Some studies have also correlated the loss of AIF to an increase in ROS production (Klein et al., 2002; Apostolova et al., 2006; van Empel et al., 2005), reinforcing the importance of AIF in maintenance of cell survival through its physiological role inside the mitochondria. Our group has shown that *in vitro*, AIF-deficient neurons have reduced survival compared to wild-type neurons due to severe mitochondrial defects, such as fragmented mitochondria, dilated cristae and reduced expression of complex I (Cheung et al., 2006). All the aforementioned models suggest that AIF is a multi-functional protein that can work differently depending on the state of the cell. Under steady-state conditions, AIF is an inner membrane mitochondrial protein essential for proper mitochondrial structure and function. Under apoptotic conditions, nuclear AIF initiates caspase-independent apoptosis. However, the exact mechanism by which AIF is maintaining neuronal survival still needs to be determined. A

striking defect in AIF-deficient neurons is the fragmented state of the mitochondria and the aberrant cristae structure (Cheung et al. 2006). Such abnormalities could suggest a novel role for AIF in the regulation of mitochondrial structure. In the following section, I will explain how mitochondrial structure is regulated and how this can impact on several functions of the organelle.

1.4. Mitochondrial dynamics

Mitochondria are dynamic organelles that move around in the cell, constantly dividing and fusing with other mitochondria. Over the last decade, the study of mitochondrial dynamics has generated much interest and we now know the impact it has on the cellular organism. There is a delicate balance between mitochondrial fission and fusion and disruption of this balance can be detrimental to the cell. For example, reducing the amount of fusion events by down-regulating members of the fusion machinery can lead to a decrease in metabolic activity and sensitization of the cell to apoptotic stimuli (Suen et al., 2008; Chen et al., 2005(b)). On the other hand, reducing the amount of fission events can limit the movement of the mitochondria within the cell, which can be deleterious for neuronal cells in particular since proper localization of the mitochondria are critical for dendritic development (Li et al., 2004). Neuronal cells are unique cell type in that their long axons can reach up to 1.5 meters in the human adult. In this setting, mitochondrial fission is an asset for efficient trafficking of mitochondria to areas with higher energy demands (Li et al., 2004; Chang et al., 2006).

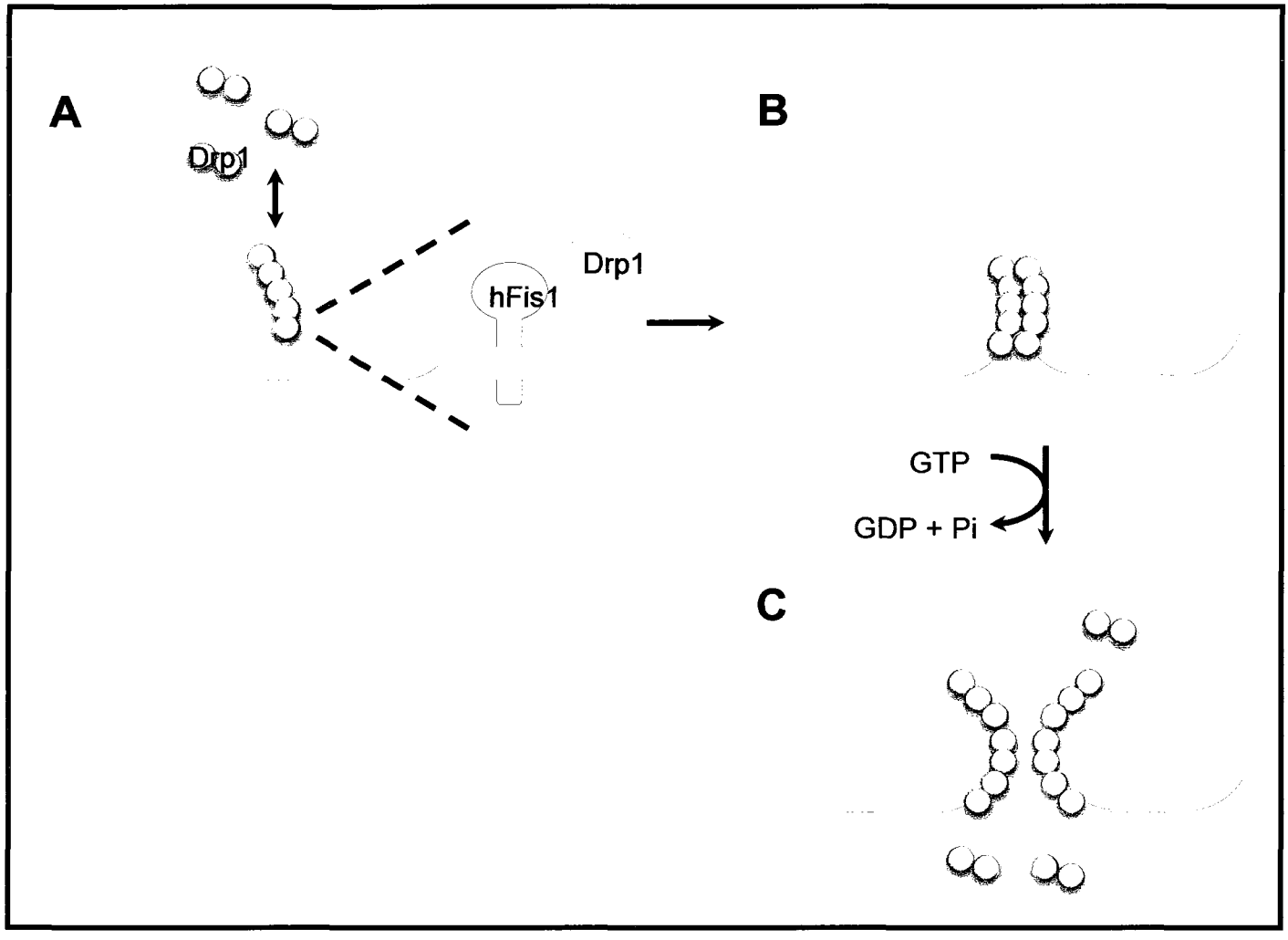
Many mitochondrial activities can be influenced by mitochondrial dynamics, including apoptotic signalling. Increased mitochondrial fission correlates with increased

sensitization to apoptosis and facilitation of cytochrome C release (Frank et al., 2001; Lee et al., 2004). Furthermore, loss of the pro-fission protein Drp1 has a negative impact on the progression of apoptosis, blocking both developmental- and age-induced cell death (Soubannier and McBride, 2009). On the contrary, various studies have demonstrated that during apoptosis mitochondrial fusion is inhibited (Karbowski et al., 2004). Enhancing mitochondrial fusion by expressing pro-fusion proteins or by blocking fission can delay the death of the cell (Jahani-Asl et al., 2007; Estaquier and Arnoult, 2007). In this section, I will describe the major proteins implicated in the regulation of mitochondrial fission and fusion and how they can affect mitochondrial structure.

1.4.1. Mitochondrial fission

Mitochondrial fission is the separation of a long mitochondrion into two smaller subunits. This event, in balance with fusion events, regulates the shape and size of the mitochondrial network. Even though mitochondrial fission has been associated with apoptosis, it is an important event implicated in vital cellular activity such as cell division, calcium regulation and development (McBride et al., 2006; Chen and Chan, 2005). In mammals, the key proteins mediating mitochondrial fission are hFis1 and Drp1 (Figure 5) (Hoppins et al., 2007; Okamoto and Shaw, 2005). hFis1 is a mitochondrial protein anchored to the outer membrane with its N-teminus localized to the cytosol. This protein clearly affects mitochondrial fission: overexpression of hFis1 results in fragmentation of the mitochondrial network and reduced levels of hFis1 result in elongation (James et al., 2003; Stojanovski et al., 2004). Nevertheless, the precise function of hFis1 in the fission process is yet to be determined.

Figure 5. Mitochondrial fission. **(A)** hFis1, located on the outer-membrane may recruit Drp1 at the site of fission. **(B)** When multiple proteins of Dmn1 are recruited to the future site of scission, they homo-oligomerize and form spirals around the mitochondria. **(C)** Constriction of the spiral enables the mitochondria to divide.

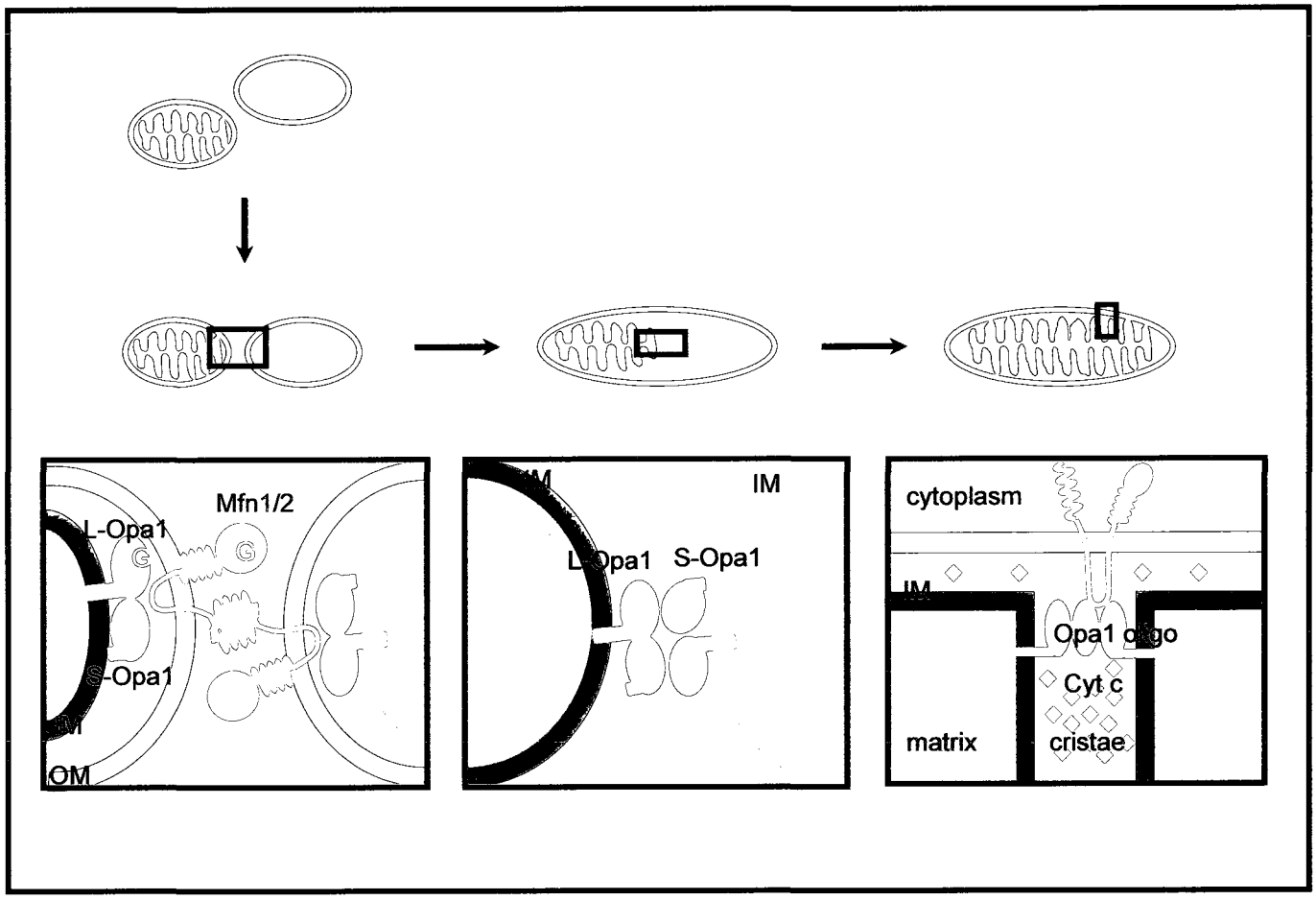

A
Drp1
hFis1
Drp1
B
GTP
GDP + Pi
C

Drp1 is a dynamin-related GTPase localized to the cytoplasm. Similarly to hFis1, blocking Drp1 activity results in mitochondrial elongation, which supports an active role of this protein in the fission process (Santel and Fuller, 2001). To induce fission, Drp1 relocates from the cytoplasm to the mitochondria and assembles in punctate structure, which will often become the fission site (Ingerman et al., 2005). The exact mechanism of mitochondrial fission in mammals still needs to be clarified, but studies performed in yeast have provided important clues on this process. In yeast, Fis1 functions to recruit the fission complex composed of Mdv1 and Caf4, which are the adaptors for Dnm1 (homologue of Drp1) to the mitochondria (Tieu and Nunnari, 2000; Griffin et al., 2005; Zhang and Chan, 2007). When multiple Dnm1 proteins are recruited to the future site of fission, they homo-oligomerize and form spirals around the mitochondria. It is thought that constriction of the spiral enables the mitochondria to divide in two (Abraham and Shaham, 2004; Roux et al., 2006). In mammals, no homologues of Mdv1 and Caf4 have yet been identified; hence Drp1 may either interact directly with hFis1 or through other adaptor proteins to mediate fission.

1.4.2. Mitochondrial fusion

Mitochondrial fusion refers to the event where two individual mitochondria will converge their outer and inner membrane to form a single mitochondrion. This ongoing event is important for many cellular aspects including development, mitochondrial metabolism and cell survival (McBride et al., 2006; Chan, 2006a). In mammalian organisms, the proteins mediating mitochondrial fusion include the dynamin related

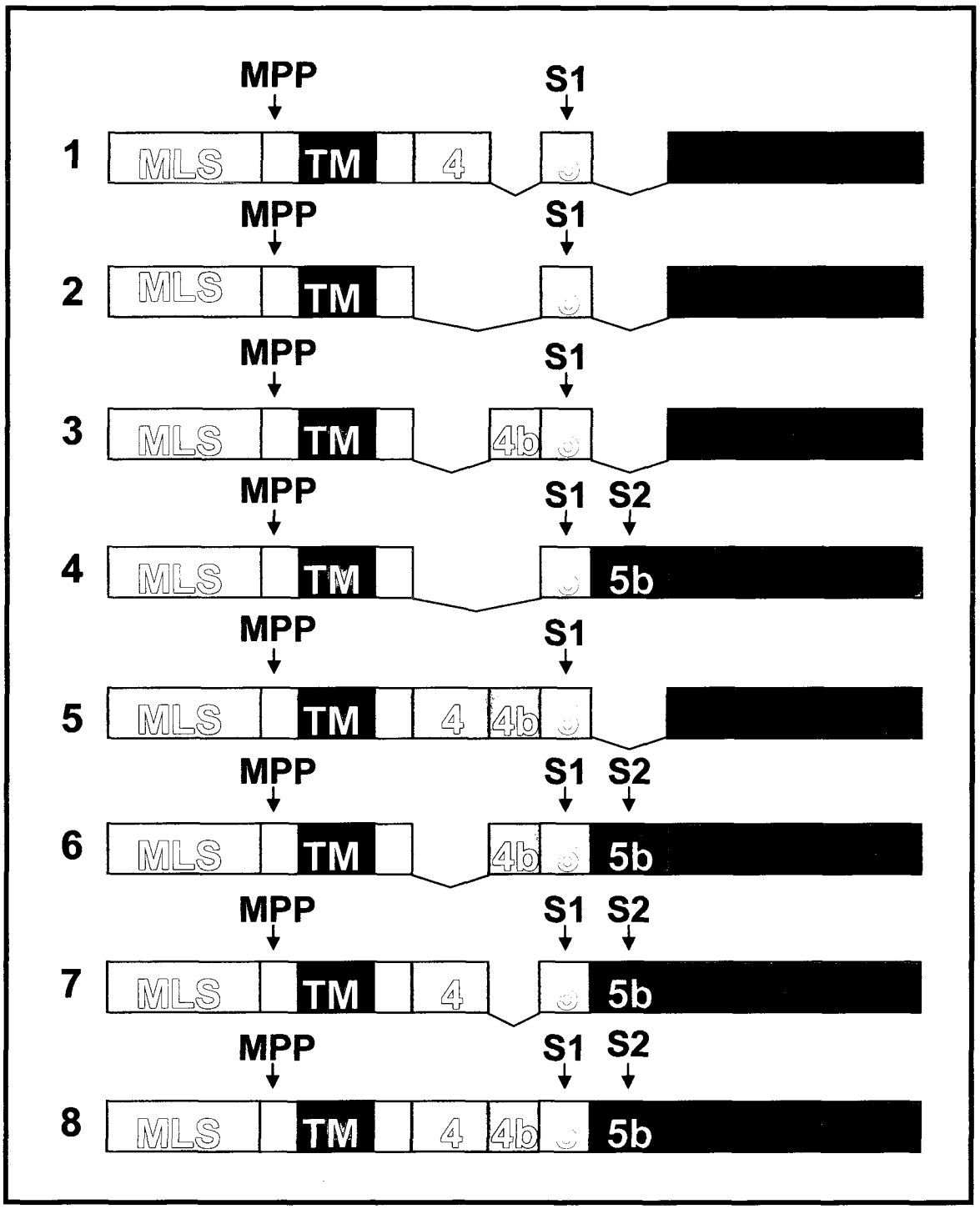
Figure 6. Mitochondrial fusion. Mitochondrial fusion requires the fusion of the outer-membrane and the inner-membrane. Through homo- and heterotypic interactions, Mfn1 and Mfn2 regulate the fusion of the outer membrane (OM). Opa1 interacts with Mfn1 to perform inner-membrane (IM) fusion.


Mfn1/2
L-Opa1
S-Opa1
OM
IM
L-Opa1
S-Opa1
cytoplasm
IM
Opa1 oligo
Cyt c
matrix
cristae

GTPase Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic Atrophy 1 (Opa1) (Figure 6). Mfn1 and Mfn2 are homologous proteins, both located on the outer membrane of the mitochondrion (Rojo et al., 2002). Even though both proteins are very similar, they have distinct functions in the cell (Cipolat et al., 2004; Ishihara et al., 2004; Chen et al., 2003). They are inserted into the mitochondrial outer membrane with their N- and C-termini located in the cytosol. They mediate fusion of the outer membrane from two adjacent mitochondria through homotypic and heterotypic interactions with their heptad repeats (coiled-coil formation domains) (Hoppins et al., 2007; Koshiba et al., 2004). Both Mfn1 and Mfn2 are vital to the organism since knockout mice of either one do not survive to birth. Cells derived from those embryos exhibit highly fragmented mitochondria with severe respiration defects and loss of their mtDNA (Chen et al., 2003). Specific deletion of Mfn2 in neuronal tissue leads to severe neurodegeneration, which underlies the importance of mitochondrial fusion in neurons (Chen et al., 2007).

Complete fusion of two mitochondria requires them to merge both the outer and inner mitochondrial membranes. Similar to outer membrane fusion, inner membrane fusion also relies on a dynamin related GTPase, more specifically on Optic atrophy 1 (Opa1) which is inserted in the inner membrane facing the inter membrane space (Cipolat et al., 2004; Meeusen et al., 2006). Loss of Opa1 can also be deleterious for neuronal tissue, causing the degeneration of retinal ganglion cells leading to blindness in autosomal dominant optic atrophy disease (Delettre et al., 2000). Morphologically, Opa1-deficient cells have fragmented mitochondria, involving this protein in the mechanism of mitochondrial fusion (Griparic et al., 2004; Olichon et al., 2003). Opa1 is transcribed as 8 different mRNA splice variants, which give rise to the long isoforms (L-Opa1) of the

Figure 7. Opa1 mRNA variants and processing. Opa1 is transcribed as 8 distinct mRNA variants that vary from alternative splicing of exon 4, 4b and 5b, forming the long isoform (L-Opa1). L-Opa1 is targeted to the mitochondria by its mitochondrial localization sequence (MLS) and is inserted in the inner-membrane with its transmembrane domain (TM). After mitochondrial import, MLS is cleaved by mitochondrial processing peptidases (MPP). Each mRNA variant can further be processed at S1 and S2, creating the short soluble form (S-Opa1). In the figure, the grey box represents the rest of Opa1 protein.


MPP
S1
1
MLS
TM
4
MPP
S1
2
MLS
TM
MPP
S1
3
MLS
TM
4b
MPP
S1
S2
4
MLS
TM
5b
MPP
S1
5
MLS
TM
4
4b
MPP
S1
S2
6
MLS
TM
4b
5b
MPP
S1
S2
7
MLS
TM
4
5b
MPP
S1
S2
8
MLS
TM
4
4b
5b

protein that are anchored to the mitochondrial inner-membrane (Figure 7). Following mitochondrial import, these isoforms can be further processed by proteases such as PARL, Yme1L and Paraplegin (Cipolat et al., 2006; Griparic et al., 2007; Song et al., 2007; Ishihara et al., 2006), to generate short soluble isoforms (S-Opa1), which are no longer attached to the mitochondria. Each long isoform possesses either one or two cleavage sites (Figure 7, S1 and S2), depending on the mRNA variant. Therefore, Opa1 is a very complex protein that can be resolved in at least 5 bands on a Western blot. The 2 heaviest bands are thought to be a mixture of long isoforms and the shorter bands represent the processed isoforms (Ishihara et al. 2006). Both L-Opa1 and S-Opa1 are required to mediate outer membrane fusion (Song et al., 2007). Although the exact mechanism of Opa1-mediated OM fusion remains to be determined, there is strong evidence that its fusion activity requires cooperation with Mfn1 (Cipolat et al. 2004). Apart from mediating OM fusion, Opa1 is also implicated in the formation and regulation of the cristae structure. In yeast, the Opa1 homologue Mgm1 is required for proper formation of the cristae and assembly of ATP synthase (Amutha et al., 2004). The importance of Opa1 was also assessed by dowregulating levels of the protein, resulting in disruption of cristae morphology (Meeusen et al., 2006; Olichon et al., 2003; Frezza et al., 2006). Opa1 was further proposed to control cristae structure by forming high molecular weight oligomers of L-Opa1 and S-Opa1 at the base of the cristae to maintain the cristae junctions in a "closed" conformation (Frezza et al., 2006). Opa1oligomers have also been implicated in apoptotic signaling, by controlling the release of cytochrome C from the intra cristae space. In the following section, I will explain how modulation of

the cristae structure is important for proper mitochondrial function and how Opa1 oligomerization can affect cristae structure during apoptotic signaling.

1.5. Cristae structure

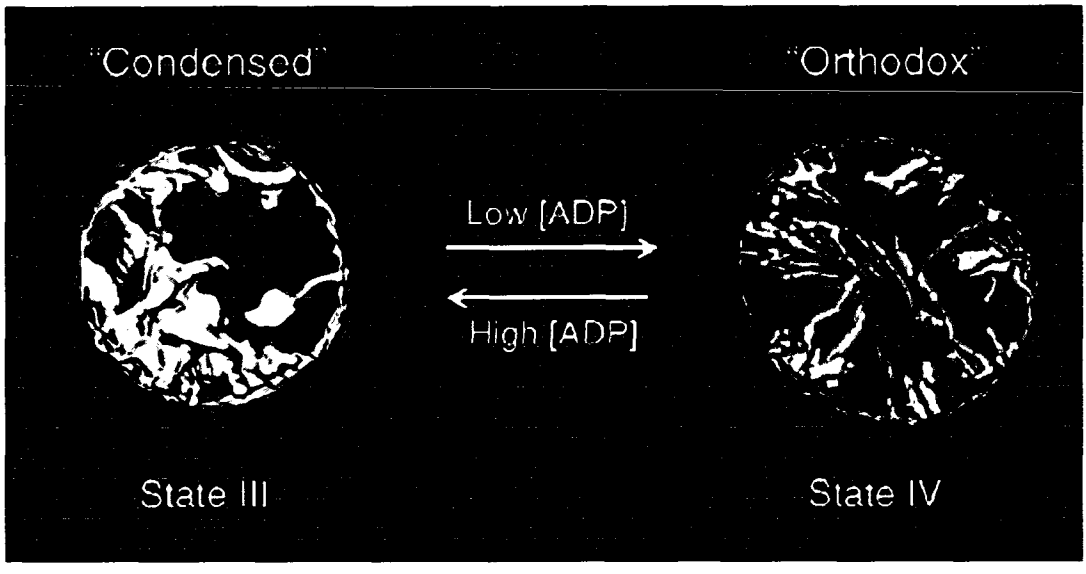
The topology of the mitochondrial inner membrane and cristae is not random but rather controlled by several proteins. Some of the pioneering experiments investigating mitochondrial cristae structure were performed by Hackenbrock in the 1960s. He was the first to correlate the metabolic state of mitochondria to the shape of the cristae (Hackenbrock, 1966). Over the years, it has become clear that the shape of the mitochondrial cristae is directly implicated in various functions of the mitochondria. If the cristae shape is different depending on the metabolic state of the cell, it is also remodelled during apoptosis signalling, linking the cristae structure to the two major roles of the mitochondria in the cell. In the following section, I will briefly describe the impact of cristae structure on mitochondrial metabolism and explain how cristae are remodelled during apoptosis and its significance for the apoptotic signalling.

1.5.1. Relevance of cristae structure for metabolism

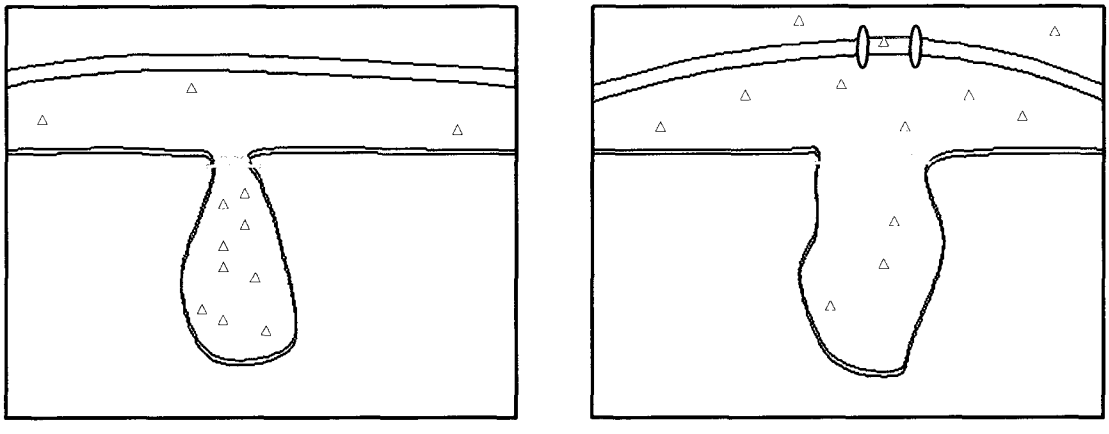
The topology of the mitochondrial inner membrane is a typical physical aspect of the mitochondria. The invagination of this membrane inside the matrix compartment is known as cristae and it is connected to the inner boundary membrane via tight tubular junctions called cristae junctions (Frey and Mannella, 2000b). The two distinct mitochondrial morphological states, known as condensed and orthodox, correspond to the variation in the structure of the cristae (Figure 8A) (Hackenbrock, 1966). The condensed

Figure 8. Cristae remodelling during metabolic change and apoptosis. (A) From Mannella, 2006. Mitochondria cristae change conformation according to the amount of ADP and substrate. State III represents the cirstae structure under high ADP (condensed) and State IV under low ADP (orthodox). **(B)** Model of cristae remodelling proposed by Frezza et al. 2006. Opa1 oligomers are disrupted during t-Bid induced apoptosis, leading to remodelling of the cristae and release of cyt C.

A



B



L-Opa1

S-Opa1

Cyt C

t-BID

state corresponds to large and wide cristae that occupy a large volume within the mitochondria, condensing the matrix. On the other hand, the orthodox mitochondria have a less dense matrix with compact cristae. Hackenbrock was the first to observe that mitochondria can switch from condensed to orthodox state depending on the availability of ADP. Mitochondria incubated with high amounts of ADP have dilated cristae, corresponding to the condensed state. Conversely, the orthodox state correlates with low levels of ADP. It was proposed by Mannella (2006) that "dilated" cristae enhances the diffusion rate of ADP when it is present in excess. However, since ADP is a substrate for the formation of ATP, computer simulations performed by Moraru and colleagues demonstrated that it can be depleted in dilated cristae (condensed state). Lower amounts of ADP and the dilated shape of the cristae limit the diffusion of the molecule, resulting in a predicted drop in ATP production (Mannella, 2006a; Mannella et al., 2001a). This observation led to the current model, suggested by (Mannella, 2006b), in which mitochondria change to the orthodox state to reduce the size of the cristae thereby increasing the diffusion of ADP, to sustain normal ATP production. How this whole process is regulated and which proteins are implicated is still unclear. However, it is evident that the topology of the cristae is important for mitochondrial respiration and ATP production.

1.5.2. Relevance of cristae structure during apoptosis

Cytochrome C is a bi functional protein indispensable for mitochondrial respiration. More that 80% of this protein is retained inside the mitochondrial cristae, probably to increase the availability of this electron transport chain substrate to enhance

ATP production. The cristae compartment connects to the inner membrane through a tight tubular structure called the cristae junction (Frey and Mannella, 2000a). During apoptosis, cytochrome C has a different function, which is to activate the caspase cascade. It was observed by Scorrano et al. (2002) that the cristae, more specifically the cristae junction, are remodelled during apoptosis (Figure 8B). This remodelling allows the diffusion of cytochrome C to the intermembrane space and increases the amount of cytochrome C released to the cytosol after permeabilization of the outer membrane, resulting in a robust activation of the caspase cascade (Scorrano et al., 2002; Frezza et al., 2006). Cristae remodelling has been observed in a variety of cell types after induction of apoptosis, underlying its importance for apoptotic signalling (Scorrano et al., 2002; Germain et al., 2005). There is evidence that this event might be regulated by the inner membrane GTPase Opa1 (Cipolat et al. 2006, Frezza et al. 2006). Reducing the levels of Opa1 using siRNA results in dilated cristae junctions, implicating this protein in the control of the cristae junction (Griparic et al. 2004). A combination of long- and short-Opa1 forms high molecular weight oligomers essential to maintain the cristae junction "closed". After treatment with t-Bid, those oligomers are degraded, which correlates with the mobilization of cytochrome C to the cytoplasm and enhancement of the caspase cascade. Overexpression of Opa1 or a disassembly-resistant mutant form of the protein (Q297V) results in significant protection against cytochrome C release and cell death (Frezza et al., 2006; Yamaguchi et al., 2008). It is highly probable that Opa1 is not the sole protein involved in cristae remodelling during apoptosis. Other proteins implicated in the maintenance of cristae structure such as Mitofilins and ATP synthase subunit e/g could also be implicated in this process (Paumard et al., 2002; Arselin et al., 2004; John

et al., 2005). Nevertheless, Opa1 oligomerization remains the checkpoint for cristae remodelling and mobilization of cytochrome C during cell death.

1.6. Structural and functional defects of AIF-deficient mitochondria

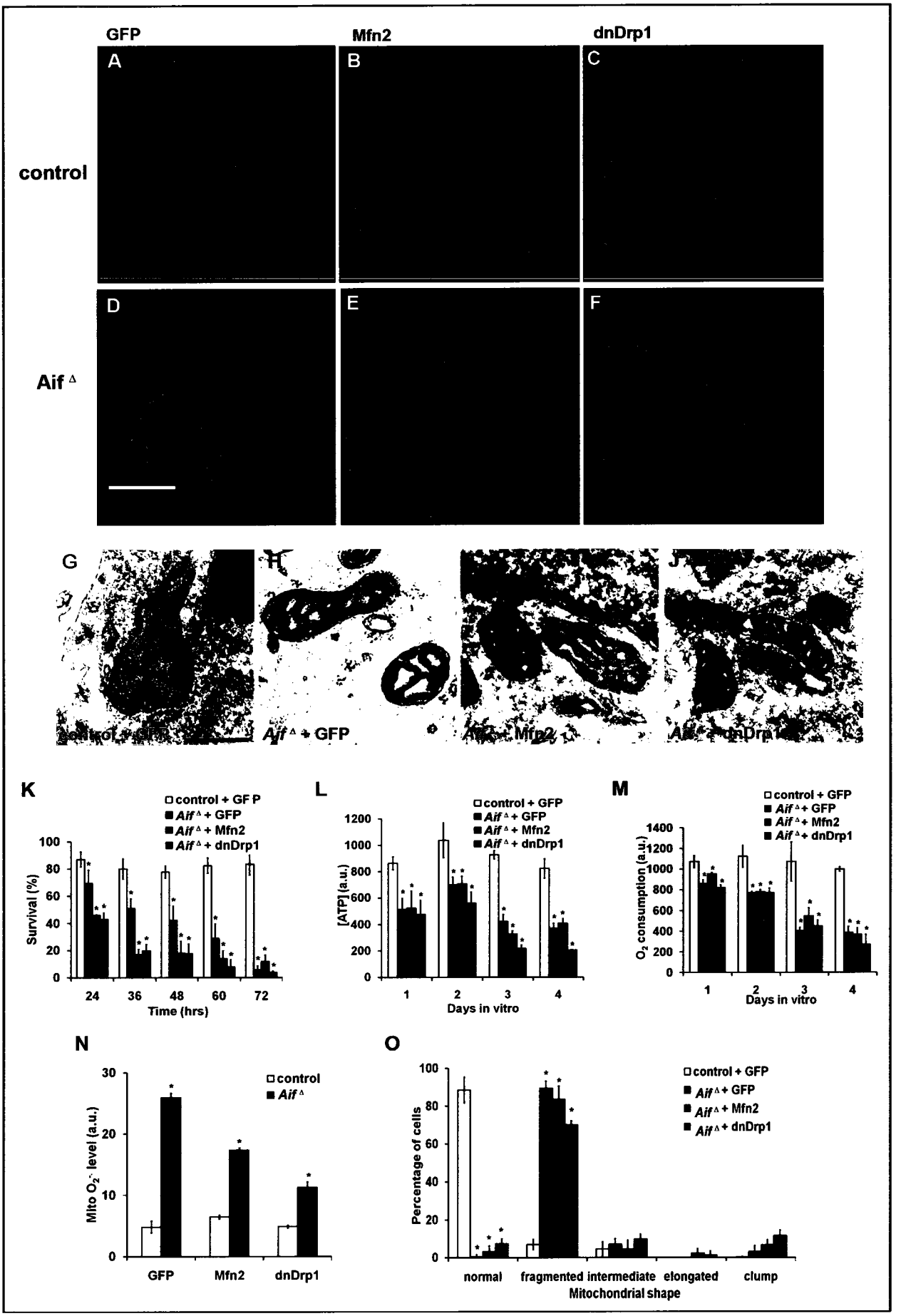
In the mitochondria, AIF also sustains cell survival by affecting mitochondrial metabolism and structure (Klein et al., 2002; Cheung et al., 2006; Vahsen et al., 2004). The importance of proper mitochondrial dynamics and cristae structure on mitochondrial function, as well as the direct impact it has on apoptotic signalling as been described above.

Our group was the first to suggest a potential role of AIF as a protein involved in maintaining mitochondrial structure, as AIF-deficient mitochondria exhibit severe fragmentation (Cheung et al. 2006). Electron microscopy of such mitochondria revealed a more profound structural defect, with balloon-shaped cristae and enlarged cristae junction. Re-introduction of a mitochondria-anchored form of AIF (N-AIF) is sufficient to completely rescue the defects caused by AIF deficiency, strengthening the idea of AIF as a structural protein. However, how AIF controls mitochondria structure is yet to be determined. One probability is that it mediate fusion similar to the mitofusins, blocking fission by modulating hFis1/Drp1 activity or implicated in the maintenance of the internal structure of the mitochondria. In the following section, I will explain the response of AIF-deficient mitochondria to different fusion-inducing conditions and describe a novel finding where AIF could functionally interact with the inner-mitochondrial membrane protein Opa1 to achieve its pro-survival activity.

1.6.1. Mitochondrial defects of $AIF^{-/-}$ are not rescued by Mfn2 and dnDrp1

$AIF^{-/-}$ neurons have fragmented mitochondria with dilated cristae, suggesting a role for AIF in maintaining mitochondrial structure. To test whether AIF modulates mitochondrial fission and fusion, two approches were taken : fusion was increased by overexpressing Mfn2 and fission was inhibited by expressing the dominant negative form of Drp1 (dnDrp1) (Frank et al., 2001). In wild type cortical neurons, enhancing mitochondrial fusion or inhibiting fission results in increased mitochondrial length (Figure 9 B-C). However, these effects were not observed in $AIF^{-/-}$ neurons, in which the mitochondria remain fragmented (Figure 9 D-F, O). Further analysis of mitochondrial structure revealed that cristae were still dilated even in the presence of Mfn2 or dnDrp1 (Figure 9 G-J). Thus, promoting mitochondrial fusion or inhibiting fission cannot rescue mitochondrial structural defects (Figure 9 O). Similarly, metabolic defects of $AIF^{-/-}$ neurons were not rescued by overexpression of Mfn2 or dnDrp1, ATP production and oxygen consumption remaining impaired (Figure 9 K-M). Interestingly, overexpression of Mfn2 or dnDrp1 could not reduce superoxide levels of $AIF^{-/-}$ back to control levels, but it was still reduced compared to $AIF^{-/-}$ with GFP (Figure 9 N). This could be a result of the fusion-independent role of Mfn2 in controlling metabolism, and inhibiting fission could mimic this effect (Pich et al., 2005; Bach et al., 2003). Finally, increased fusion or inhibited fission could not rescue survival of $AIF^{-/-}$ neurons. All this evidence demonstrate that AIF does not promote cell survival

Figure 9. Mfn2 and dnDrp1 can not rescue the mitochondrial structure and metabolism defects of AIF-/- neurons. (A-F) Representative images of mitochondria by cytochrome c staining from wild-type and AIF-/- neurons infected with Mfn2, dnDrp1 and GFP control. **(G-J)** TEM images of wild-type neurons with GFP and AIF-/- expressing Mfn2, dnDrp1 and GFP control. **(K)** Quantitative analysis of cell death of control neurons expressing GFP and AIF-/- expressing Mfn2, dnDrp1 and GFP control (n=3). **(L)** Measurement of ATP production of AIF-/- neurons expressing Mfn2, dnDrp1 and GFP control compared to wild-type expressing GFP (n=3). **(M)** Measurement of Oxygen consumption of wild-type neurons expressing GFP and AIF-/- expressing Mfn2, dnDrp1 and GFP control (n=3). **(N)** Analysis of mitochondrial ROS production in wild-type and AIF-/- neurons expressing Mfn2, dnDrp1 and GFP control (n=3). **(O)** Quantitative analysis of control and $AIF^{-/-}$ neurons expressing Mfn2, dnDrp1, and GFP control with the indicated mitochondrial shape (n=3). * $p<0.05$ compared to control + GFP (from Cheung E., Pilon-Larose K. et al.)

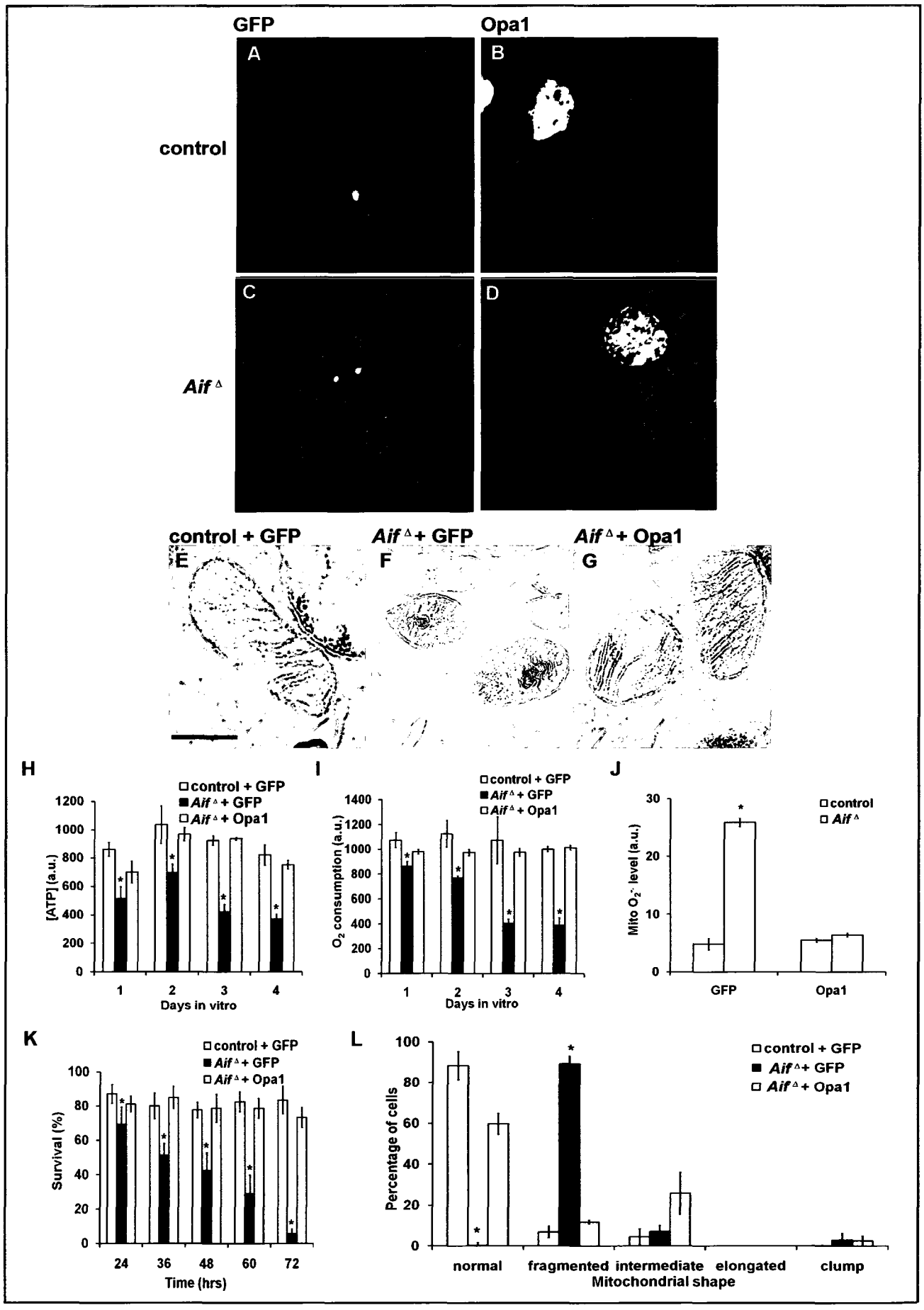

GFP
Mfn2
dnDrp1
A
B
C
control
D
E
F
Aif Δ
G
H
J
control + GFP
Aif Δ + GFP
Aif Δ + Mfn2
Aif Δ + dnDrp1
K
control + GFP
Aif Δ + GFP
Aif Δ + Mfn2
Aif Δ + dnDrp1
Survival (%)
Time (hrs)
L
[ATP] (a.u.)
Days in vitro
M
O2 consumption (a.u.)
Days in vitro
N
control
Aif Δ
Mito O2- level (a.u.)
GFP
Mfn2
dnDrp1
O
Percentage of cells
normal
fragmented
intermediate
elongated
clump
Mitochondrial shape

by modulating mitochondrial fusion or fission, since over-expression of neither Mfn2 nor dnDrp1 could rescue cell survival, mitochondrial structure and function.

1.6.2. Opa1 rescues the defects of AIF-deficient mitochondria

AIF deficient cells exhibit severe mitochondrial defects and impaired survival. It seems unlikely that AIF ensures mitochondrial integrity and cell survival by modulating mitochondrial fusion or fission. Therefore, it would be possible that AIF sustains neuronal survival by maintaining mitochondrial structure. It could functionally interact with a protein responsible for regulating mitochondrial architecture and in the absence of AIF, this protein's function would be impaired, resulting in the structural defects observed. Opa1 would be an interesting candidate since it promotes fusion by interacting with Mfn1 and is responsible for maintaining inner membrane structure by forming oligomers with its long (L-Opa1) and its soluble short (S-Opa1) forms (Cipolat et al., 2004, Meeusen et al., 2006). Loss of Opa1 induces defects similar to AIF deficiency. Indeed, $Opa1^{-/-}$ cells have fragmented mitochondria with dilated cristae as well as respiration defects leading to cell death (Chen et al., 2005, Griparic et al., 2004). These similarities between AIF and Opa1 suggest that they may be involved in a common pathway to regulate mitochondrial function. Over-expression of Opa1 in $AIF^{-/-}$ cells can rescue mitochondria structure (Figure 10 A-G, L). Also, mitochondria in $AIF^{-/-}$ neurons expressing Opa1 have tighter and more organized cristae (Figure 10 E-G). Hence, it seems that Opa1 on its own has the potential to rescue structural defects of AIF deficient neurons (Figure 10 L). Correspondingly, Opa1 over-expression could also rescue oxygen consumption, ATP production and cell survival in $AIF^{-/-}$ neurons (Figure 10 H, I and K).

Figure 10. Opa1 rescues AIF-/- mitochondrial and metabolic defects. (A-D) Representative images of mitochondria by cytochrome c staining from wild-type and AIF-/- neurons infected with Opa1 and GFP control. **(F-H)** TEM images of wild-type neurons expressing GFP and AIF-/- neurons expressing Opa1 or GFP control. **(I)** Measurement of ATP production of AIF-/- neurons expressing Opa1 and GFP control compared to wild-type expressing GFP (n=3). **(J)** Measurement of Oxygen consumption of wild-type neurons expressing GFP and AIF-/- expressing Opa1 and GFP control (n=3). **(K)** Analysis of mitochondrial ROS production in wild-type and AIF-/- neurons expressing Opa1 and GFP control (n=3). **(L)** Quantitative analysis of cell death of control neurons expressing GFP and AIF-/- expressing Opa1 and GFP control (n=3). **(M)** Quantitative analysis of control and $AIF^{-/-}$ neurons expressing Opa1 and GFP control with the indicated mitochondrial shape (n=3). * $p<0.05$ compared to control + GFP (from Cheung E., Pilon-Larose K., et al.)

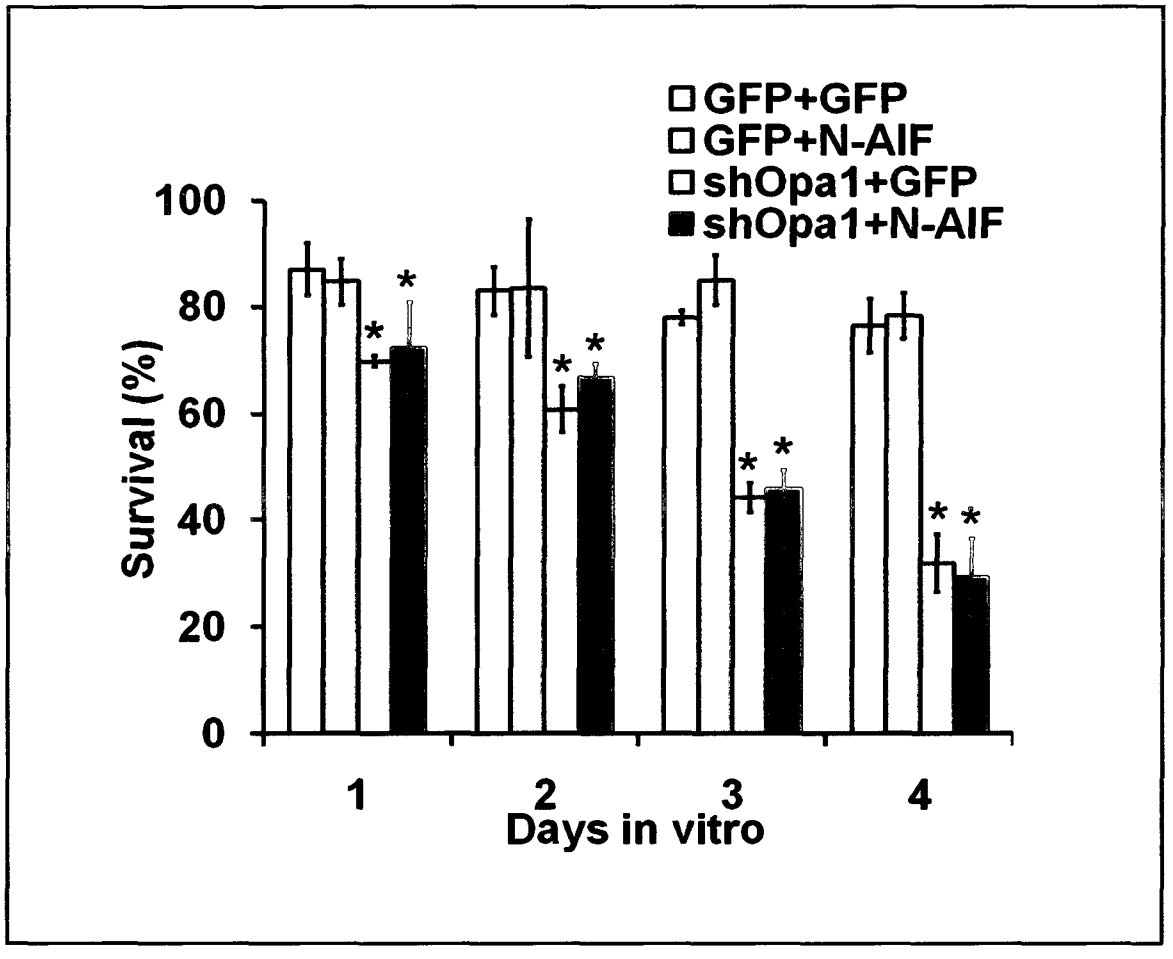

GFP
Opa1
A
B
control
C
D
Aif Δ
control + GFP
Aif Δ + GFP
Aif Δ + Opa1
E
F
G
H
[ATP] (a.u.)
Days in vitro
I
O2 consumption (a.u.)
Days in vitro
J
Mito O2· level (a.u.)
control
Aif Δ
GFP
Opa1
K
Survival (%)
Time (hrs)
L
Percentage of cells
normal
fragmented
intermediate
elongated
clump
Mitochondrial shape

Importantly, superoxide levels are significantly reduced by overexpressing Opa1, suggesting that the increase in ROS observed in $AIF^{-/-}$ cells is due to the loss of Opa1 activity in these cells (Figure 10 J). Therefore, this functional interaction between AIF and Opa1 suggests that AIF pro-survival function is to maintain mitochondrial structure.

1.6.3. Opa1 acts downstream of AIF

Overexpression of Opa1 can rescue defects of $AIF^{-/-}$ cells, suggesting that Opa1 works downstream of AIF. Conversely, if AIF acted upstream of Opa1, overexpression of AIF in $Opa1^{-/-}$ neurons would be nonfunctional. Knockdown of Opa1 in neurons results in impaired cell survival. N-AIF, a mitochondria-anchored variant of AIF that is permanently tethered to the mitochondria inner-membrane, was demonstrated to promote cells survival in AIF-deficient cells and delay apoptosis in wild-type neurons (Cheung et al. 2006). Expression of this AIF variant in the Opa1 knockdown neurons could not rescue cell survival (Figure 11). Hence, this suggests that AIF acts upstream of Opa1 within the same pathway to promote cell survival by controlling mitochondrial structure.

Figure 11. Opa1 is downstream of AIF. (A) Quantitative analysis of cell death of wild-type neurons expressing N-AIF and GFP control and wild-type neurons with a knock-down of Opa1 expressing N-AIF or GFP control (n=5).


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

1.7. Rationale

Mitochondria are vital organelles in the cell. Apart from their central role as an energy producer, mitochondria are also relaying stations for apoptosis signalling. Several mitochondrial proteins have both pro-survival and pro-death functions, such as cytochrome C and Apoptosis Inducing Factor. AIF was first described as a pro-apoptotic protein but it became increasingly clear that this protein also play a pro-survival role inside the mitochondria, as it is required to maintain proper mitochondrial structure and function. As discussed above, mitochondria are crucial for both ATP production and cell death signalling. AIF deficient mitochondria are highly fragmented, with defective cristae and impaired metabolism. Such abnormalities cannot be rescued by enhancing fusion or by blocking fission, demonstrating a profound structural defect in the absence of AIF in the mitochondria. The mitochondrial GTPase Opa1 can rescue both metabolic and structural defects of AIF deficient mitochondria. Furthermore, it seems that Opa1 acts downstream of AIF in a common pathway. However, the exact mechanism by which AIF maintains mitochondria structure and metabolism through Opa1 is yet to be determined.

1.8. Statement of hypothesis and objectives

Since in the absence of AIF mitochondria exhibit severe structural and metabolic defects that can be rescued by overexpression of Opa1, we hypothesize that AIF acts upstream of Opa1 in a common pathway to regulate mitochondrial structure and function by modulating Opa1 activity via physical interaction. To test this hypothesis we set up the following objectives:

Objective #1: Confirm that AIF acts upstream of Opa1 in a common pathway to regulate mitochondrial structure and function

Given our preliminary results suggesting that AIF functions upstream of Opa1, we will use primary cortical neurons in which Opa1 will be knocked down by siRNA technology to analyze the protective potential of Opa1 and AIF after induction of apoptosis. Cytochrome c release and nuclear morphogenesis will be used as a measure of cell death after the treatment.

Objective #2: Assess whether AIF physically interacts with Opa1

After confirming that AIF functions upstream of Opa1, we will perform co-immunoprecipitation assays on isolated mitochondria from wild-type cells or cells overexpressing N-AIF-GFP and c-myc Opa1 to determine if AIF and Opa1 are physically interacting under steady-state conditions. We will also repeat this assay on crosslinked mitochondria to investigate for interaction between AIF and Opa1 oligomers.

Objective #3: Elucidate the mechanism by which AIF regulates Opa1 function

AIF-deficient neurons exhibit dysfunctional cristae structure, which can be attributed to a defect in Opa1 oligomerization (Frezza et al. 2006). Since Opa1 overexpression rescues the defects observed in $AIF^{-/-}$ cortical neurons, we will analyze the levels of Opa1 oligomers by gradient gel electrophoresis in AIF knockout neurons and in neurons overexpressing Opa1. We will also investigate how AIF influences Opa1 oligomerization during cell death.

Objective #4: Evaluate the protective potential of the interaction between AIF and Opa1 during apoptosis

We will investigate the protective potential of the interaction between AIF and Opa1 after induction of cell death using primary cortical neurons overexpressing the proteins of interest via adenoviral infection.

Objective #5: Identify other factors that can influence Opa1 oligomerization

After identifying how AIF affects Opa1 oligomerization, we will investigate whether other factors such as the metabolic state of the cell can affect the levels of Opa1 oligomerization. For this, we will perform *in vitro* assays on fresh mitochondria isolated from mouse liver, followed by gradient gel analysis.

METHODS

2.1. Mice

The AIF floxed mouse was first described by (Joza et al., 2005). Mice were maintained on C57/BL6 and FVB/N mixed backgrounds and littermates were used for all experiments. In this work, $AIF^{flox/flox}$ females were bred with Foxg1-cre males (Hebert and McConnell, 2000) to generate telencephalon-specific AIF knock-out embryos (indicated as AIF^{Δ}). To analyze the genotype of the parents and embryos, PCR analysis was performed. For the Foxg1-cre allele, the primers cre 3B (TGACCAGAGTCATC CTTAGCG) and cre 5B (AATGCTTCTGTCCGTTTGCC) yield a band around 700 bp if the cre is present. For the AIF floxed allele, primers 1303 fwd (GTAGATCAGTT GGCCAGAAACTC) and 1903 rvs (GGATTAAAGGCATGTGCCAACACG) yield a band at 600bp for the wild-type allele and 700bp for the floxed allele.

2.2. Cortical neuronal cultures

Cortical neurons are cultured according to the protocol described in Cregan et al., 2004 from embryos at embryonic days 14.5 and 15.5. Cortices were dissociated from the embryo and, if not specified, were pooled together in a solution supplemented with 0.50 mg/ml of trypsin (Sigma). Cortices were rotated gently 15 minutes at 37°C, trypsinization was stopped by adding a solution containing 0.2mg/ml trypsin inhibitor (Roche) and 0.2mg/ml DNase I (Roche). Neurons were centrifuged for 5 minutes at 3000 rpm and the cell pellet was resuspended in a trituration solution containing 0.2mg/ml of trypsin inhibitor and 0.25mg/ml of DNase I. Neurons were triturated by pipeting gently up and down 20 times with a flame polished glass pipette. After trituration, cells were incubated at room temperature 10 minutes to sediment all possible debris and the supernatant was

centrifuged for 5 minutes at 3000 rpm. The cell pellet was resuspended in Neurobasal media (Invitrogen) supplemented with B27 (Invitrogen), N2 (Invitrogen), L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen). Neurons were plated in Nunc 35mm dishes ($1.5x10^6$ cells/dish), 60 mm dishes ($6x10^6$ cells/dish) or 10 cm dishes ($1x10^7$ cells/dish) coated with poly-D Lysine (BD Biosciences). Only when specified, cortices from each embryo were cultured individually and remaining tissue was used to genotype and select appropriate neurons for the experiment.

2.3. Primary culture of AIF knock-out Mouse Embryonic Fibroblasts (MEFs)

$AIF^{flox/flox}$ female was paired with an $AIF^{flox/Y}$ male. Embryos were taken for dissection at E13.5 and each were cultured individually, as described by FERO Lab (http://labs.fhcrc.org/fero/protocols/MEFs.html). For all individual embryos, brain and liver were discarded, keeping only muscle and bones in a 10ml tissue culture dish. Tissue was chopped into small pieces; 4ml of 1X trypsin was then added and incubated at 37°C for 15 minutes. Following incubation, 5 ml of DMEM (WISENT) supplemented with 10% FBS (WISENT) and 5% penicillin/streptomycin (Invitrogen) was added to inhibit the trypsin. Cells were triturated by pipetting up and down 20 times with a 5 ml pipette and divided into 3-10cm plates. Cells were incubated overnight and washed with PBS the next day to discard dead cells. When cells reached 80% confluency, they were divided into 2-10 cm plates and incubated 24 hrs before freezing (FBS supplemented with 10% DMSO). The genotype of each individual embryo was assessed by PCR analysis as described above. After thawing, primary MEFs were maintained in normal culture conditions and were passaged up to 7 times. To create AIF knockouts, MEFs were

infected with an adenovirus expressing cre-gfp and incubated 72 hours before the experiment was performed.

2.4. Cell transfection, adenoviral infection and cell death assay

HEK293T cells were transfected with the c-myc Opa1 construct (gift from Dr Luca Scorrano) using Lipofectamine 2000 (Invitrogen). cDNA carrying N-AIF (Cheung et al., 2006) Opa1, Mfn2, Fis1, shOpa1 (gift from Dr Heidi M. McBride), Cre and Bcl-2 (Cregan et al., 2000) were transformed in recombinant adenoviral vectors using the AdEasy system (He et al., 1998). Viruses were constructed, purified and tittered as described in Cregan et al., 2000 by Steve Callaghan and Jason MacLaurin. The $AIF^{flox/flox}$ MEFs were passaged twice before infection with 150 MOI of the appropriate virus. Cells were harvested for protein analysis after 72 hours of infection. For neuronal cultures, the cells were infected with 100 MOI of the appropriate virus at the time of plating. After 48 to 72 hours of infection, cells were harvested for protein analysis. For the cell death assays, the neurons were treated with 10 mM camptothecin (Sigma) or 50 μM hydrogen peroxide (Sigma) 48 hours post-infection.

2.5. Cell viability and immunostaining

Neurons were seeded onto glass coverslips coated with poly-D Lysine and fixed with 4% PFA for 30 minutes. Coverslips were washed twice with PBS and the immunostaining was performed or coverslips were stored at 4°C. Cells were permeabilized for 10 minutes with a solution containing 0.4% Triton X-100 and 10% normal goat serum (NGS). Primary antibody was prepared in 0.4% Triton X-100 and 2% NGS and was

added for 1 hours (mouse anti-GFP (Abcam, 1:100), mouse anti-cytochrome C (BD Pharminigen, 1:500), mouse anti-6A7 Bax (BD Bioscience, 1:100) and rabbit anti-active caspase 3(Cell Signaling, 1:250)). Cells were washed with PBS and incubated for 1 hour with a secondary antibody (anti-mouse and anti-rabbit Alexa Fluor 488/Alexa Fluor 594). Hoechst stain (Sigma) was added to visualize the nucleus and the coverslips were mounted using Gel Mount Aqueous mounting medium (Sigma). Cell death was determined by the typical nuclear/chromatin condensation revealed by Hoechst staining as described in (Cregan et al., 2002).

2.6. Mitochondrial isolation from cells

Mitochondrial isolation for the immunoprecipitation experiments was performed as described previously (Yu et al., 2002; Cheung et al., 2006). Cells were washed with PBS, resuspended in 500 µl of hypotonic buffer (10 mM HEPES-HCl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$ supplemented with 1X commercially available proteinase inhibitors cocktail (CLAAAP, Sigma) and incubated on ice for 30 minutes. After incubation, cells were broken with 50 strokes in a Dounce homogenizer and samples were centrifuged at 3000 rpm for 5 minutes to pellet the nuclei and unbroken cells. The supernatant was further centrifuged at 10 000 rpm for 10 minutes to pellet the heavy membrane fraction enriched in mitochondria. The supernatant from this centrifugation is the cytoplasmic fraction. The mitochondria-enriched pellet was washed in 500 µl of isotonic buffer (hypotonic buffer with 0.25 M sucrose supplemented with 1X proteinase inhibitors, centrifuged at 10 000 rpm for 10 minutes and pellet was either frozen at -80°C or used right away for an experiment. For gradient gel analysis of Opa1 oligomers, mitochondria were isolated

according to the protocol described in (Germain et al., 2002). Neurons were washed in PBS, resuspended in 100 µl of isotonic buffer (HIM buffer : 200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) and cells were broken by pipeting up and down with a 25G needle 15 times. The homogenate was centrifuged at 1000 rpm for 10 minutes to discard nuclei and cellular debris. The supernatant was centrifuged at 10 000 rpm for 10 minutes to pellet the heavy membrane fraction containing mitochondria. The pellet was washed with 500ul of isotonic buffer and the final pellet was resuspended in HIM or frozen at -80°C.

2.7. Mitochondrial isolation from tissue

For each experiment, a CD-1 mouse was sacrificed and the liver rinsed in PBS to eliminate excess blood. The tissue was cut into small pieces, inserted in a Dounce homogenizer filled with HIM buffer and 10 strokes were given. Homogenate was then centrifuged at 1000 rpm for 10 minutes at 4°C to pellet cellular debris and nuclei. The pellet was discarded and supernatant was centrifuged at 10 000 rpm for 10 minutes at 4°C. The pellet was washed with HIM and centrifuged again at 1000 rpm. Supernatant was then centrifuged at 10 000 rpm for 10 minutes and the pellet, containing the heavy membrane fraction enriched in mitochondria were resuspended in HIM (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) or CMRM (0.25M sucrose and 10 mM HEPES pH to 7.4 with KOH, 2.5 mM ATP, 12.5 mM NaSuccinate, 0.2 mM ADP and 5 mM K_2HPO_4 pH to 7.4).

2.8. Western blot analysis

For this work, the following antibodies were used: goat anti-AIF D20 (Santa Cruz, 1:5000), mouse anti-Opa1 (BD Bioscience, 1:3000), mouse anti-mtHSP70 (ABR Bioreagents, 1:10 000), mouse anti-Actin (Sigma, 1:10 000), mouse anti-cytochrome C (BD Bioscience, 1:500), mouse anti-DLP1(BD Transduction, 1:500) and rabbit anti-GFP (1:2000, Abcam). For Western blot analysis, protein concentration was determined by the Bio-Rad Bradford assay. The samples were analyzed by SDS-PAGE then transferred to a nitrocellulose membrane followed by blotting with the appropriate primary antibody. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) were added and luminescent signal was visualized with enhanced chemiluminescence (Fisher).

2.9. Co-immunoprecipitation

Fresh mitochondria were isolated following the protocol described above and the mitochondrial pellet was resuspended in 500 µl of RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% NaDOC, 0.1% SDS and 5 mM EDTA) supplemented with 1X CLAAAP protease inhibitor. Mitochondria were lysed by rotating gently at 4°C for 45 minutes followed by centrifugation at 13 000 rpm for 20 minutes. The supernatant was incubated with 1µg of the appropriate antibody; c-myc (Invitrogen), GFP (Abcam), Opa1 (BD Bioscience), for 3 hours at 4°C. Protein G agarose beads (Sigma) were washed 3 times in TBS, resuspended at a 50% slurry and 30 µl was added to each samples. After 2 hours incubation, beads were centrifuged gently and supernatant discarded. The beads were washed 4 times with TBS and the proteins were eluted by resuspending the samples

in 40 µl of 1X Western Loading Buffer followed by boiling for 5 minutes. Proteins were analyzed on a 10% acrylamide gel.

2.10. Co-immunoprecipitation after protein cross-linking

Mitochondria isolated from mouse liver were resuspended in isotonic buffer to a concentration of 1µg/µl and 1mM of Disuccinimidyl suberate (DSS) (Pierce) or 1-Ethyl-3-[3-dimethyl-aminopropyl]carbodiimide Hydrochloride (EDC) (Fisher) were added for 30 minutes at room temperature. DSS was quenched with 1M Tris pH 7.5 and EDC with 100 mM β-mercaptoethanol. Samples were centrifuged and pellets lysed in RIPA buffer supplemented with 1X protease inhibitors. Proteins were incubated with Opa1 antibody or AIF antibody for 3 hours. Protein G sepharose beads (GE Healthcare) were washed 3 times in TBS, resuspended at a 50% slurry and 30 µl was added to each samples. To maximize the amount of protein pulled down, the samples were incubated overnight at 4°C. Protein were eluted as described above and analyzed on a 6% acrylamide gel.

2.11. Opa1 oligomerization assay on isolated mitochondria

The protocol for measurement of Opa1 oligomers was adapted from (Frezza et al., 2006). Mitochondria were isolated from cells as described above and resuspended in isotonic buffer. The amount of mitochondria present in each sample was normalized with a Bradford assay. For each experiment, 15 µg-20 µg of mitochondria were resuspended in isotonic buffer at a final concentration of 0.5 µg/µl and EDC was added to a final concentration of 1 mM. Proteins were cross-linked for 30 minutes at room temperature

followed by quenching of the EDC with 10 mM β-mercaptoethanol for 4 minutes. Samples were centrifuged at 10 000 rpm for 10 minutes to pellet the mitochondria. After centrifugation, the supernatant was discarded, the pellet was resuspended in 30 µl of 1X LDS buffer (Invitrogen) and boiled for 5 minutes. Samples were analyzed on a 3-8% Tris-acetate gradient gel (Invitrogen) followed by Western blotting.

2.12. Opa1 oligomer assay on isolated mitochondria from CGNs

Cortical granule neurons (CGNs) culture was performed as described in (Jahani-Asl et al., 2007). Culture, viral infection and treatment of the cells were performed by Arezu Jahani-Asl. The cells were infected with ad-calpastatin or GFP controls at 5 DIV at a multiplicity of infection (MOI) of 100. Neurons were treated with 100 µM NMDA at 7 DIV for 1 hr followed by switching to conditioned media. For PD studies, neurons were treated with 100 µM PD 3 hrs prior to treatment with NMDA. Samples were collected at 12 hrs following treatment with NMDA and the lysates were subject to mitochondrial fractionation followed by Opa1 oligomerization assay.

2.13. In vitro Opa1 oligomerization assay

For this assay, the mitochondria were freshly isolated from a mouse liver following the protocol described above. Isolated mitochondria were resuspended in HIM (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA) or CMRM (0.25 M sucrose and 10 mM HEPES pH to 7.4 with KOH, 2.5 mM ATP, 12.5 mM NaSuccinate, 0.2 mM ADP and 5 mM K_2HPO_4 pH to 7.4) to a final concentration of 0.5 µg/µl. NAD^+ or NADH (Sigma) was added and samples were incubated on a 37°C heating block for 30

minutes. After the incubation period, EDC was added to a final concentration of 1 mM and samples were incubated at room temperature for 30 minutes. The cross-linker was quenched by the addition of 100 mM of β-mercaptoethanol and samples were analyzed on a gradient gel followed by Western blotting. For the rotenone (Sigma) and carbonylcyanide-3-chlorophenylhydrazone (CCCP) (Sigma) experiment, 100 nM of rotenone and 10 μM of CCCP were added simultaneously with the NAD^+/NADH.

2.14. Quantifications and statistical analysis

For cell death analysis, percentage of neurons with mitochondrial cyt *c* and percentage of neurons with active caspase 3, a minimum of 500 cells for each independent experiment was scored at the indicated time points. Only cells infected with the adenovirus containing the constructs were counted in the cell death analysis. The data represent mean values +/- s.d. from at least three independent experiments (n=3) unless otherwise noted.

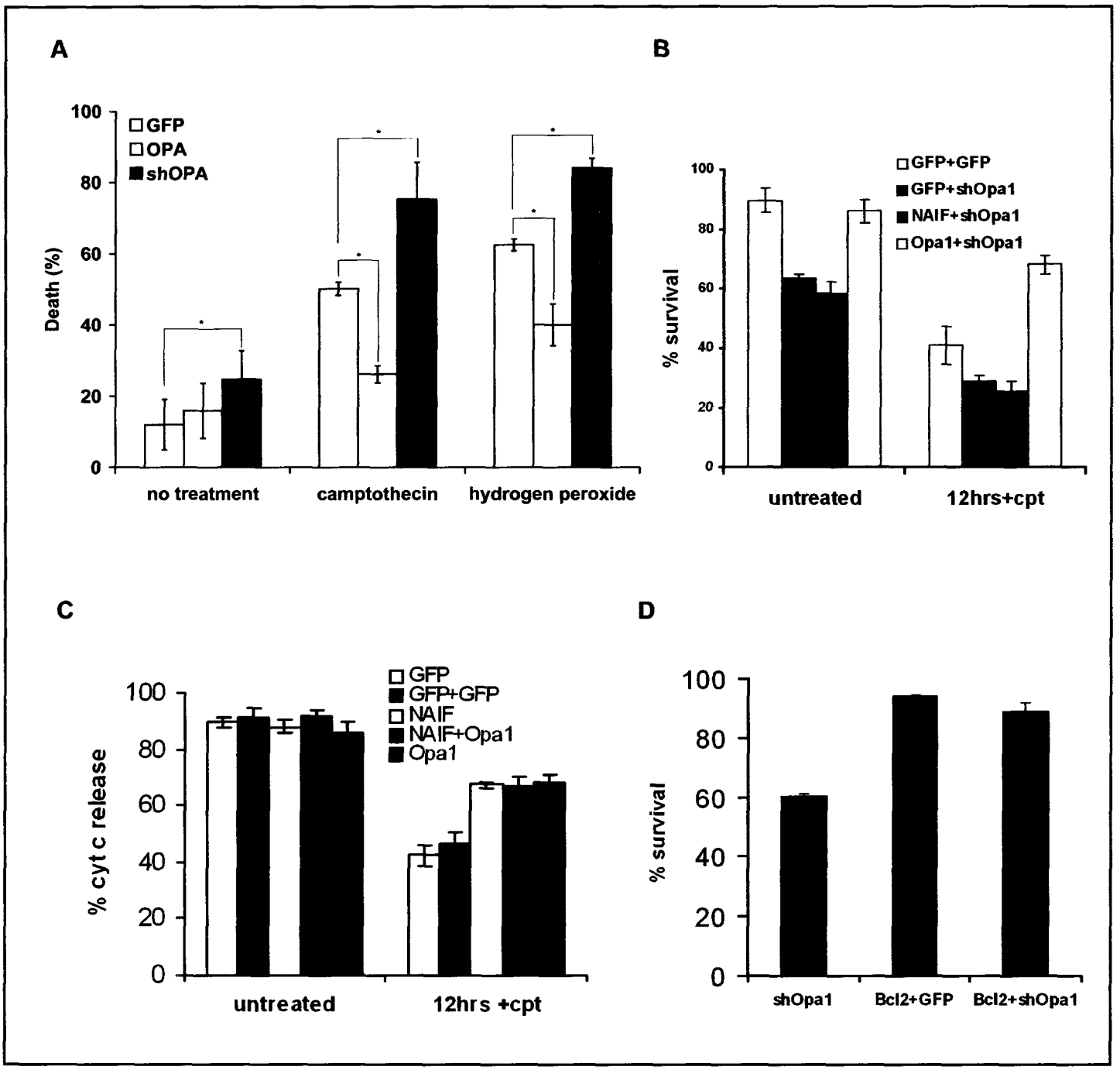
RESULTS

3.1. AIF is upstream of Opa1 in a common pathway crucial for the regulation of mitochondrial structure and function.

Together, the rescue of metabolic function, mitochondrial structural defects and improved cellular survival upon overexpression of Opa1 in AIF knockout neurons, indicate that the two proteins functionally interact in a common pathway. Furthermore, when Opa1 levels are reduced in wild-type cortical neurons, cellular survival is impaired. Since the expression of an anchored form of AIF targeted to the mitochondria is not able to rescue the survival of Opa1 knockout neurons, we postulated that AIF is upstream of Opa1 in a common pathway regulating mitochondrial structure and function. To consolidate this hypothesis, we performed a cell death assay on cortical neurons in which Opa1 was either overexpressed or downregulated. Since Opa1 is proposed to be downstream of AIF, elevating the levels of Opa1 should protect against apoptosis. Conversely, decreasing the amount of Opa1 should sensitize the cells to an apoptotic insult. Our results revealed that overexpression of Opa1 significantly protected the cells against apoptosis following camptothecin and hydrogen peroxide treatments (Figure 12 A). On the other hand, knocking down the amount of Opa1 greatly sensitized the neurons to both DNA damage and reactive oxygen species (ROS)-induced cell death (Figure 12 A). Hence, when AIF is released from mitochondria after an apoptotic insult, elevated levels of Opa1 increases cellular survival, and reduced levels of Opa1 sensitize neurons to cell death. This data supports our hypothesis that the two proteins are working through the same pathway and that Opa1 indeed fonctions downstream of AIF.

It has been previously reported by our group that overexpression of a mitochondria-anchored form of AIF (N-AIF) protects cortical neurons against

Figure 12. AIF is upstream of Opa1. (A) Quantitative analysis of cell death of wild-type neurons infected with Opa1, shOpa1 or GFP control after induction of apoptosis with camptothecin and hydrogen peroxide (n=3). **(B)** Quantitative analysis of cell death of wild-type neurons infected with GFP and Opa1 knock-down neurons infected with Opa1, N-AIF and GFP control after camptothecin treatment (n=3 embryos). **(C)** Quantitative analysis of cytochrome c release of wild-type neurons expressing N-AIF, Opa1, N-AIF+Opa1 compared to GFP control after camptothecin treatment (n=3 embryos). **(D)** Quantitative analysis of cell death of wild-type neurons expressing Bcl2+GFP and Opa1 knock-down neurons expressing Bcl2 after 3 days in vitro (n=3 embryos).


A
100
80
60
40
20
0
Death (%)
GFP
OPA
shOPA
no treatment
camptothecin
hydrogen peroxide
B
100
80
60
40
20
0
% survival
GFP+GFP
GFP+shOpa1
NAIF+shOpa1
Opa1+shOpa1
untreated
12hrs+cpt
C
100
80
60
40
20
0
% cyt c release
GFP
GFP+GFP
NAIF
NAIF+Opa1
Opa1
untreated
12hrs +cpt
D
100
80
60
40
20
0
% survival
shOpa1
Bcl2+GFP
Bcl2+shOpa1

camptothecin-induced cell death (Cheung et al., 2006). However, we were curious to see if N-AIF would retain its protective capacity in the absence of Opa1. Our experiments revealed that survival of Opa1 knockdown neurons was rescued by replacing Opa1 but N-AIF, similar to GFP control, failed to rescue the cells (Figure 12B). Furthermore, N-AIF did not offer any protection against camptothecin-induced cell death even though Opa1 overexpressing neurons did exhibit an increased survival, indicating that the presence of Opa1 is required for the protective activity of AIF (Figure 12B). Taken together, this experiment strengthens our hypothesis that AIF functions upstream of Opa1 on a common pathway.

It could be argued that knockdown of Opa1 irreversibly damages the neurons and therefore failure of N-AIF to rescue the survival of these cells may be due to non-specific effects from dying neurons. To control for this possibility, we overexpressed the anti-apoptotic protein Bcl-2, which would not be expected to rescue irreversibly damaged cells caused by loss of Opa1 but would block activation of the cell death pathway. Bcl-2 could rescue the survival of Opa1 knockdown cells back to wild-type levels (Figure 12 D), therefore, the inability of N-AIF to rescue survival of Opa1 knockdown cells is most likely a direct consequence of AIF requiring Opa1 to fulfill its biological function.

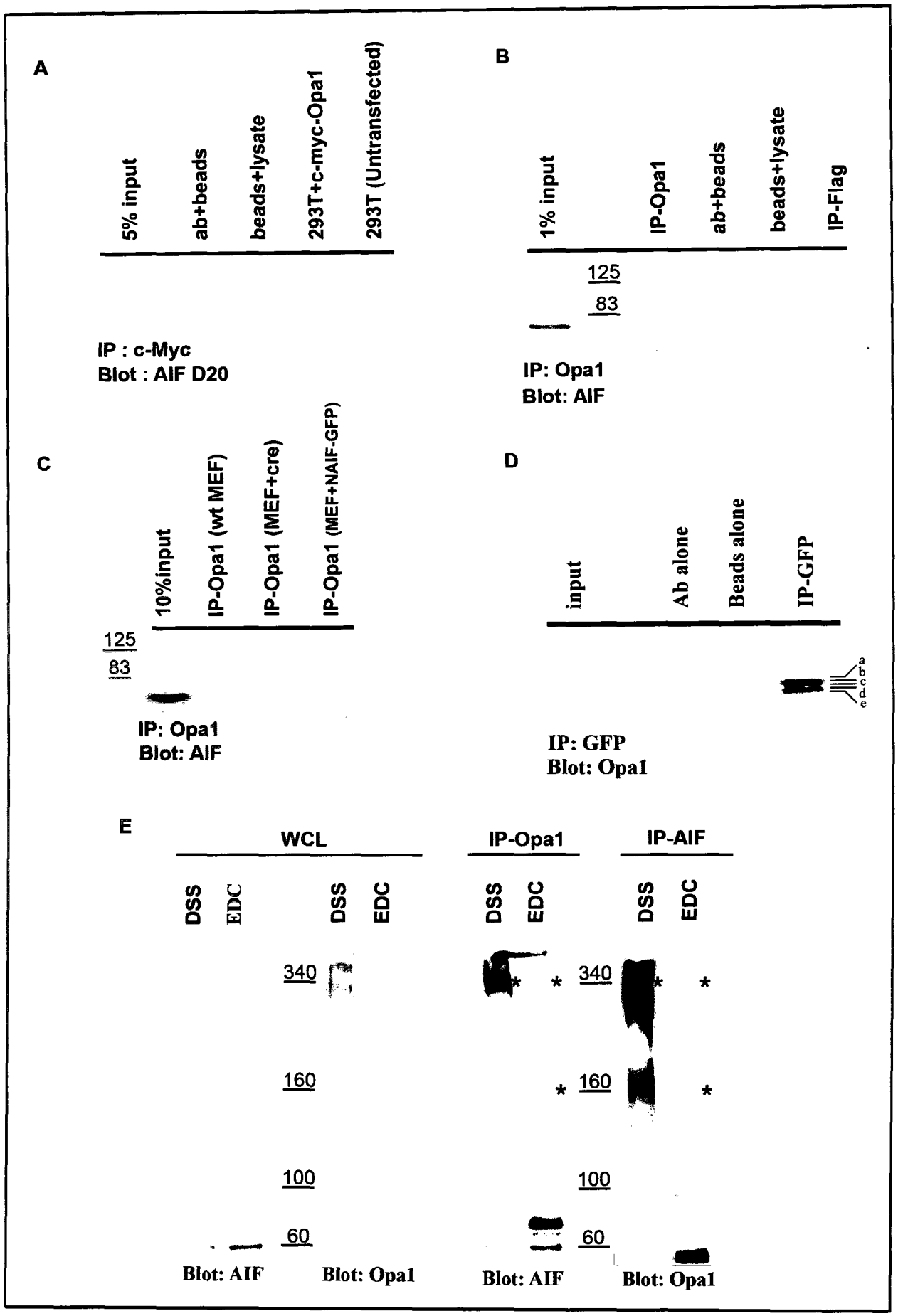
We know that N-AIF and Opa1 can independently delay cytochrome c release from mitochondria after a cell death insult (Cheung et al., 2006; Frezza et al., 2006) and Figure). However, it would be of interest to assess the protective activity of Opa1 and N-AIF when overexpressed together. If AIF and Opa1 are indeed in the same pathway, there should not be an additive protection against cytochrome c release. Around 70% of the neurons expressing N-AIF or Opa1 retained their mitochondrial pool of cytochrome C

after 12 hours treatment with camptothecin compared to 45% for the neurons expressing the GFP control (Figure 12C). Similarly, the neurons expressing N-AIF and Opa1 together exhibited the same level of protection as the neurons expressing the two proteins independently (Figure 12C), supporting the interpretation that the two proteins are working on a common pathway. Taken together, during apoptosis, we show that overexpression of Opa1 is protective but knockdown of Opa1 sensitizes the cells to death. Furthermore, we demonstrate that N-AIF is not protective in the absence of Opa1 and overexpression of Opa1 and AIF together do not show additive protection. Thus, we are proposing that AIF is upstream of Opa1 on a common pathway that is important in regulation of mitochondrial structure and function as well as apoptotic signaling.

3.2. AIF physically interacts with Opa1.

We have identified a strong functional interaction between AIF and Opa1 and both seem to be part of a common pathway important for the regulation of mitochondrial structure and function. Next, we asked whether the two proteins also physically interact. We first assessed this possibility by transfecting HEK293T cells with a myc-tagged construct of Opa1 and performed a co-immunoprecipitation assay. Immunoprecipitation with anti-myc antibody pulled down endogenous AIF with Opa1 (Figure 13 A). To validate the physiological relevance of this result, we then assessed if endogenous Opa1 and AIF could also physically interact in cortical neurons. Immunoprecipitation of Opa1 from isolated mitochondria was able to co-precipitate endogenous AIF (Figure 13 B). We successfully repeated this result using mitochondria isolated from HEK293T and MEFs, indicating that AIF interacts with Opa1 in various cell types under physiological

Figure 13. AIF physically interact with Opa1. (A) Immunoprecipitation of c-myc Opa1 in HEK 293T cells followed by western blotting against AIF. **(B)** Immunoprecipitation of endogenous Opa1 in cortical neurons followed by western blotting against AIF. **(C)** Immunoprecipitation of endogenous Opa1 in $AIF^{flox/flox}$ MEFs, $AIF^{flox/flox}$ + cre and $AIF^{flox/flox}$ +N-AIF-GFP followed by western blotting against AIF. **(D)** Immunoprecipitation of GFP tagged N-AIF in HEK293T cells followed by western blotting for Opa1. Opa1 isoforms are identified from a-e. **(E)** Mitochondria from a mouse liver were crosslinked with DSS or EDC. Immunoprecipitation of Opa1 was followed by western blotting against AIF and immunoprecipitation of AIF was followed by western blotting against Opa1. High molecular complexes of AIF and Opa1 are indicated by *.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5% input
ab+beads
beads+lysate
293T+c-myc-Opa1
293T (Untransfected)
IP : c-Myc
Blot : AIF D20
B
1% input
IP-Opa1
ab+beads
beads+lysate
IP-Flag
125
83
IP: Opa1
Blot: AIF
C
10%input
IP-Opa1 (wt MEF)
IP-Opa1 (MEF+cre)
IP-Opa1 (MEF+NAIF-GFP)
125
83
IP: Opa1
Blot: AIF
D
input
Ab alone
Beads alone
IP-GFP
a
b
c
d
e
IP: GFP
Blot: Opa1
E
WCL
IP-Opa1
IP-AIF
DSS
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340
160
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340
160
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Blot: AIF
Blot: Opa1
Blot: AIF
Blot: Opa1

conditions (data not shown). To confirm the specificity of this interaction, we developed an AIF-inducible knockdown system using primary mouse embryonic fibroblast (MEFs) derived from $AIF^{flox/flox}$ embryos. Infection on those cells with an adenoviral Cre, induces recombination of the AIF floxed allele within infected cells, knocking-out expression of the AIF protein. Under those conditions, immunoprecipitation of Opa1 was able to pull down endogenous AIF in control MEFs (Figure 13C) but failed to precipitate AIF in the AIF-deficient MEFs (Figure 13C). Furthermore, after infection with an adenoviral N-AIF-GFP, both wild-type AIF and the GFP-tagged protein were co-immunoprecipitated with Opa1 (Figure 13 C). This assay further validates the specificity of the interaction between the two proteins and suggests that AIF influences Opa1 activity by physically interacting with the protein.

Opa1 is a very complex protein with eight different mRNA splice variants which will give rise to 8 long isoforms of the protein. The latter can undergo further proteolytic cleavage at one or two different sites giving rise to the short isoforms of Opa1 (Delettre et al., 2001). The eight different Opa1 mRNA splice variants and subsequent proteolytic cleavage products can be resolved in at least 5 bands on a Western blot. The 2 heaviest bands are thought to be a mixture of long isoforms and the shorter bands represent processed isoforms (Ishihara et al., 2006). We were curious to see if AIF was interacting with both long and short Opa1 and if this interaction was specific to only one isoform. To assess this question, HEK293T cells were infected with N-AIF-GFP followed by an immunoprecipitation against GFP. Western blotting for Opa1 showed the typical 5 band pattern, indicating that AIF interacts with both the long and short forms of Opa1 (Figure 13 D). If AIF was interacting with one single splice variant of Opa1, only one long

isoform and one or two short isoforms would be co-immunoprecipitated with AIF, depending on which splice variant. Hence, we can also say that the interaction between the two proteins is not specific to only one variant of Opa1, although we cannot conclude specifically which splice variants are interacting with AIF. Hence, this new interaction between Opa1 and AIF provides evidence of a direct interaction but does not explain how the two proteins function to maintain mitochondrial structure and function. The following studies are designed to answer this important question.

3.3. AIF physically interacts with Opa1 oligomers.

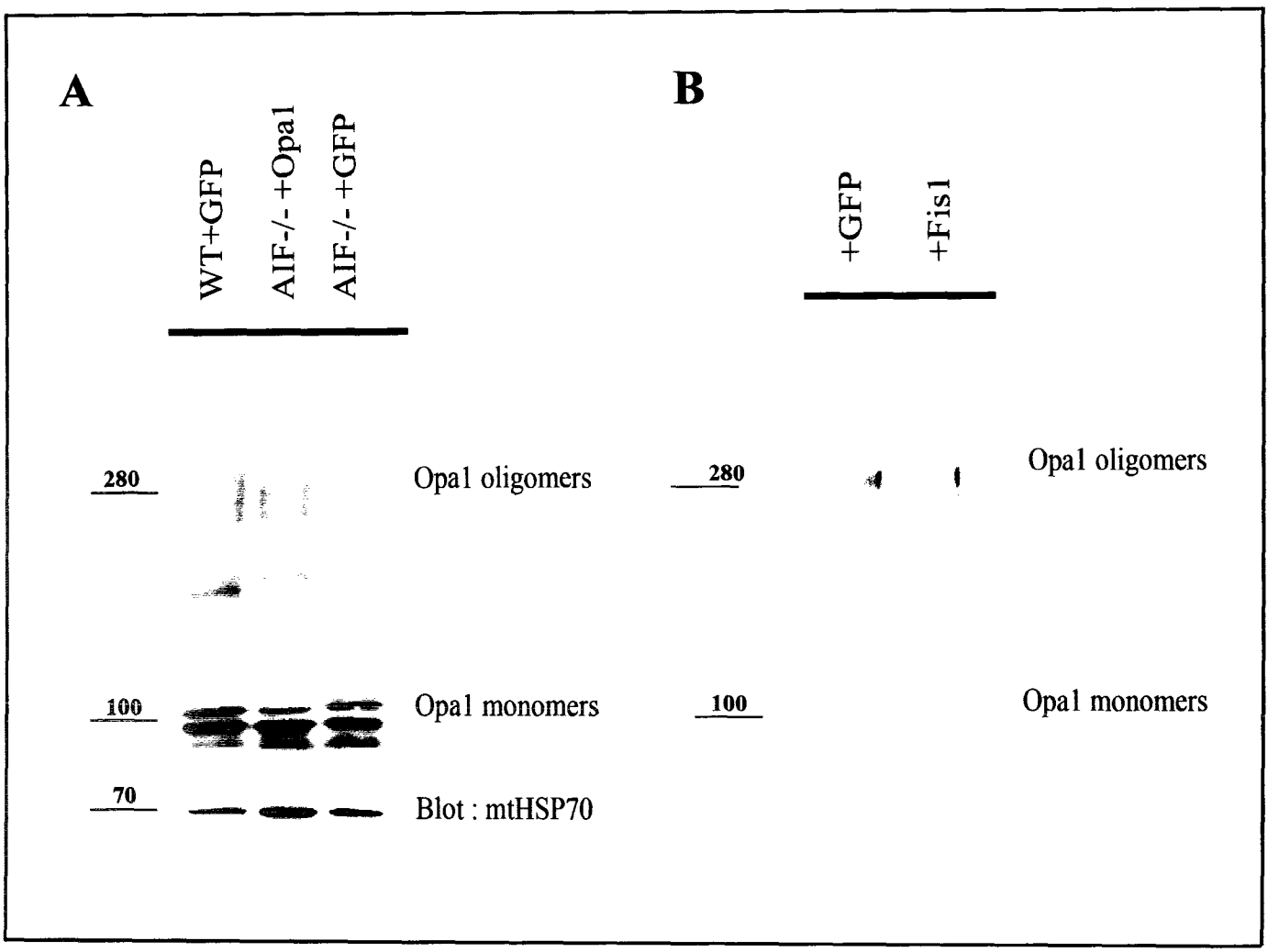
Apart from its monomeric form, it was previously reported that the long and short isoforms of Opa1 can assemble to create high molecular weight oligomers (Frezza et al., 2006). It was also proposed that those oligomers were formed at the base of the cristae and functioned to maintain the cristae junction in a closed conformation and prevent diffusion of intra cristae proteins to the inter-membrane space (Figure 8B). Since we have shown that AIF can interact with the monomeric form of Opa1, we were interested to see if it could also interact with the oligomeric complexes of Opa1. To test this possibility, freshly isolated mitochondria from a mouse liver were incubated with the primary amine crosslinker disuccinimidyl suberate (DSS). Solubilized proteins were then immunoprecipitated against Opa1 followed by Western blotting for AIF. Two bands enriched in AIF migrated at approximately 160 kDa and 300 kDa. The lower molecular weight band represents the monomeric form of Opa1 interacting with AIF and the high molecular weight band represents the oligomeric form of Opa1 binding to AIF (Figure 13 E). To confirm these results, we repeated the experiment with the zero-length crosslinker

1-ethyl-3-[3-dimethyl-aminopropyl]carbodiimide hydrochloride (EDC). Similar bands enriched in AIF were observed, further confirming that Opa1 oligomers also interact with AIF (Figure 13E). Also, when we immunoprecipitated AIF after crosslinking with DSS and EDC, we could see Opa1 enriched bands migrating at the same molecular weight (Figure 13E). All together, these results demonstrate that AIF can not only interact with the monomeric form of Opa1 but can also bind to the oligomeric complexes. This suggests that AIF may be modulating Opa1 activity by physically interacting with both forms of the protein.

3.4. AIF is implicated in the formation of Opa1 oligomers.

We next questioned the mechanism by which AIF controls Opa1 activity to maintain the mitochondrial structure: How may a direct interaction between AIF and Opa1 modulate Opa1 function? Previous studies have indicated that Opa1 forms oligomers which are essential to maintain cristae junctions while the absence of Opa1 results in mitochondrial cristae malformation and defective oxidative phosphorylation (Griparic et al., 2004; Frezza et al., 2006; Herlan et al., 2003). Since we have previously shown that AIF deficiency results in cristae malformation and here we show that AIF functions upstream of Opa1, we asked whether mitochondrial AIF may be required for the oligomerization of Opa1. To test this, mitochondria from wild type and AIF-/- neurons infected with GFP were subjected to cross-linking treatment with EDC, gradient gel electrophoresis and Western blot analysis. As previously described, Opa1 oligomers were clearly visible at 260-280 kDa as well as the monomeric protein (Figure 14 A)

Figure 14. AIF stabilizes Opa1 oligomers. (A) Mitochondria from wild-type neurons infected with GFP and AIF-/- neurons expressing Opa1 or GFP control were crosslinked with EDC and analyzed by gradient gel electrophoresis followed by western blotting against Opa1. **(B)** Mitochondria from wild-type neurons expressing Fis1 or GFP control were analyzed as described above.


A
WT+GFP
AIF-/- +Opa1
AIF-/- +GFP
280
Opa1 oligomers
100
Opa1 monomers
70
Blot : mtHSP70
B
+GFP
+Fis1
280
Opa1 oligomers
100
Opa1 monomers

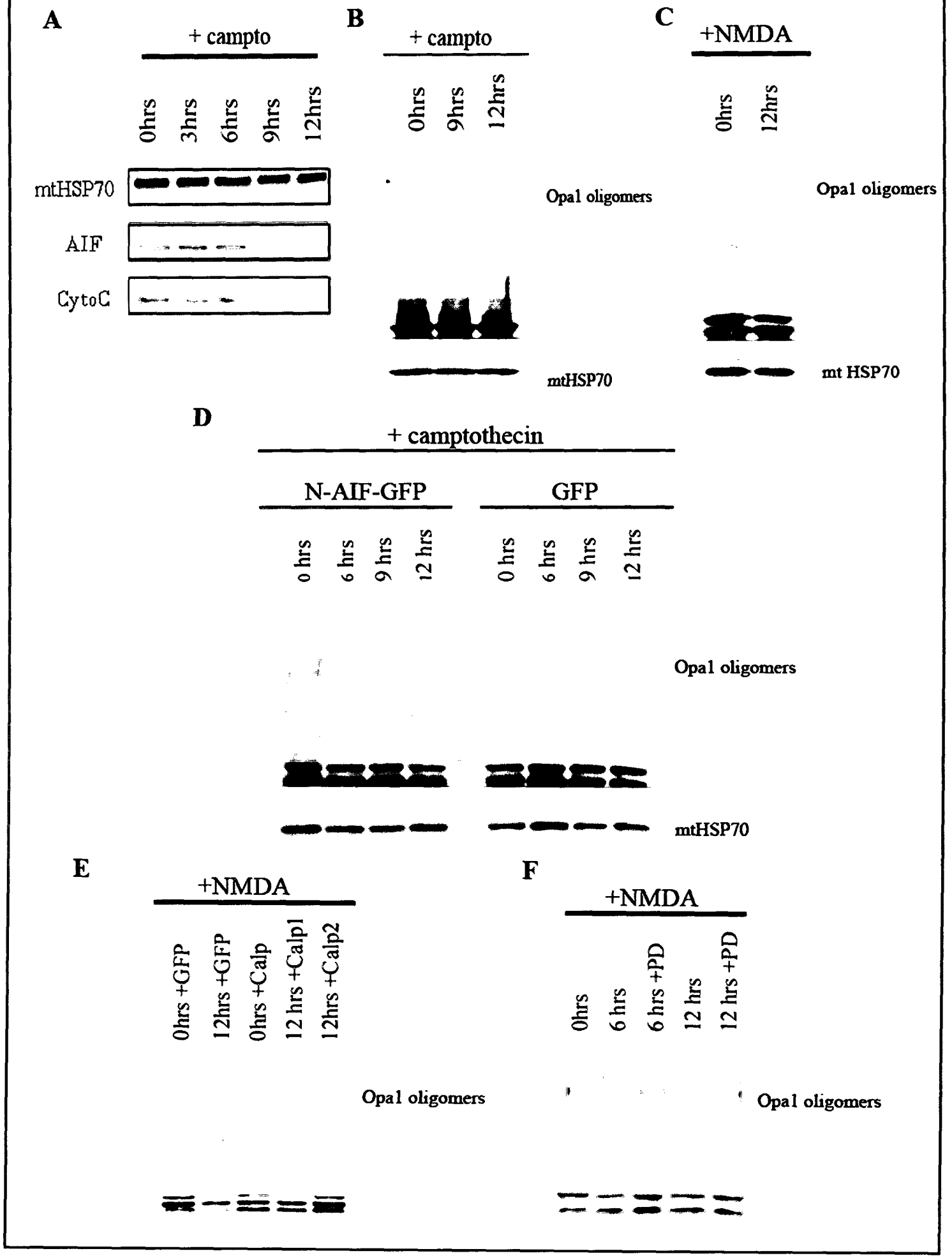
(Frezza et al., 2006). Unlike wild-type cells, crosslinking extracts from AIF-/- mitochondria revealed that they are almost completely depleted of high molecular weight Opa1 complexes (Figure 14 A, lane 3). Overexpression of Opa1 in the AIF-/- neurons significantly restored the oligomers, which explains why Opa1 can rescue the defects observed in AIF-/- neurons (Figure 14 A, lane 2). Since AIF-/- mitochondria are highly fragmented, one important control was to assess if Opa1 oligomers were lost due to the absence of an essential protein, AIF, or because of excessive fragmentation. To answer this question, we overexpressed the pro-fission protein Fis1 in wild-type cortical neurons to promote mitochondrial fission. Levels of Opa1 oligomers were similar in Fis1-expressing neurons and GFP controls (Figure 14 B). This confirms that the loss of oligomerization observed in AIF-/- neurons is caused by loss of the AIF protein and not a secondary effect due to mitochondrial fragmentation. Therefore, our results indicate that AIF is required for the oligomerization of Opa1 and that loss of AIF results in the instability and degradation of Opa1 oligomers.

3.5. AIF influence Opa1 oligomers during apoptosis signaling.

During apoptotic signaling, disruption of Opa1 oligomers is a critical step for efficient cytochrome C release (Frezza et al., 2006; Yamaguchi et al., 2008). If Opa1 oligomers are maintained after the induction of apoptosis, it can result in significant protection against cell death. However, the mechanism by which Opa1 oligomers are disrupted during apoptotic signaling remains unclear. In this study, we demonstrated that AIF is upstream of Opa1 in a common pathway regulating mitochondrial structure and that it physically interacts with Opa1 to maintain the stability of Opa1 oligomers under

normal conditions. To further investigate the relationship between the two proteins, we analyzed how AIF influences Opa1 under apoptotic conditions. Treatment of cortical neurons with camptothecin, a DNA-damage inducing molecule, results in gradual release of AIF and cytochrome C from the mitochondria (Figure 15A). After 12 hours of treatment, only a small fraction of AIF remains inside the mitochondria (Figure 15A). Similarly, there is a significant reduction in the amount of Opa1 oligomers 12 hours after treatment with camptothecin (Figure 15B). This result suggests that, during apoptotic signaling, release of AIF from the mitochondria leads to instability of the Opa1 oligomers and gradual degradation. To further confirm this observation, we also analyzed the response of Opa1 oligomers on NMDA-treated cortical granular neurons (CGNs). Activation of NMDA receptors increases the intra-cellular concentration of Ca^{2+} which in turns leads to intra-mitochondrial calcium overload, disruption of ATP production and production of free radicals (Dugan et al., 1995). Similar to camptothecin-treated cortical neurons, levels of Opa1 oligomers were decreased after a 12 hours treatment with NMDA (Figure 15C). Hence, disruption of Opa1 oligomers seems to be a general event after induction of different modes of apoptosis, which could be attributable to the release of AIF from the mitochondria and subsequent instability of the oligomers. To reinforce our hypothesis of the potential role of AIF in stabilizing Opa1 complexes, we next asked whether increased levels of mitochondrial AIF (N-AIF), which is not released during cell death (Cheung et al., 2006), could prevent the breakdown of the Opa1 oligomers during death signaling. We measured Opa1 oligomerization in neurons expressing N-AIF and in control neurons expressing GFP following camptothecin treatment. In the presence of anchored AIF, a reduction in the degradation of the Opa1 oligomers compared to the

Figure 15. AIF stabilize Opa1 oligomers during cell death. (A) Western blot of isolated mitochondria from wild-type neurons after a time course treatment with camptothecin. **(B)** Gradient gel analysis of Opa1 oligomers from cortical neurons after a time course treatment with camptothecin. **(C)** Gradient gel analysis of Opa1 oligomers from CGNs after a time course treatment with NMDA. **(D)** Gradient gel analysis of Opa1 oligomers from cortical neurons infected with N-AIF-GFP or control GFP after a time course treatment with camptothecin. **(E)** Gradient gel analysis of Opa1 oligomers from CGNs infected with Calpastatin (Calp1= 30MOI, Calp2= 100MOI) or control GFP after a time course treatment with NMDA. **(F)** Gradient gel analysis of Opa1 oligomers from CGNs treated with PD followed by a time course treatment with NMDA.


A
+ campto
0hrs
3hrs
6hrs
9hrs
12hrs
mtHSP70
AIF
CytoC
B
+ campto
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12hrs
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mtHSP70
C
+NMDA
0hrs
12hrs
Opa1 oligomers
mt HSP70
D
+ camptothecin
N-AIF-GFP
GFP
0 hrs
6 hrs
9 hrs
12 hrs
0 hrs
6 hrs
9 hrs
12 hrs
Opa1 oligomers
mtHSP70
E
+NMDA
0hrs +GFP
12hrs +GFP
0hrs +Calp
12 hrs +Calp1
12hrs +Calp2
Opa1 oligomers
F
+NMDA
0hrs
6 hrs
6 hrs +PD
12 hrs
12 hrs +PD
Opa1 oligomers

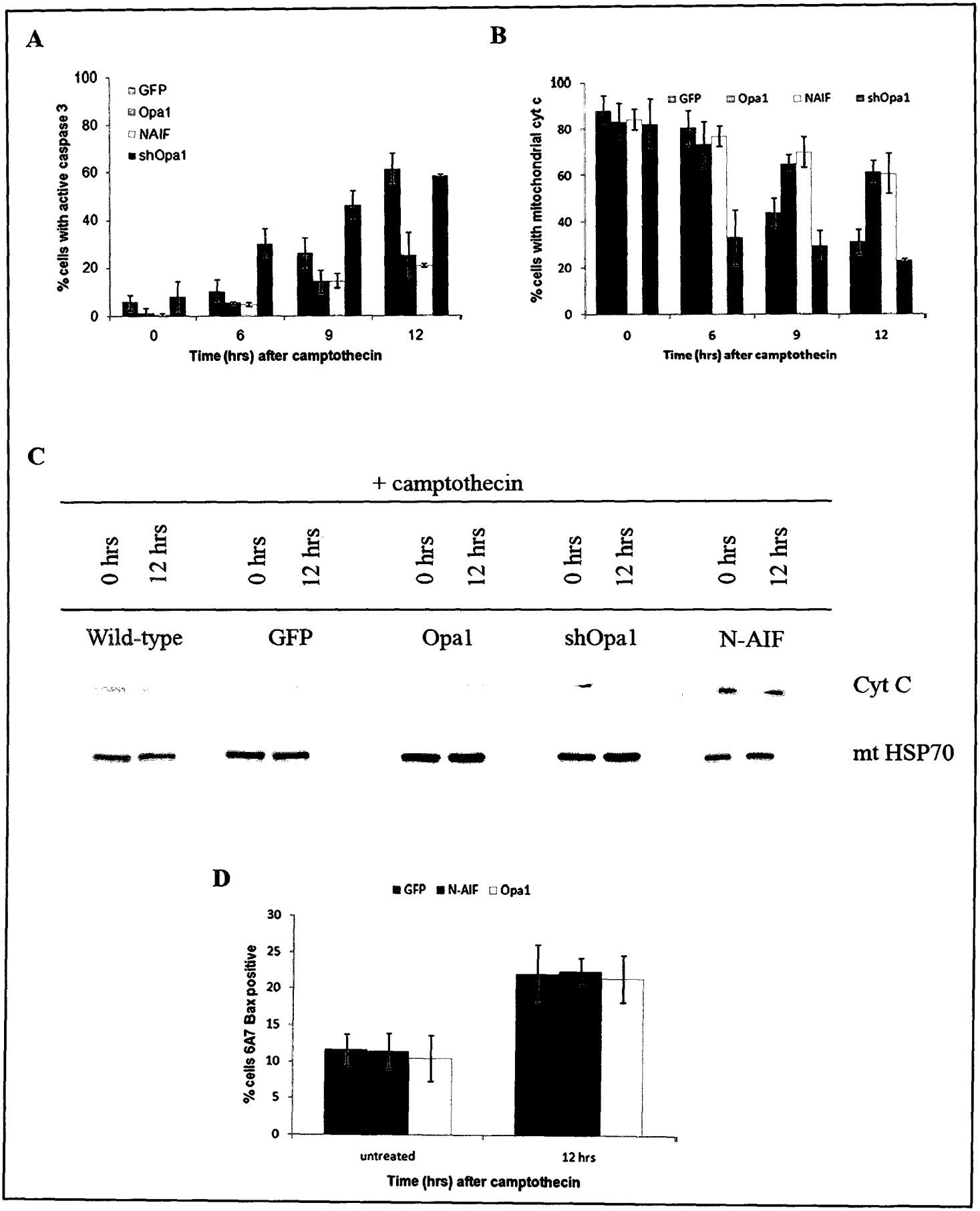
GFP control was observed (Figure 15D). Thus, when a portion of AIF is retained inside the mitochondria after the induction of cell death, Opa1 oligomers are more stable and degrade less rapidly. It was recently reported that activation of calpain I is responsible for cleaving AIF after induction of apoptosis and enabling it to translocate to the nucleus (Cao et al., 2007). In the same study, it was also shown that calpastatin, a general inhibitor of calpains, was sufficient to prevent AIF release from the mitochondria. Based on those results, we analyzed the stability of Opa1 complexes in CGNs infected with an adenovirus expressing calpastatin after a 12 hour treatment with NMDA. According to our hypothesis, maintaining AIF inside the mitochondria by inhibiting calpain activation should also result in stabilization of Opa1 complexes after induction of apoptosis. Control GFP-infected CGNs exhibited a decrease in the levels of Opa1 oligomers after 12 hour NMDA treatment (Figure 15E). However, CGNs infected with a low MOI of calpastatin virus exhibited a small protection against NMDA-induced Opa1 complexes degradation (Figure 15E, 12hrs + Calp1). Furthermore, increasing the multiplicity of infection to 100 MOI resulted in even stronger protection of the oligomers (Figure 15E, 12hrs + Calp2). Hence, similar to increasing the amount of intra-mitochondrial AIF, inhibiting AIF release from the mitochondria also results in stabilization of Opa1 complexes. To corroborate this result, we also treated CGNs with PD150606, a specific calpain inhibitor compound that blocks AIF release from the mitochondria during apoptosis, as recently shown in (Norberg et al., 2008). Six hours after the addition of NMDA, there was no difference in the levels of Opa1 oligomers between the untreated CGNs and those treated with PD150606 (Figure 15F). However, after 9 hours, there was a significant protection of the Opa1 complexes in the presence of PD150606 (Figure 15F). This result strengthens

our initial observation that inhibiting AIF release stabilizes Opa1 oligomers. Taken together, increasing the amount of intra-mitochondrial AIF or inhibiting AIF release during apoptosis results in stabilization of Opa1 complexes. Hence, these results confirm that the physical interaction between AIF and Opa1 functions to stabilize Opa1 oligomers under both steady-state and apoptotic conditions.

3.6. AIF stabilization of Opa1 oligomers delays cytochrome C release.

Throughout this work, we established a novel role of AIF in maintaining mitochondrial structure and function through its physical interaction with Opa1 to enhance the stability of Opa1 oligomers. The physiological relevance of this interaction under steady-state conditions is very clear since AIF deficient cortical neurons exhibit severe mitochondrial defects and compromised survival that can only be rescued by overexpression of Opa1. However, the consequence of this interaction between the two proteins under apoptotic conditions remains to be determined. As proposed by Frezza et al., 2006, disruption of Opa1 oligomers is required for efficient release of the intra-cristae pool of cytochrome C. They also suggested that overexpression of Opa1 protects against cell death by delaying cytochrome C release. Similarly, it was demonstrated by our group that N-AIF can also delay cytochrome c release and activation of caspase-3, protecting cortical neurons against apoptosis (Cheung et al., 2006). Based on our new results, we can now link the two proteins in the same pathway and explain how overexpression of either AIF or Opa1 can efficiently protect against apoptotic insults. Elevating the levels of mitochondrial AIF stabilizes Opa1 complexes during apoptosis (Figure 15 D).

Figure 16. AIF stabilization of Opa1 oligomers delays cytochrome c release. (A) Quantitative analysis of cytochrome c release of wild-type neurons infected with N-AIF, Opa1, shOpa1 and GFP control after a treatment with camptothecin (n=3). **(B)** Quantitative analysis of caspase-3 activation of wild-type neurons infected with N-AIF, Opa1, shOpa1 and GFP control after a treatment with camptothecin (n=3). **(C)** Western blot of the mitochondrial fraction from neurons infected with N-AIF, Opa1, shOpa1 and GFP control after a 12hrs treatment with camptothecin. **(D)** Quantitative analysis of Bax activation (6A7 Bax) of wild-type neurons infected with N-AIF, Opa1, shOpa1 and GFP control after a 12hrs treatment with camptothecin (n=3 embryos).


A
100
80
60
40
20
0
% cells with active caspase 3
GFP
Opa1
NAIF
shOpa1
0
6
9
12
Time (hrs) after camptothecin
B
100
80
60
40
20
0
% cells with mitochondrial cyt c
GFP
Opa1
NAIF
shOpa1
0
6
9
12
Time (hrs) after camptothecin
C
+ camptothecin
0 hrs
12 hrs
0 hrs
12 hrs
0 hrs
12 hrs
0 hrs
12 hrs
0 hrs
12 hrs
Wild-type
GFP
Opa1
shOpa1
N-AIF
Cyt C
mt HSP70
D
GFP
N-AIF
Opa1
30
25
20
15
10
5
0
% cells 6A7 Bax positive
untreated
12 hrs
Time (hrs) after camptothecin

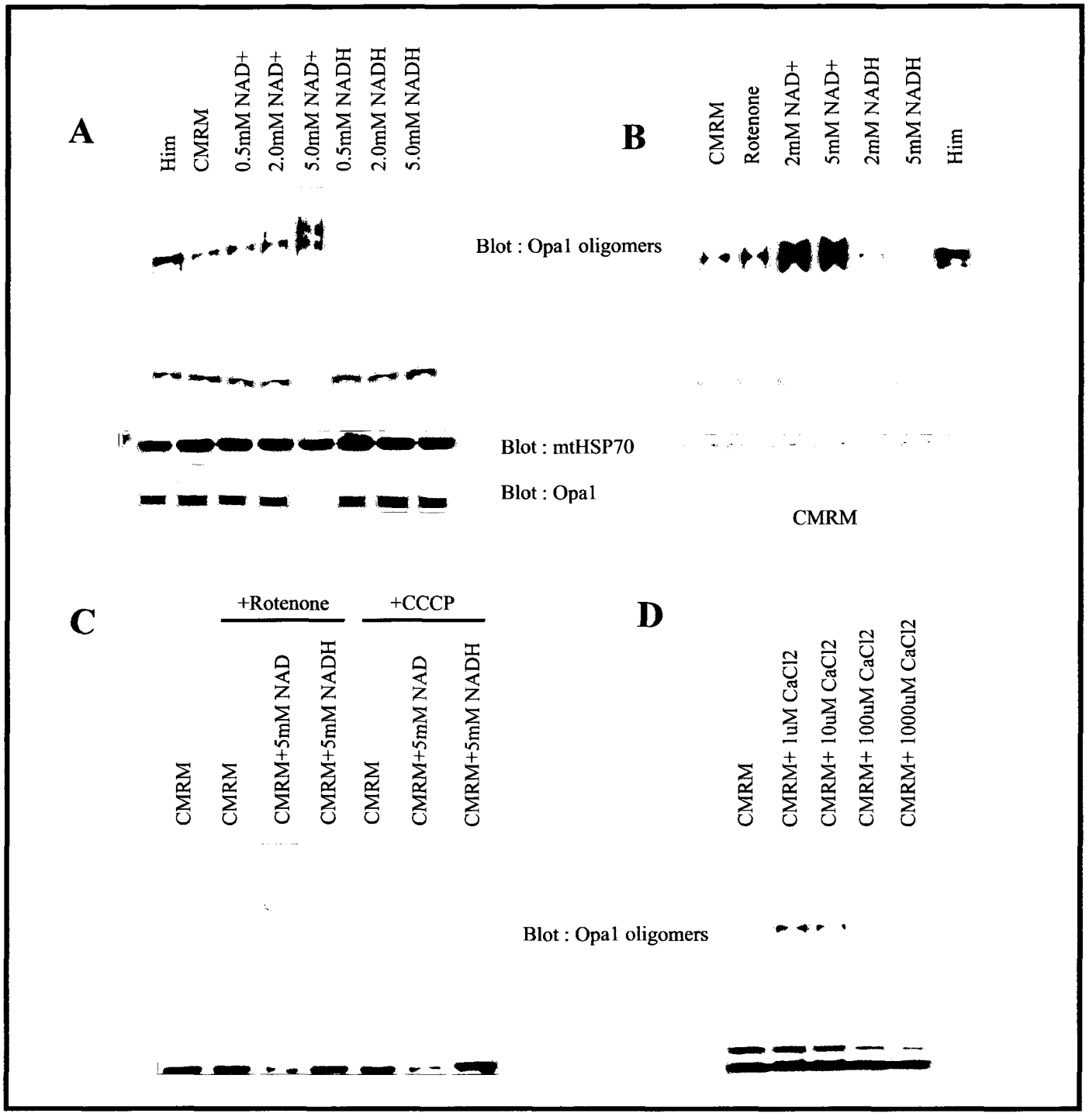
As a direct consequence, the rate of cytochrome C release is significantly reduced in N-AIF infected neurons compared to GFP control as shown by immunostaining and Western blotting techniques (Figure 16 A and C). Overexpression of Opa1 decreased cytochrome C release to the same extent as N-AIF and knock-down of Opa1 increased the rate of cytochrome C release (Figure 16 A). Our studies revealed that a lower rate of cytochrome C release correlated with a delay in the activation of caspase-3 (Figure 16 B). Hence, we propose that overexpression of AIF during cell death can offer significant protection by maintaining the stability of the Opa1 complexes and therefore delaying cytochrome C release. However, it could be argued that N-AIF protects against cell death by blocking Bax activation and subsequent cytochrome c release instead of stabilizing Opa1 oligomers. To test this possibility, we measured the activation of Bax (6A7Bax) in neurons infected with N-AIF after a treatment with camptothecin. N-AIF infected neurons displayed similar levels of 6A7Bax compared to Opa1 and GFP-overexpressing cells (Figure 16 D). This important result confirms that AIF protects against apoptosis by increasing the stability of Opa1 oligomers during apoptosis which in turn delays cytochrome C release and activation of caspase-3.

3.7. Opa1 oligomers are influenced by mitochondria metabolism.

Since we have shown that AIF is one factor that can influence Opa1 oligomers and structure of the mitochondria, we were curious to see if there were other factors that could also modulate the oligomeric state of Opa1. Mitochondrial metabolism is closely related to mitochondrial structure, as reported several years ago by Hackenbrock (1966). For example, mitochondria changing from a condensed state to an orthodox state

correlates with elevated levels of ADP, indicating a state of metabolic stress (Hackenbrock, 1966). To assess how Opa1 oligomers respond to mitochondrial metabolism, we incubated liver mitochondria in an isotonic buffer (HIM) or in an energizing buffer containing ATP and succinate (CMRM), followed by protein cross-linking and gradient gel analysis. The amount of Opa1 oligomers from the sample incubated in the HIM buffer is significantly greater than the sample incubated in CMRM (Figure 17A, lane 1 and 2). Thus, it seems that Opa1 oligomers are somehow sensitive to metabolism, as energizing the mitochondria can affect the quantity of oligomerized Opa1. To further pursue how the oligomers respond to the metabolic state, isolated mitochondria were incubated with NAD^+ or NADH. NADH, an essential component for ATP production, is a by-product of the Krebs cycle. When the electron transport chain (ETC) is active, NADH is being oxidized to NAD^+ at the level of complex I. Hence, in a cellular organism, high levels of NADH indicate that the cell has sufficient energy whereas high levels of NAD^+ acts as a sign of metabolic stress. To our great surprise, Opa1 oligomers also responded to NAD^+/NADH; increasing in the presence of NAD^+ and decreasing in the presence of NADH (Figure 17A). This response was also dependent on the amount of NAD^+/NADH, as shown by the gradual increase and decrease after NAD^+/NADH treatment. Since Opa1 is encoded by nuclear DNA and that our experiments were performed on isolated mitochondria, we can say that Opa1 oligomers are modified through post-translational processes and does not require *de novo* synthesis of the protein. To determine if a functional electron transport chain (ETC) is required for the response of Opa1 oligomers to a metabolic signal, we incubated the samples with rotenone, a complex I inhibitor. This compound did not seem to have a

Figure 17. The metabolism influences Opa1 oligomers. (A) Gradient gel analysis of Opa1 oligomers from isolated mitochondria incubated in HIM or CMRM supplemented with NAD+ or NADH. **(B)** Analysis of Opa1 oligomers from isolated mitochondria incubated in HIM (leftt blot) or CMRM (right blot) supplemented with rotenone and NAD+ or NADH. **(C)** Analysis of Opa1 oligomers from isolated mitochondria incubated in HIM (left blot) or CMRM (right blot) supplemented with rotenone, CCCP and NAD+ or NADH. **(D)** Gradient gel analysis of Opa1 oligomers from isolated mitochondria incubated in CMRM supplemented with $CaCl_2$.


A
Him
CMRM
0.5mM NAD+
2.0mM NAD+
5.0mM NAD+
0.5mM NADH
2.0mM NADH
5.0mM NADH
Blot : Opa1 oligomers
Blot : mtHSP70
Blot : Opa1
B
CMRM
Rotenone
2mM NAD+
5mM NAD+
2mM NADH
5mM NADH
Him
CMRM
C
+Rotenone
+CCCP
CMRM
CMRM
CMRM+5mM NAD
CMRM+5mM NADH
CMRM
CMRM+5mM NAD
CMRM+5mM NADH
D
CMRM
CMRM+ 1uM CaCl2
CMRM+ 10uM CaCl2
CMRM+ 100uM CaCl2
CMRM+ 1000uM CaCl2
Blot : Opa1 oligomers

significant effect on Opa1 oligomers (Figure 17 B and C). Also, they do not affect the capacity of the oligomers to respond to NAD^+/NADH treatment (Figure 17 B and C). Hence, it may be possible that a functional ETC is not directly affecting Opa1 oligomerization.

We were also interested to assess if a functional membrane potential was necessary for the oligomeric response of Opa1 to metabolic signals, since it is the driving force for ATP production. Treatment of isolated mitochondria incubated in HIM or CMRM with CCCP, a mitochondrial uncoupler, did not affect the capacity of the oligomers to respond to NAD^+/NADH treatment, suggesting that the mitochondrial membrane potential may not directly affecting Opa1 oligomerization (Figure 17 C). Although these results are very preliminary, it seems that Opa1 oligomers have evolved a specific response to the metabolic state of the cell that is directly dependent on the availability of NAD+ and NADH. Calcium is yet another important factor which could affect mitochondrial metabolism, and an increase in intra-mitochondrial calcium concentration can activate oxidative phosphorylation (Territo et al., 2001a; Territo et al., 2001b). Hence, if we incubate isolated mitochondria in CMRM supplemented with Ca^{2+}, ATP production will be activated, which should consume the intra-mitochondrial stock of NADH and elevate the amount of NAD+. If this is true, we should see an increase in the amount of Opa1 oligomers. Incubation of energized mitochondria with Ca^{2+} indeed increased the amount of Opa1 oligomers, further demonstrating that there is a link between mitochondrial metabolism and the oligomeric state of Opa1 (Figure 17 D). In conclusion, the results of our experiments have revealed that Opa1 oligomers stability under steady-state and apoptotic conditions is influenced by the presence of the

mitochondrial protein AIF but the amount of oligomers can vary depending on the metabolic state of the cell.

DISCUSSION

4.1. Summary of major findings

AIF-deficient cells exhibit highly fragmented mitochondria with dilated and unorganized cristae. As a consequence, mitochondrial metabolism as well as cellular survival, is greatly compromised. We have identified a novel role of AIF in regulating mitochondria structure and function by physically interacting with Opa1. AIF presence inside the mitochondrial inner-membrane seems to stabilize Opa1 oligomers under steady-state and apoptotic conditions. Since Opa1 oligomerization has been proposed to be important for proper cristae structure, we suggest that AIF influences mitochondrial cristae structure by maintaining the stability of Opa1 oligomers. In the absence of AIF, Opa1 oligomers are degraded, leading to aberrant cristae structure and exacerbated mitochondrial fragmentation. Since proper cristae structure is an important factor for mitochondrial metabolism, we think that loss of Opa1 oligomers due to the lack of AIF could result in dramatically impaired mitochondrial respiration, explaining the low ATP production and oxygen consumption of AIF knock-out neurons. Apart from AIF, we found that the metabolic state of the cell can also influence the amount of Opa1 oligomerization. Addition of excessive amounts of NAD^+ to isolated mitochondria enhances Opa1 oligomerization. On the other hand, elevated amount of NADH leads to a decrease in Opa1 oligomers. This response to NADH and NAD^+ seems to be independent of a functional ETC and membrane potential, since rotenone and CCCP did not significantly alter the response of Opa1 oligomers to the metabolic cues. Hence, the work presented here would be the first to propose that Opa1 oligomerization requires the presence of AIF, and that Opa1 oligomers levels are modulated by cell metabolism.

4.2. AIF modulates mitochondria structure through Opa1

It has been well demonstrated that the mitochondrial network can be modulated by the expression of members of the fission and fusion machinery (reviewed in Chan, 2006b). However, the severe structural and metabolic defects of AIF-deficient mitochondria were not rescued by promoting fusion or blocking fission (Figure 9). Instead, $AIF^{-/-}$ neurons could only be rescued by Opa1 (Figure 10). We believe that the ability of Opa1 to rescue the defects observed in the absence of AIF mainly relies on its capacity to control cristae structure. AIF and Opa1 function on a common pathway to regulate mitochondria structure. AIF cannot protect against cell death in the absence of Opa1, consistent with the interpretation that it acts upstream of Opa1 in this pathway (Figure 11 and 12). Since Opa1, but not Mfn2, can rescue both the structure of the cristae and mitochondria fragmentation in $AIF^{-/-}$ neurons, we suggest that AIF and Opa1 act upstream of Mfn2 and that Opa1 oligomers are essential for Mfn2 to stimulate mitochondrial fusion.

Since its discovery, AIF has been suggested to promote cell survival through various mechanisms. For example, it has been proposed to act as a ROS scavenger, a complex I and III stabilizer and even a member of the ETC (Klein et al., 2002; Vahsen et al., 2004; van Empel et al., 2005; Urbano et al., 2005; van Empel et al., 2006; Palmisano et al., 2007). Indeed, AIF does contain a FAD and NADH binding domains, which could mediate its oxydoreductase activity. However, a recent *in vitro* study by (Churbanova and Sevrioukova, 2007) indicated that AIF has a very low ability to transfer electrons. Hence, this conflicts with the idea of AIF being a member of the electron transport chain or being involved in the production of $O_2^{\cdot-}$. Also, in the same study, AIF could not reduce $O_2^{\cdot-}$,

arguing against its role as a ROS scavenger. Consistent with the work of Churbanova et al. 2007, we have preliminary results suggesting that AIF does not simply act as a mitochondrial ROS scavenger. Treatment of AIF-/- neurons with a free radical scavenger NAC (N-acetylcysteine) is not sufficient to completely rescue mitochondrial function and cell survival in the absence of AIF. This suggests that AIF acts beyond ROS scavenging in the mitochondria (Cheung, data not shown). It has been recently shown that AIF is part of complex I in heart mitochondria (Palmisano et al., 2007). If AIF is an integral member of complex I, however, it would be difficult to understand how AIF could also stabilize complex III, as suggested by Vahsen et al., 2004. A study performed by our group proposed a very different role for AIF. We identified a potential role for AIF as a mitochondrial structural protein (Cheung et al. 2006). We were the first to observe that AIF deficient mitochondria were highly fragmented with aberrant cristae structure. Re-introduction of a mitochondria-tethered variant of AIF (N-AIF) is sufficient to rescue the abnormal mitochondrial structure and the survival of these neurons.

In the present work, we propose that AIF modulates mitochondria structure by physically interacting with Opa1 monomers and oligomers (Figure 13). Although the amount of AIF being pulled down with Opa1 in the absence of crosslinker is somewhat low, this small interaction is important as our results indicate that AIF can modulate Opa1 function. We propose that the interaction between AIF and Opa1 might be transient or that only one AIF protein interacts with multiple Opa1 proteins. AIF-deficient cortical neurons exhibit a drastic reduction in the amount of Opa1 high molecular weight complexes, implying that AIF is required either for the formation or the stability of Opa1 oligomers. Since infection of AIF knock-out neurons with an adenovirus expressing Opa1

could rescue the levels of Opa1 oligomers back to wild-type levels, AIF is not required for Opa1 to oligomerize. Therefore, it is more likely that the role of AIF is to maintain the stability of the oligomers after their formation (Figure 14A).

Since enhancing fusion or blocking fission could not rescue AIF-/- neurons, we suggest that Opa1 rescues AIF deficient mitochondria by reforming oligomers rather than a direct consequence of Opa1 fusogenic activity. Under steady-state conditions, AIF can physically interact with Opa1 monomers and oligomers (Figure 13) to stabilize Opa1 oligomerization, which in turn maintains proper inner-membrane structure. Nevertheless, there are some questions remaining such as the mechanism by which mitochondrial fragmentation is rescued. Whether AIF regulates mitofusins activity through Opa1 or if Opa1 oligomers have a role in mediating fusion remains to be determined. Frezza et al. 2006 suggested that Opa1 oligomers can mediate formation cristae structure independently from mitochondrial fusion. However, the role of Opa1 oligomers in mediating fusion has not yet been assessed. Opa1 requires Mfn1 to complete inner-membrane fusion (Cipolat et al., 2004; Meeusen et al., 2006) and both short and long forms of Opa1 are required to efficiently promote fusion (Song et al., 2007). Since Opa1 oligomers are formed from both long- and short-Opa1, it is tempting to speculate that Opa1 oligomers may also be involved in inner-membrane fusion. Hence, it is possible that AIF has a dual function inside the mitochondria by affecting Opa1 oligomers: (a) it could regulate mitochondrial cristae structure, and (b) it may control mitochondrial inner-membrane fusion.

Another important question that needs to be addressed is how this interaction between AIF and Opa1 rescues mitochondrial metabolism. AIF has been suggested to

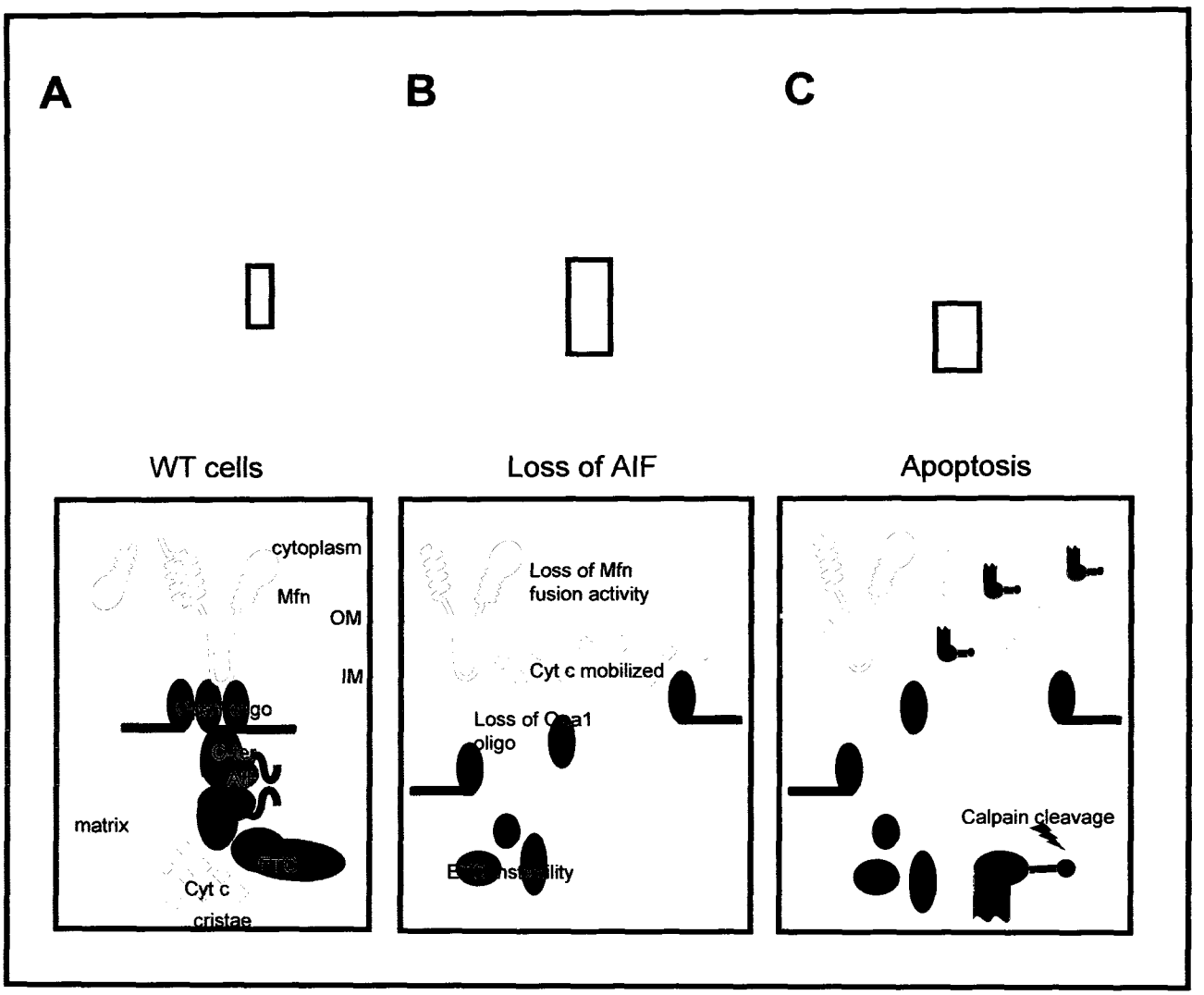
stabilize complex I and III and to participate in the electron transport chain by acting in proton transfer. Although AIF proton transferring activity has been recently disputed (Churbanova and Sevrioukova, 2007), we do not exclude the possibility that it acts as a stabilizer of Complex I and III. However, does it perform this role by directly binding to the complexes or by modulating the shape of the cristae? Although the relationship between cristae structure and mitochondrial metabolism is not well understood, there is evidence that cristae structure can influence mitochondrial metabolic activity (Hackenbrock, 1966; Mannella et al., 2001b; Zick et al., 2009). In our work, overexpression of Opa1 in AIF-deficient neurons can restore normal mitochondrial metabolism, which we attributed to the rescue of the cristae structure. Nevertheless, the mechanism by which Opa1 oligomers rescue mitochondrial respiration and ATP production remains unclear. One possibility might be that they simply rescue the structure of the cristae, which is required for proper assembly and stability of the electron chain complexes. Alternatively, AIF and Opa1 could be part of a large multi-complex composed of multiple structural proteins and complexes from the electron transport chain. Opa1 deficient mitochondria exhibit similar metabolic defects as $AIF^{-/-}$, such as elevated ROS production and decreased ATP production (Chen et al., 2005; Griparic et al., 2004; Herlan et al., 2003). Hence it is possible that AIF and Opa1 collaborate to stabilize ETC and ATP production. On the other hand, Opa1 oligomers may be necessary to complete mitochondrial fusion, and by rescuing Opa1 oligomerization in AIF-deficient mitochondria, mitochondrial fusion would be restored. Complexes of the electron transport chain are formed of multiple subunits, which are encoded by nuclear and mitochondrial DNA. One role of mitochondrial fusion is to exchange mtDNA between

the organelles, to ensure proper complementation and reparation (Chan, 2006a), (Sato et al., 2009). If fusion is blocked, a mitochondrion lacking or having mutant mtDNA would not be able to form correct ETC complexes and ATP production would be compromised (Chen et al., 2007). In AIF deficient neurons, rescue of Opa1 oligomerization may allow mitochondrial fusion and reparation of mutant mtDNA, resulting in functional ETC complexes. Although very complex, this novel role of AIF as a stabilizer of Opa1 oligomers demonstrates that mitochondria structure and dynamics are closely related to the metabolic activity (Figure 18).

4.3. AIF protects against apoptosis by delaying cristae remodelling

During the progression of apoptosis, one important step is the permeabilization of the mitochondrial outer membrane and the subsequent release of cytochrome C. Once in the cytosol, this protein initiates the caspase cascade. Inside the mitochondria, more than 80% of the cytochrome C pool is located inside the cristae, where it participates in ATP production. During cell death, mitochondrial cytochrome C is mobilized by remodelling of the structure of the cristae which is a well described event during apoptosis (Frezza et al., 2006; Scorrano et al., 2002; Germain et al., 2005). After an apoptotic insult, such as BIK or t-Bid treatment, both mitochondria cristae and the cristae junctions widen. This coincides with increased mobilization of cytochrome C to the inter-membrane space and its ensuing release to the cytoplasm following permeabilization of the outer membrane (Figure 8 B). As proposed by Frezza et al. 2006, Opa1 oligomers are responsible for maintaining the cristae junction in a "closed" conformation, keeping the bulk fraction of cytochrome C inside the cristae where it is used for

Figure 18. Model for AIF control of the mitochondria structure. (A) Under steady-state conditions, AIF interact with Opa1 to stabilize the oligomerization, maintaining proper cristae structure. In turn, this leads to proper functioning of the ETC and normal ATP production. Opa1 oligomers can also participate in mitochondrial fusion through the mitofusins. **(B)** When AIF is absent, Opa1 oligomers are unstable and degrade. This causes aberrant cristae structure and ETC dysfunction, leading to a decrease in ATP production. Loss of Opa1 oligomers also reduces mitochondrial fusion, causing excessive fragmentation. **(C)** During apoptosis, cleavage of AIF from the IM disrupts the interaction with Opa1, causing oligomers instability and degradation. This enhances the mobilization of cyt C to the IMS and subsequent release after MOMP.


A
B
C
WT cells
Loss of AIF
Apoptosis
cytoplasm
Mfn
OM
IM
matrix
Cyt c
cristae
Loss of Mfn
fusion activity
Cyt c mobilized
Calpain cleavage

mitochondrial respiration. According to this model, disruption of Opa1 oligomers during cell death results in cristae remodelling and release of cytochrome C. Inhibiting the breakdown of Opa1 complexes during apoptosis can protect against cell death by slowing down the release of the bulk fraction of cytochrome C from the intra-cristae space, and thereby delaying the activation of the caspase pathway.

We previously reported that a mitochondria-anchored form of AIF (N-AIF) can hinder cytochrome C release during apoptosis (Cheung et al. 2006), however, the mechanism by which AIF maintains cytochrome C inside the mitochondria was not clear. Since overexpression of Opa1 can rescue Opa1 oligomerization and delay cytochrome C release in apoptotic cells (Frezza et al. 2006, Figure 16 A and C), we asked whether AIF could stabilize Opa1 oligomers during cell death progression. We showed that Opa1 oligomers disruption during excitotoxicity (Figure 15 B-F) could be decreased by blocking AIF release (Figure 15 D-F). Therefore, in a physiological injury model, AIF can function as a pro-survival protein if retained inside the mitochondria.

Based on these previous observations, we predicted that stabilization of Opa1 oligomers by AIF would translate in a delay in cytochrome C release. DNA-damage was chosen as the apoptotic insult since AIF has been shown to translocate to the nucleus under those conditions (Wang et al., 2004; Cregan et al., 2002; Cregan et al., 1999). Expression of a mitochondria-tethered AIF variant could delay Opa1 oligomers disruption (Figure 15 D), showing that AIF can stabilize Opa1 oligomers under steady-state and apoptotic conditions. While the endogenous AIF could translocate to the nucleus, N-AIF remained inside the mitochondria and maintained the levels of Opa1 oligomers. When we analyzed cytochrome C release by immunochemistry and Western

blotting, we observed that the release of cytochrome C was much slower in neurons overexpressing Opa1 and N-AIF, even though active Bax levels were similar to control neurons (Figure 16 A-C). Hence, we suggest that N-AIF protects against cell death by delaying the mobilization of cytochrome C to the IMS by maintaining Opa1 oligomers during apoptotic signalling. Consistent with this, we also observed delayed caspase 3 activation, further demonstrating apoptosis is reduced in the mitochondria overexpressing N-AIF or Opa1 (Figure 16 B). Importantly, N-AIF could not protect against camptothecin treatment when the levels of Opa1 were decreased by siRNA infection (Figure 12 B). Hence, AIF requires the presence of Opa1 to perform its pro-survival role, providing further support to our model by which AIF could protect against an apoptotic insult by stabilizing Opa1 oligomers and by delaying cytochrome C release.

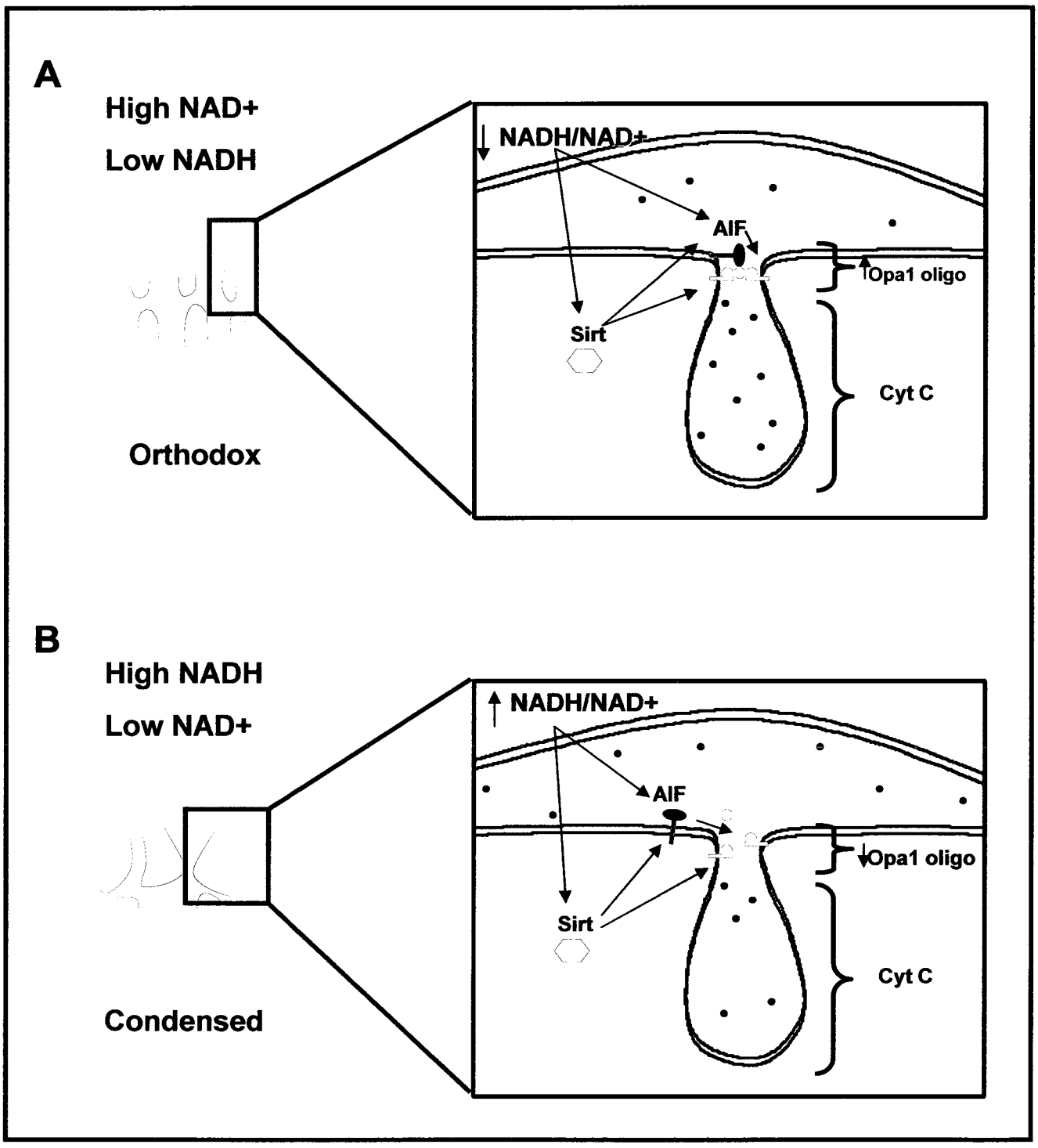
4.4. Metabolism affects the oligomeric state of Opa1

The shape of the mitochondria varies according to the metabolic state of the cell. Following an increase in intracellular ADP levels, mitochondria change their cristae structure from orthodox to condensed state (Hackenbrock, 1966). This metabolic-dependent variation in cristae structure is believed to enhance ATP production by enlarging and connecting the cristae, therefore increasing the diffusion rate of substrates. Similarly, when ADP levels are low, cristae volume decreases (ie orthodox state) to keep the diffusion rate steady. There are many proteins that play a role in controlling cristae structure, including Opa1. Since this protein can promote inner-membrane fusion as well as control the configuration of cristae junction, Opa1 would be an excellent candidate to execute the change from the orthodox to the condensed state. Opa1 has been shown to

regulate cristae structure by forming high molecular weight oligomers, and we demonstrate here that this process requires the presence of AIF. However, it remains unclear whether Opa1 oligomers levels are modulated during metabolic changes. In the present study, we demonstrate that Opa1 oligomerization is influenced by the metabolic state of the cell. We hypothesize that lower levels of oligomers correspond with larger cristae structure (similar to condensed state), as more oligomers would reflect a more thin and compact cristae (similar to orthodox state). This seems to correlate well with the observations described by Hackenbrock, whereby the mitochondria transform from the orthodox to the condensed state under excess amount of ADP and NADH.

Here we show that levels of Opa1 oligomers seem to be directly affected by the amount of NAD^+ and NADH (Figure 17 A). Those molecules are essential substrates for Electron Transport Chain, where NADH is oxidized to NAD^+ at complex I. Therefore, Opa1 can either directly sense NADH/NAD^+ levels or there is another mitochondrial protein that signals to Opa1 to influence its oligomerization. Incubation with rotenone did not affect Opa1 response to NADH and NAD^+, which could suggest that the activity complex I is not directly involved in modulating Opa1 oligomerization (Figure 17 B and C). Similar results were observed when mitochondria were uncoupled with CCCP (Figure 17 C). Therefore we hypothesized that mitochondrial cristae structure is dictated by the amount of substrate, more specifically the NADH/NAD^+ ratio. Under low amounts of NADH and ADP, mitochondria would increase the levels of Opa1 oligomers to reduce the volume of their cristae, switching to the orthodox state. As proposed by one group (Mannella, 2006a; Mannella et al., 2001a), this reduction in intra-cristae volume would increase the diffusion rate of the substrates, enabling the mitochondria to sustain efficient

Figure 19. Model of cristae remodelling during metabolic variation. (A) When the levels of ETC substrates such as NADH are low, mitochondria cristae will adopt an orthodox shape to enhance the diffusion rate of cyt C inside the cristae. To modulate the structure of the cristae, they increase Opa1 oligomerization. AIF could directly modulate Opa1 oligomerization by sensing the levels of NADH. Mitochondrial sirtuins could also sense the levels of NAD+ and propagate the signal directly to Opa1 or indirectly through AIF. **(B)** When the levels of substrates are high, mitochondria will switch to the condensed shape by reducing the levels of Opa1 oligomers. AIF and mitochondrial sirtuins could be implicated in this process.


A
High NAD+
Low NADH
Orthodox
NADH/NAD+
AIF
Opa1 oligo
Sirt
Cyt C
B
High NADH
Low NAD+
Condensed
NADH/NAD+
AIF
Opa1 oligo
Sirt
Cyt C

ATP production. Although this is yet to be confirmed by electron microscopy, we hypothesize that Opa1 levels correlate with the shape of the cristae. But how do Opa1 oligomers sense the levels of NADH and NAD^+ since structure analysis of the Opa1 protein did not reveal NADH or NAD^+ binding sites? It is probable that a NADH/NAD^+-binding mitochondrial protein signals to Opa1. Since we showed that Opa1 oligomerization is dependent on AIF and that the latter can directly bind to NADH, it is possible that AIF can serve as a metabolic sensor. However, we do not exclude the possibility that another sensor protein carries the message to Opa1 either through AIF or in an AIF-independent manner. Potential candidates include the mitochondrial sirtuins, Sirt-3 and -5 which are protein deacetylases and Sirt-4 which is an ADP-ribosyl transferase. The enzymatic activity of these proteins reflects the energy status of the cell since it depends on the NADH/NAD^+ ratio, since their catalytic activity requires hydrolyzation of NAD+ to NADH. Yet, very few targets of the mitochondrial sirtuins have been identified. However, a recent mass spectrometry study of mitochondrial proteins revealed that more than 130 proteins are acetylated, making them excellent substrates for the sirtuins (Kim et al., 2006). Since modulation of Opa1 oligomers levels does not require *de novo* synthesis of the protein, it would be possible that Opa1 undergoes rapid metabolic-dependent post-translational modification. For example, deacetylation of Opa1 when NAD+ levels are high could favour its oligomerization of the protein.

Another possibility could be that sirtuins indirectly modulate Opa1 oligomerization by acting on AIF, which would in turn propagate the signal to Opa1. Nevertheless, modulation of Opa1 oligomerization depending on the metabolic state of

the cell could be the missing link that would finally reunite cristae structure and mitochondrial metabolism, perhaps explaining how mitochondria modulate their shape according to the availability of substrates (Figure 19).

4.5. Future directions

4.5.1. Assess whether Opa1 oligomerization levels are responsible for determining mitochondrial respiration state

Hackenbrock reported that mitochondria change their internal structure according to the metabolic state of the cell. Excess of ADP and substrates force the mitochondria to adopt a condensed shape, which correlates with high energy production. However, how mitochondria can alter their inner-membrane structure according to the availability of substrate remained unknown. Therefore, it was not clear whether the transition from the orthodox to condensed shape was simply an artefact or if it was a precise and controlled event to optimize ATP production. We identified Opa1 as a potential candidate for the modulation of cristae structure according to the metabolic state of the cell. Therefore, it would be interesting to investigate whether Opa1 oligomerization status also correlates with the cristae shape dictated by the metabolism. Isolated mitochondria incubated with an excess of NADH exhibit low levels of Opa1 oligomers. Electron microscopy of mitochondria under such conditions needs to be performed in order to analyze the shape of the cristae when levels of Opa1 oligomers are low, to see if it correlates with the condensed shape described by Hackenbrock. Similarly, we would expect that NAD^+ reverts the mitochondria to the orthodox state by enhancing Opa1 oligomerization. This

would be the first direct evidence that Opa1 is dictating the metabolic-dependent shape of the cristae by modulation of its oligomerization.

4.5.2. Identify the pathway that signals the metabolic state to Opa1 oligomers

In this thesis, we demonstrated that modulation of Opa1 oligomers levels is dependent on the amount of NADH/NAD^+. Since our assays were performed on isolated mitochondria, modulation of Opa1 oligomerization does not require *de novo* synthesis of the protein. Opa1 does not have NADH or NAD^+ binding domains, therefore we suspect that another mitochondrial protein is responsible to sense NADH/NAD^+ levels and modulate Opa1 oligomerization. One possible candidate would be AIF, as we have demonstrated that it is involved in Opa1 oligomerization by stabilizing the complexes. AIF can directly bind NADH but does not exhibit a good proton transfer activity, which makes it a great candidate as a sensor of NADH levels. To determine how AIF affects the metabolic-dependent response of Opa1 oligomerization, one could create liver-specific AIF knock-out by crossing $AIF^{flox/flox}$ mice with mice carrying Cre driven by the albumin promoter (Postic et al., 1999). Incubation of AIF-deficient mitochondria with excess amounts of NADH or NAD^+ followed by analysis of Opa1 oligomers could reveal whether AIF has an additional role in modulating its levels depending on the metabolism. Mutagenesis of AIF's NADH-binding domain and subsequent analysis of Opa1 oligomerization could also reveal if AIF acts as a sensor of NADH to propagate the metabolic signal to Opa1. As we have previously mentioned, other possible candidates for such role are the mitochondrial sirtuins, Sirt-3, -4 and -5, since their enzymatic activity depends on the availability of NAD^+. It could be possible that one of the

mitochondrial sirtuins catalyzes a post-translational modification either directly on Opa1 or on AIF to influence the oligomerization of Opa1. Inhibiting the activity of those enzymes by incubating isolated mitochondria with nicotinamide would provide an indication of those proteins being implicated in modulating Opa1 oligomerization (Porcu and Chiarugi, 2005). Furthermore, one could knock-down specific sirtuins with commercially available siRNA to identify which one is involved in the modification of Opa1 oligomerization. Mass spectrometry may also be used to determine if Opa1 or AIF are acetylated under specific metabolic conditions and if this acetylation is removed when we change the metabolic settings. Finally, it will also be important to assess if Opa1 or AIF are substrates of specific mitochondrial sirtuins by performing an ELISA using an antibody directed against acetylated lysine (as described in Schlicker et al., 2008).

4.6. Conclusions

Mitochondria are vital organelles common to all eukaryotes. By producing the majority of the cellular ATP, they serve as the pillar of life for the cellular organism. Perhaps paradoxically; they are also central players in the induction of cell death. Understanding how mitochondria switch from sustaining life to inducing death could be a very powerful tool for modern medicine. This work provides one potential therapeutic candidate to influence on both life and death functions of the mitochondria. AIF, by regulating Opa1 oligomers, could be the protein linking cell survival and apoptosis. Inhibiting the binding of AIF to Opa1 in cancer cells would destabilize Opa1 oligomers, leading to the collapse of the cristae structure and excessive mitochondrial fragmentation. Consequently, mitochondrial metabolism would be deeply affected, producing low

amounts of ATP and high levels of ROS. By altering energy production and elevating the amount of free radicals, the survival of the cell would be greatly compromised. On the other hand, maintaining AIF interaction with Opa1 in injured cells could both sustain mitochondrial metabolism and delay the activation of apoptosis. During acute neuronal injury such an ischemic event, ATP production is severely impaired and collapse of the mitochondrial network drive the neuron to death. Maintaining the interaction between AIF and Opa1 under those conditions could restore proper mitochondrial structure and metabolic activity, as well as delaying the activation of apoptosis by hindering the release of cytochrome C. This short moment of grace might be sufficient for neurons to recuperate from the injury and interrupt the progression of cell death. Nevertheless, the novel function of AIF proposed in this thesis provides a clue on how mitochondria structure and dynamics can affect both life and death functions of the organelle and reveals how mitochondria structure fluctuate to promote apoptosis or metabolic efficiency.

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