Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death?

Céline Candé[1], Francesco Cecconi[2], Philippe Dessen[1] and Guido Kroemer[1,*]

[1]Centre National de la Recherche Scientifique, UMR1599, Institut Gustave Roussy, 39 rue Camille-Desmoulins, F-94805 Villejuif, France
[2]Department of Biology, University of Rome Tor Vergata, Roma, Italy
*Author for correspondence (e-mail: kroemer@igr.fr)

Journal of Cell Science 115, 4727-4734 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00210

Summary

Numerous pro-apoptotic signal transducing molecules act on mitochondria and provoke the permeabilization of the outer mitochondrial membrane, thereby triggering the release of potentially toxic mitochondrial proteins. One of these proteins, apoptosis-inducing factor (AIF), is a phylogenetically old flavoprotein which, in healthy cells, is confined to the mitochondrial intermembrane space. Upon lethal signaling, AIF translocates, via the cytosol, to the nucleus where it binds to DNA and provokes caspase-independent chromatin condensation. The crystal structures of both human and mouse AIF have been determined, and the fine mechanisms accounting for its oxidoreductase activity and its electrostatic interaction with double-stranded DNA have been elucidated. Importantly, the apoptogenic and oxidoreductase functions of AIF can be dissociated. Thus, mutations that abolish the AIF-DNA interaction suppress AIF-induced chromatin condensation, yet have no effect on the NADH oxidase activity. Recent studies suggest AIF to be a major factor determining caspase-independent neuronal death, emphasizing the central role of mitochondria in the control of physiological and pathological cell demise.

Key words: Apoptosis, Caspases, Cell death

Introduction

Although the seminal paper by Kerr, Wyllie and Currie (Kerr et al., 1972), who coined the word ‘apoptosis’, stated that only nuclei but not cytoplasmic organelles would undergo major modifications in the dying cell, recent evidence suggests that no cellular compartment is spared (Ferri and Kroemer, 2001). Mitochondria are particularly affected early during the apoptotic process, and they are now thought to act as central regulators of cell death. Indeed, several pro-apoptotic signal transduction and damage pathways converge on mitochondria to induce mitochondrial membrane permeabilization (MMP), and this phenomenon is under the control of Bcl-2-related proteins (Kroemer and Reed, 2000). MMP can occur as a result of a primary caspase activation but it often results from caspase-independent pathways (Green and Kroemer, 1998). Moreover, MMP leads to the activation of caspases and caspase-independent death effectors, mainly through the release of soluble intermembrane proteins that are normally secured behind the outer mitochondrial membrane. One caspase-activating intermembrane protein is cytochrome *c* (Liu et al., 1996), which triggers the proteolytic maturation of caspases within the apoptosome, the caspase activation complex including Apaf-1, caspase-9 and caspase-3 (Budijardjo et al., 1999). Other caspase activators include Hsp10 (which stimulates the apoptosome), Smac/DIABLO and HtrA2 (which both inhibit caspase-inhibitory IAP proteins). Caspase-independent effects can be attributed to ‘apoptosis-inducing factor’ (AIF) (Susin et al., 1999), endonuclease G, as well as HtrA2, which also posesses a serine protease activity (Ravagnan et al., 2002). Here, we summarize recent progress on the structure and function of AIF.

AIF: a phylogenetically old flavoprotein with a glutathione reductase-like fold

The mammalian AIF precursor contains an N-terminal mitochondrial localization sequence (MLS, residues 1-100) and a large C-terminal part (121-610) that shares similarity with bacterial oxidoreductases (Susin et al., 1999). AIF homologs are also found in invertebrates, including insects, nematodes, fungi, and plants, meaning that the AIF gene has been conserved throughout the eukaryotic kingdom (Fig. 1). In humans, one AIF homolog (AMID/PRG3), which is distantly related to AIF (Fig. 1), has been recently described to exert a pro-apoptotic function (Ohiro et al., 2002; Wu et al., 2002). According to conflicting reports, AMID/PRG3 may either be associated with the outer mitochondrial membrane (Wu et al., 2002) or localized in the non-mitochondrial cytoplasma (Ohiro et al., 2002).

The mature form of AIF (57 kDa) is generated by cleaving of the MLS, after import into the mitochondrial intermembrane space. Because it can stably bind FAD, AIF falls in the category of flavoproteins. AIF displays NAD(P)H oxidase as well as monodehydroascorbate reductase activities (Miramar et al., 2001). The overall crystal structure of mature mouse AIF has been recently resolved at 2.0 Å resolution. AIF displays a glutathione-reductase-like fold, with an FAD-binding domain (aa 122-262 and 400-477), an NADH-binding domain (263-399), and a C-terminal domain (478-610) that bears a small AIF-specific insertion (509-559) not found in glutathione reductase (Fig. 2a). The amino acids interacting with FAD and NADH have been mapped precisely, and the mutants E313A and K176A have been shown to reduce FAD binding (Mate et al., 2002). Human mature AIF (which is 92% identical to

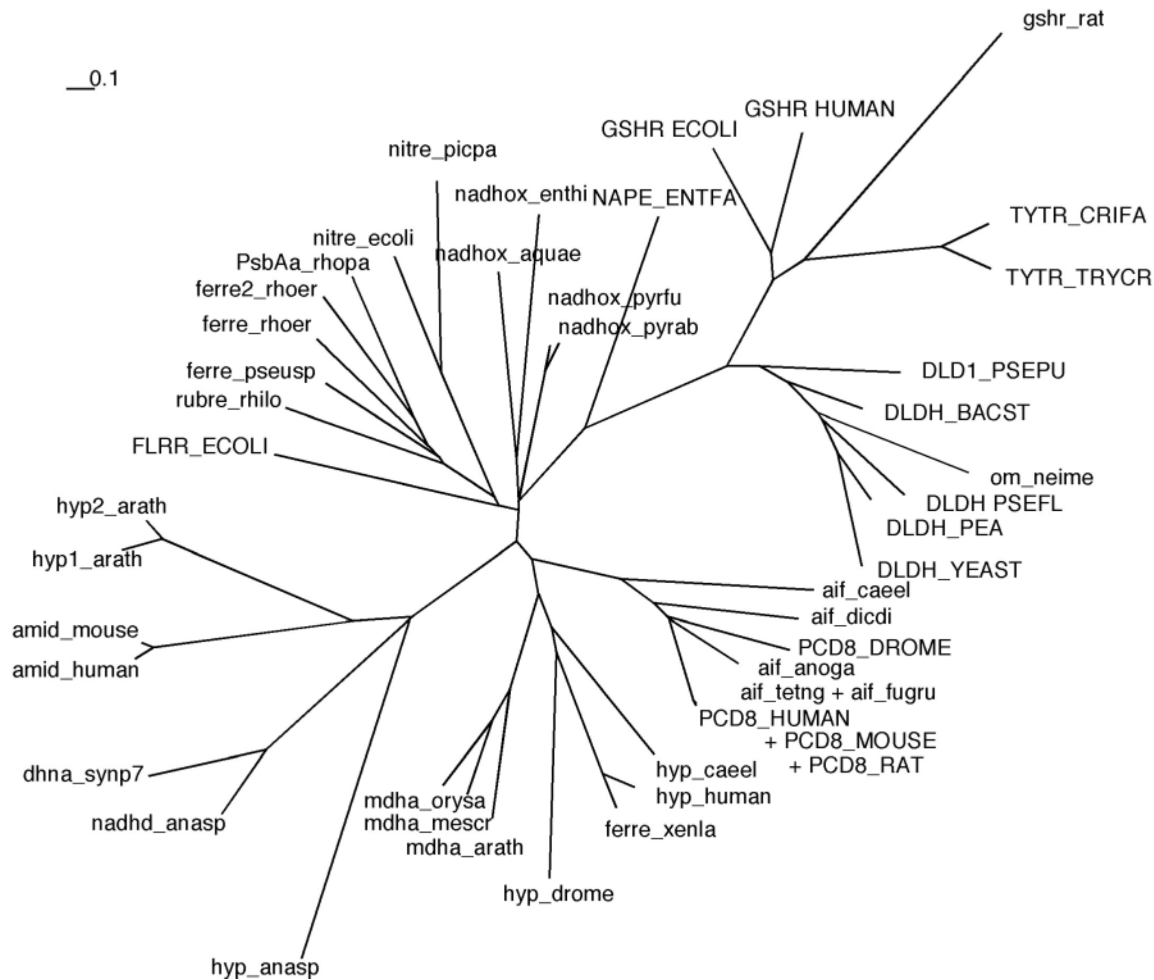

Fig. 1. Phylogenetic tree of the common pyridine nuleotide disulphide oxidoreductase domain. The limits are taken on the mouse AIF gene (offical name PCD8_MOUSE, positions 120 to 477). The alignment and the phylogenetc tree have been performed with the Clustal program, and the figure was produced with Treeview. Species names have been abbreviated according to the Swiss-Prot nomenclature. Upper case letters indicate the Swiss-Prot identification, while lower case letters correspond to mnemonic identification. aif_anoga, aif_tetng, AIF (*Tetraodon nigroviridis*); aif_caeel, Q9U229 (*C. elegans*); aif_disdi, Q9GRX6, AIF (*D. discoideum*); aif_fugru, AIF (*Fugu rubripes*); amid_human, AMID (*Homo sapiens*); amid_mouse, AMID (*Mus musculus*); dhna_synp7, Q935X8 (*Synechococcus* sp.); DLD1_PSEPU, dihydrolipoamide dehydrogenase (*Pseudomonas putida*); DLDH_BACST, dihydrolipoamide dehydrogenase (*Bacillus stearothermophilus*); DLDH_PEA, dihydrolipoamide dehydrogenase (*Pisum sativum*); DLDH_PSEFL, oxoglutarate dehydrogenase complex (*Pseudomonas fluorescens*); DLDH_YEAST, dihydrolipoamide dehydrogenase (*Saccharomyces cerevisiae*); EAA12325, (*Anopheles gambiae*); ferre_pseusp, Q52437, ferredoxin reductase (*Pseudomonas* sp.); ferre_rhoer, O69367, ferredoxin reductase (*Rhodococcus erythropolis*); ferre_xenla, O42346, neurula-specific ferrodoxin reductase-like protein (*Xenopus laevis*); FLRR_ECOLI, flavorubredoxin reductase (*E. coli*); GSHR_ECOLI, glutathione reductase (*E. coli*); GSHR_HUMAN, glutathione reductase (*H. sapiens*); gshr_rat, O89049, thioredoxin reductase (*R. norvegicus*); hyp_anasp, Q8YRY5 (Anabaena sp.); hyp_caeel, Q19655 (*C. elegans*); hyp_drome, O77266 (*D. melanogaster*); hyp_human, Q96NN9 (*H. sapiens*); hyp1_arath, Q9LXP4 (*Arabidopsis thaliana*); hyp2_arath, Q9C574 (*A. thaliana*); mdha_mescr, Q93YG1, monodehydroascorbate reductase (*Mesembryanthemum crystallinum*); mdha_orysa, Q8S3R2, putative cytosolic monodehydroascorbate reductase (*Oryza sativa*); mdha-arath, P92947, monodehydroascorbate reductase (*A. thaliana*); nadhd_anasp, Q8YPU6, NADH dehydrogenase (*Anabaena* sp.); nadhox_aquae, O67007, NADH oxidase (*Aquifex aeolicus*); nadhox_enthi, Q8WR54, NADH oxidase (*Entamoeba histolytica*).; nadhox_pyrab, Q9V0X9, NADH oxidase (*Pyrococcus abyssi*); nadhox_pyrfur, Q8U0Q3, NADH oxidase (*Pyrococcus furiosus*); NAPE_ENTFA, NADH peroxidase (*Enterococcus faecalis*); nitre_ecoli, Q8XEE3, nitrite reductase (NAD(P)H) subunit. (*E. coli*); nitre_picpa, Q9URM3, nitrite reductase (*Pichia angusta*); om_neime, OUTER MEMBRANE PROTEIN (*Neisseria meningitidis*); PCD8_DROME, AIF (*Drosophila melanogaster*); PCD8_HUMAN, AIF (*H. sapiens*);PCD8_MOUSE, AIF (*Mus musculus*); PCD8_RAT, AIF (*Rattus norvegicus*); PsbAa_rhopa, Q9XDW7 (*Rhodopseudomonas palustris*); rubre_rhilo, Q98BL3, rubredoxin reductase (*Rhizobium loti*); TYTR_CRIFA, trypanothione reductase (*Crithidia fasciculata*); TYTR_TRYCR, trypanothione reductase (*Trypanosoma cruzi*).

mouse AIF) has a very similar crystal structure, resembling that of oxidoreductases (Ye et al., 2002). Regardless of the presence or the absence of NAD(P)H and/or FAD (which is the essential prosthetic group of the oxidoreductase), AIF can induce nuclear apoptosis (Loeffler et al., 2001; Miramar et al., 2001). Similarly, the AIF-related protein AMID/PRG3 induces apoptosis even after deletion of large parts of the protein that share homology with the flavoprotein domain of AIF (Ohiro et

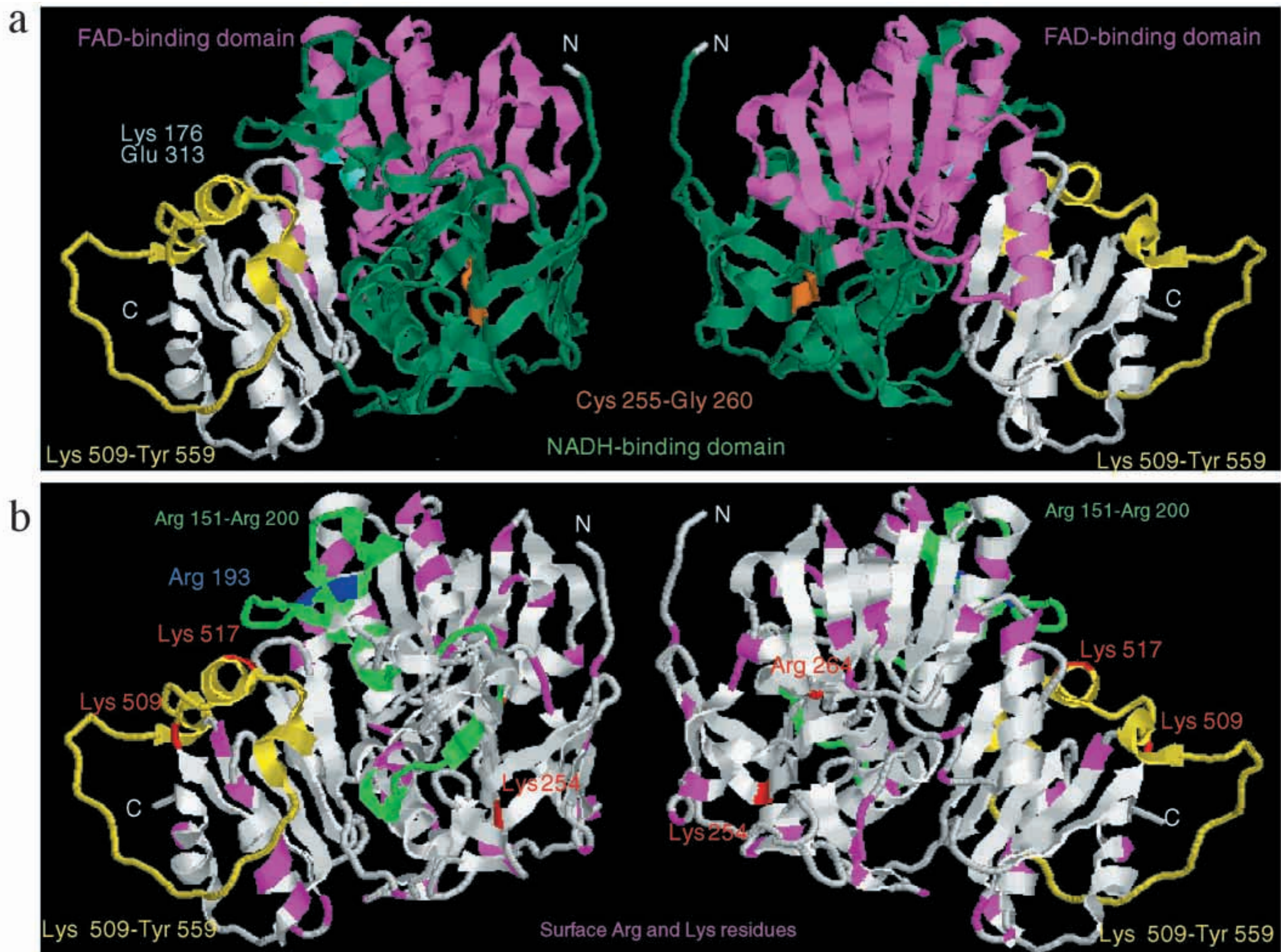

Fig. 2. Functional domain organization of AIF. (a) Domains of AIF important for its oxidoreductase function. The FAD-binding and NADH-binding domains of AIF are depicted. In addition, an AIF-specific insertion (509-559) not found in other proteins of this family has been marked. Residues whose mutation affect FAD binding (Lys176, Glu313, Cus255-Gly260) are indicated. (b) Domains of AIF important for its apoptogenic function. Amino acids with positive charges exposed at the surface are indicated. The surface-exposed area of AIF recognized by a neutralizing antibody (151-200) is also marked. Gain-of-function mutations are indicated in blue, while loss-of-function mutations are in red. Note that this model has been built on the crystal structure of mouse AIF, while data on gain-of-function and loss-of-function mutations of positively charged surface arginines and lysines have been obtained from human AIF.

al., 2002; Wu et al., 2002). Together, these data strongly suggest that the oxidoreductase function of AIF is not required for its apoptogenic action.

The redox reaction catalyzed by AIF in mitochondria in the living cell still remains elusive. Based on its similarity to prokaryotic oxidoreductases, it has been speculated that AIF might interact with the cytochrome bc1 complex, which catalyzes the electron transfer from ubihydroquinone to cytochrome *c* in the mitochondrial respiratory chain (Mate et al., 2002). Thus, AIF can catalyze the reduction of cytochrome *c* in the presence of NADH in vitro, meaning that cytochrome *c* is a possible electron acceptor for AIF (Miramar et al., 2001). Alternatively or in addition, AIF might fulfill some yet-to-be-characterized antioxidant function at the mitochondrial level.

AIF: a bifunctional protein attacking DNA

After an apoptotic insult, the mitochondrial outer membrane is permeabilized, and AIF translocates to the cytosol and the nucleus, where it induces peripheral chromatin condensation, as well as high-molecular-weight (50 kbp) DNA fragmentation. Translocation of AIF to the nucleus appears to be a general feature of apoptosis in mammalian cells (Cande et al., 2002). When cells are transfected with an AIF deletion mutant in which the MLS has been removed (AIFΔ1-100), AIF accumulates in the extramitochondrial compartment and in particular in nuclei, and this results in chromatin condensation and cell death (Loeffler et al., 2001). Moreover, the addition of recombinant AIF to purified nuclei suffices to cause chromatin condensation in vitro (Susin et al., 1999). These observations suggest that the mitochondrio-nuclear translocation of AIF participates in the apoptotic process.

The crystal structure of human AIF revealed the presence of a strong positive electrostatic potential at the AIF surface (Fig. 2b), despite its having a calculated neutral isoelectric point. Recombinant human and mouse AIF interacts with DNA, both

in vitro (in gel retention assays) and in vivo, in dying cells, where endogenous AIF becomes co-localized with DNA at an early stage of nuclear morphological changes, as indicated by immune electron microscopy. The electrostatic interaction between AIF and DNA is independent of the DNA sequence. Structure-based mutagenesis showed that DNA-binding-defective mutants of AIF, which were obtained by replacing positively charged residues by alanines, failed to induce cell death. The potential DNA-binding site identified from mutagenesis coincided remarkably well with computational docking of a DNA duplex to the AIF protein (Ye et al., 2002). Together, these findings suggest that DNA binding by AIF is required for its apoptogenic function, at least at the nuclear level. Two of the mutations that completely blocked the capacity of AIF to interact with DNA and to induce chromatin condensation (K255A, R265A and K510A,K518A), conserved NADH oxidase activity (Ye et al., 2002), thus confirming that the oxidoreductase and apoptosis-inducing activities of AIF can be fully dissociated. Note that one mutation that effectively abolished DNA binding (K510A,K518A) affects the C-terminal insertion in AIF (residues 509-559) not found in glutathione reductase (Fig. 2b), thus underscoring the probable relevance of this protein to apoptosis.

How AIF induces chromatin condensation and DNA fragmentation remains, however, an conundrum. Three possibilities can be envisaged. First, AIF could itself have some cryptic nuclease activity. Second, the interaction of AIF with DNA may increase the susceptibility of DNA to latent nucleases. Third, AIF might recruit downstream nucleases to induce partial chromatinolysis.

AIF: its complex relationship to caspases

The idea that AIF can induce caspase-independent death is based on several pieces of evidence. The mitochondrio-nuclear translocation of AIF is in a caspase independent fashion, at least in some examples of apoptosis [e.g. when cell death is induced by staurosporin (Susin et al., 1999) or by HIV infection (Ferri et al., 2000) and caspase activation is suppressed by the addition of chemical caspase inhibitors]. Similarly, the translocation of AIF can be observed in vitro in cells in which there is no caspase activation, owing to knockout of Apaf-1, caspase-9 or caspase-3 (Susin et al., 2000). This AIF translocation also occurs in vivo in mice lacking Apaf-1, which fail to activate caspases (Cecconi et al., 1998; Yoshida et al., 1998). In such mice, the interdigital web persists transiently during embryonic development, although interdigital cells eventually die without caspase activation, which allows generation of correctly formed toes (Cecconi et al., 1998; Yoshida et al., 1998). Additional evidence in favor of the caspase independency of interdigital cell death is that addition of a chemical caspase inhibitor to explanted embryonic limbs fails to inhibit cell death in vitro, although it does inhibit (caspase-dependent) chromatin condensation in inderdigital cells (Chautan et al., 1999). Importantly, it appears that, in dying interdigital *Apaf-1*$^{-/-}$ cells, AIF is overexpressed and translocates to the nucleus (Fig. 3). Similar observations have been obtained in *Apaf-1*$^{-/-}$, *caspase-9*$^{-/-}$ or *caspase-3*$^{-/-}$ embryoid bodies, in which AIF translocates from mitochondria to the nucleus when inner mass cells die during cavitation (Joza et al., 2001).

Microinjection or transfection of *Apaf-1*$^{-/-}$, *caspase-9*$^{-/-}$ or *caspase-3*$^{-/-}$ cells with AIF protein or AIFΔ1-100 cDNA, respectively, also induces cell death without caspase activation, but with some features of apoptosis, such as phosphatidylserine exposure, partial chromatin condensation and cellular shrinkage (Loeffler et al., 2001; Susin et al., 2000). In vitro, both purified natural AIF and recombinant AIF protein affect the structure of chromatin and cause large-scale DNA fragmentation in purified nuclei, in a fashion that is not influenced by chemical caspase inhibitors (Susin et al., 2000).

Together, these data indicate that AIF can act as a caspase-independent death effector. However, there is crosstalk between AIF and the caspase cascade at several levels. When caspase activation occurs early during apoptosis, for instance in CD95-triggered cell death, the release of AIF is secondary to activation of caspase-8 (Susin et al., 1997). Similarly, in etoposide-induced apoptosis, the activation of caspase-2 occurs upstream of MMP and presumably upstream of the release of AIF (Lassus et al., 2002; Robertson et al., 2002). Activated caspases and the caspase-activated protein *t*-Bid can trigger the release of AIF from purified mitochondria (Zamzami et al., 2000). Conversely, AIF can trigger the release of cytochrome *c* from isolated mitochondria in vitro (Susin et al., 1999). In several paradigms of cell death induction, AIF is released from mitochondria before cytochrome *c* (Daugas et al., 2000; Susin et al., 1999; Yu et al., 2002), and neutralization of AIF (by microinjection of an antibody or by knockout) (Ferri et al., 2000; Joza et al., 2001; Yu et al., 2002) can prevent cell death, as well as the mitochondrial release of cytochrome *c*. This suggests that, at least in some cases, AIF can be required for the cytochrome-*c*-dependent caspase activation cascade. However, in other examples of cell death, mitochondria release AIF well after cytochrome *c* (Cregan et al., 2002), which underlines the idea that different modes of MMP can operate in apoptosis. Another level of crosstalk between AIF and caspases may exist at the level of Hsp70. AIF interacts with Hsp70, an inhibitor of Apaf-1-dependent caspase activation (Ravagnan et al., 2001). Theoretically, AIF thus could indirectly (via Hsp70) de-inhibit the caspase cascade.

AIF and caspases may thus cooperate in the cell death cascade, and their contribution may depend on the specific apoptosis-inducing stimulus and perhaps the cell type. In several cases, it appears that the simultaneous neutralization of caspases and AIF is required to prevent hallmarks of apoptosis such as chromatin condensation. This applies for instance to staurosporin-induced death of mouse embryonic fibroblasts (Susin et al., 2000), to menadione-induced death of embryonic stem cells (Joza et al., 2001) or to p53-dependent death of cortical neurons (Cregan et al., 2002).

AIF: ontogeny recapitulates phylogeny?

Phylogenetic comparisons are widely used in the field of apoptosis to weight the relative importance of death pathways and to apprehend the 'original' death machinery. Thus, the essential contribution of caspases to developmental cell death in *Caenorrhabditis elegans* has been used as an argument in favor of the importance of caspases for mammalian cell death (Kaufmann and Hengartner, 2001). As mentioned above, several mitochondrial AIF homologs have been found in higher eukaryotes, including in kingdoms that lack clear-cut

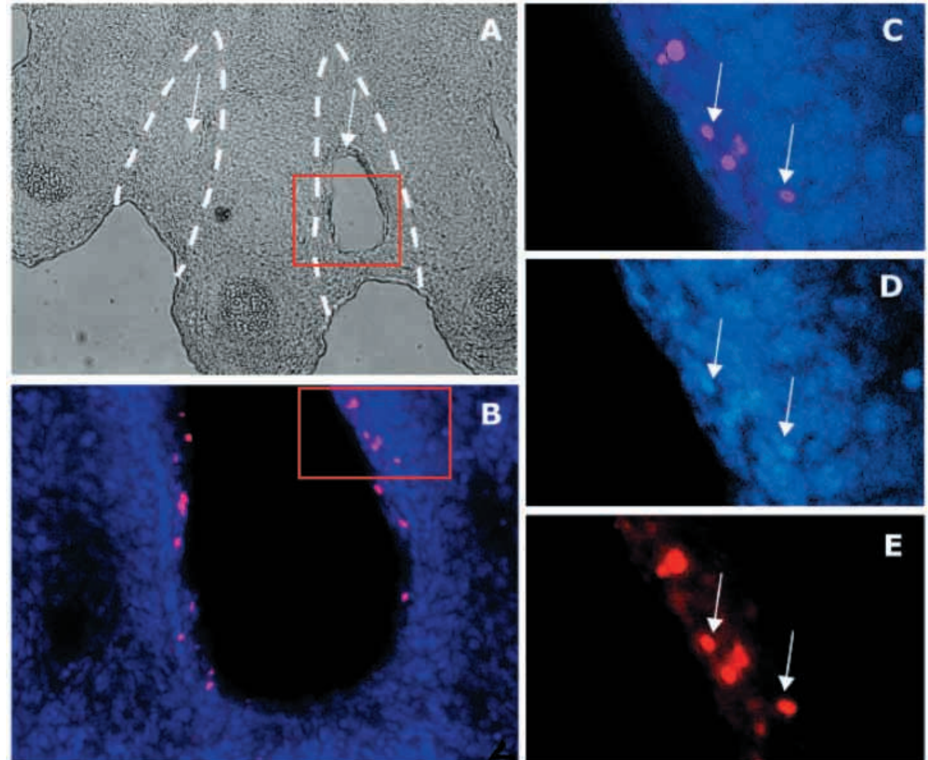

Fig. 3. Mitochondrio-nuclear AIF translocation in the interdigital cells of the mouse embryo. (A) Persistent interdigital web from an *Apaf*1$^{-/-}$ embryo (E15.5). The interdigital webs are marked by a dashed line. Holes are forming by Apaf1-independent death. The red insert is shown at higher magnification in B. (B) Double staining with Hoechst 33324 (blue) and an anti-AIF antibody (red). (C-E) Further magnification of the rectangle shown in B. Note that condensed nuclei (D) contain AIF (E) within the same cells (overlay in C). Arrows in C, D and E point to the same dying cells.

homologues of caspases such as plants and fungi. Intriguingly, an AIF homolog normally localizes to mitochondria and translocates to nuclei during developmental cell death of the slime mold *Dictyostelium discoideum*. Since recombinant *D. discoideum* AIF can induce chromatin condensation in purified human HeLa nuclei (Arnoult et al., 2001), AIF might have conserved its function throughout the evolution of programmed cell death. It is important to note that *D. discoideum* is normally unicellular, yet can aggregate to a pluricellular organism upon starvation. Programmed cell death occurs only in a specialized subset of differentiated stalk cells, after formation of the metozoan structure, and thus exemplifies the very first example of ‘altruistic’ death. The genome of *D. discoideum* does not contain any obvious homolog of animal caspases, although it does contain more distantly related caspase homologues, the so-called para-caspases (Aravind et al., 2001). Moreover, apoptosis of *D. discoideum* cells proceeds normally in the presence of chemical caspase inhibitors (Olie et al., 1998). It will be important, however, to provide definitive proof of the involvement of AIF in *D. discoideum* cell death (and that of other model organisms) by deleting the corresponding gene.

The genetic inactivation of mitochondrial apoptogenic factors has been employed to assess their relative contributions to apoptosis in mice. Thus, inactivation of Smac/DIABLO (an inhibitor of inhibitors of apoptosis (IAPs), which inhibit caspases) has no detectable phenotype; lack of caspase-9 causes brain hyperplasia with perinatal lethality; lack of cytochrome *c* results in a severe mitochondriopathy with deficient apoptosis and embryonic death on day 10. These phenotypes are relatively weak compared with that of the double knockout of the two pro-apoptotic Bcl-2 family proteins Bax and Bak, which participate in the apoptotic permeabilization of mitochondrial membranes (Table 1). The most severe phenotype results from the inactivation of AIF, which abolishes cavitation, an apoptosis-mediated process indispensable for early embryonic morphogenesis, before gastrulation. Note that cavitation-associated apoptosis does not appear to require caspase activation, since it occurs normally in the presence of the caspase inhibitor Z-VAD.fmk, as well as in embryos lacking Apaf-1, caspase-9 or caspase-3 (Joza et al., 2001). Thus, the absence of AIF is embryonically lethal, at a very early stage, and abrogates the first wave of caspase-independent programmed cell death occurring during mammalian development, shortly after formation of the pluricellular embryo. Whether the phenotype of the AIF knockout is a consequence of some sort of mitochondriopathy (assuming that AIF has a normal, presumably redox-related mitochondrial function) is currently unknown. An indirect argument against this possibility is furnished by the observation that AIF-deficient embryonic stem cells can differentiate into cells from all three germ layers both in vitro and in vivo. Thus ES cells injected into immunodeficient mice develop histologically undistinguishable teratocarcinomas, irrespective of the status of the *AIF* gene, which suggests that AIF is not generally required for proliferation and differentiation in vivo (Joza et al., 2001). To resolve definitively the question of whether the redox activity of AIF influences cell death control, it will be important to perform knock-in mutations in the *AIF* gene that affect either its redox activity or its DNA binding.

AIF thus appears to be involved in programmed cell death at early stages of ontogeny and phylogeny. However, this hypothesis requires further experimental evidence.

Involvement of AIF in pathological apoptosis

The mitochondrio-nuclear translocation of AIF has thus far been encountered in all models of regulated cell death in which it has been studied. In some models of apoptosis, AIF appears to be particularly important. This may apply to the death of postmitotic neurons, which contributes to the pathogenesis of both acute and chronic (neurodegenerative) diseases. Acute neuronal apoptosis can be induced by trauma, hypoglycemia

Table 1. Characteristics of knockout mice

Knockout	Phenotype	References
Bax, Bak	Perinatal lethal; neurological defects, persistence of interdigital webs, imperforate vagina, excess numbers of cells in CNS and hemopoietic system; mice are resistant to Fas-induced liver degeneration; MEFs are resistant to a wide range of apoptotic stimuli including tBid transfection, growth factor deprivation, staurosporine, etoposide, UV irradiation and ER stress.	Lindsten et al., 2000 Wei et al., 2001
Apaf-1	Perinatal lethal; defective neuronal apoptosis, brain malformations, craniofacial abnormalities, retinal hyperplasia; ES cells, MEFs and thymocytes undergo delayed cell death in response to cytotoxic drugs; normal negative selection in thymocytes.	Yoshida et al., 1998 Cecconi et al., 1998 Hara et al., 2002
Caspase-3	Lethality at 3-5 weeks of age; defective neuronal apoptosis; T cells resistant to antigen-induced death; abnormal apoptotic morphology in dying cells.	Kuida et al., 1996 Woo et al., 1998
Caspase-9	Perinatal lethal; impaired neuronal apoptosis; ES cells, MEFs and thymocytes generally resistant to intrinsic death stimuli such as DNA damage in vitro.	Kuida et al., 1998 Hakem et al., 1998
Cytochrome c	Lethality after E10; MEF and ES cells resistant to UV irradiation, staurosporine and growth factor withdrawal.	Li et al., 2000
Smac/DIABLO	No discernible phenotype.	Okada et al., 2002
AIF	Embryoid bodies fail to undergo apoptosis-mediated cavitation. ES cells are partially resistant to serum deprivation (in the absence of Z-VAD.fmk) as well as to menadione (in the presence of Z-VAD.fmk. ES cells die normally in response to staurosporin, etoposide, UV irradiation, azide and anisomycin in vitro.	Joza et al., 2001

or transient ischemia and involves cytotoxic agents as diverse as excitotoxins, reactive oxygen species (ROS) and DNA damage. The translocation of AIF has been observed in several paradigms of neuronal apoptosis, including death of photoreceptors induced by retinal detachment (Hisatomi et al., 2001), neuronal cell death induced in vivo by brain trauma (Zhang et al., 2002) and death of cortical neurons induced in vitro by exposure to heat-inactivated *Streptococcus pneumoniae* (Braun et al., 2001), hydrogen peroxide, peroxynitrite (Zhang et al., 2002), the topoisomerase I inhibitor camptothecin, infection with a p53-expressing adenovirus (Cregan et al., 2002), or the excitotoxin *N*-methyl-D-aspartate (NMDA) (Yu et al., 2002). The DNA-damage-induced AIF translocation and apoptosis has been shown to depend on the presence of p53 and its transcriptional target Bax (Cregan et al., 2002). NMDA-induced apoptosis, conversely, has been shown to rely on the presence of poly(ADP-ribose) polymerase (PARP) (Yu et al., 2002). The molecular mechanisms accounting for PARP-dependent AIF translocation are obscure. It has been speculated that the PARP-mediated depletion of NAD might activate AIF translocation (Yu et al., 2002).

In several instances, microinjection of an antibody recognizing a surface-exposed domain of AIF (residues 151-200, Fig. 2b) prevents cell death but caspase inhibition alone has no beneficial effect on neuronal survival (Braun et al., 2001; Yu et al., 2002; Zhang et al., 2002). Assuming that the anti-AIF antibody has no additional effects (for instance on AIF-related proteins), this suggests that AIF contributes to cell killing. Similarly, local injection of Z-VAD.fmk fails to inhibit photoreceptor apoptosis induced by retinal detachment, although injection of nerve cell growth factor prevents the mitochondrio-nuclear translocation of AIF in photoreceptors and maintains the photoreceptors functional, as far as can be judged from electroretinograms (Hisatomi et al., 2002). In a model of neurotrauma, the translocation of AIF in selected brain areas could be correlated with genomic DNA degradation to ~50 kbp fragments (which is a hallmark of AIF-mediated nuclear apoptosis), although the cells lacked oligonucleosomal DNA fragmentation (which is mediated by caspase-activated DNase) (Zhang et al., 2002). Thus, AIF could be involved in several paradigms of pathogenic cell death. The conditional or neuron-specific knockout of AIF should clarify to what extent AIF does indeed contribute to neuronal cell loss.

Perspectives

Apoptosis has been conceptually tied to the activation of catabolic hydrolases in two waves. During the 80s, it was generally assumed that DNases were responsible for execution of the cell. When this notion turned out to be wrong, during the 90s, most investigators became convinced that caspases were responsible for cell death. Indeed, in many cases, caspase inhibition delays and attenuates morphological features of apoptosis such as chromatin condensation. Oligonucleosomal DNA fragmentation, one of the historic hallmarks of apoptosis, is entirely caspase dependent. Nonetheless, it has become increasingly clear that pharmacological inhibition or genetic disruption of caspase activation pathways in mammals frequently fails to confer true cytoprotection against environmental stress, pathogenic insults or developmental cell death. It will be important to investigate which caspase-independent death effector is rate limiting for cell killing, in which circumstances and, in particular, to what extent and through which exact mechanisms AIF contributes to apoptotic signaling and cell death execution. It will also be tempting to explore the possibility of modulating AIF function, by generating small AIF-activating or AIF-inhibiting molecules, the aim being to use such compounds for the therapeutic manipulation of apoptosis. The recent elucidation of the crystal structure of AIF should facilitate this task.

This work has been supported by a special grant form the Ligue Nationale contre le Cancer, as well as by grants from ANRS and European Commission (QLG1-1999-00739 to G.K.). F.C. is an Assistant Telethon Scientist. We are grateful to Elisabeth Ferraro (Rome) for the AIF immunofluorescence experiment.

References

Aravind, L., Dixit, V. M. and Koonin, E. V. (2001). Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. *Science* **291**, 1279-1284.

Arnoult, D., Estaquier, J., Tatischeff, I., Tissier, J. P., Grodet, A., Dellinger,

M., Kahn, A., Ameisen, J. C. and Petit, P. X. (2001). On the evolutionary conservation of the cell death pathway: mitochondrial release of an apoptosis inducing factor during Dictyostelium discoideum cell death. *Mol. Biol. Cell* **12**, 3016-3030.

Braun, J. C., Novak, R., Murray, P. J., Eischen, C. M., Susin, S. A., Kroemer, G., Halle, A., Weber, J. R., Tuomanen, E. I. and Cleveland, J. L. (2001). Apoptosis-inducing factor mediates microglial and neuronal apoptosis causes by pneumococcus. *J. Infect. Dis.* **184**, 1300-1309.

Budijardjo, I., Oliver, H., Lutter, M., Luo, X. and Wang, X. (1999). Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol.* **15**, 269-290.

Cande, C., Cohen, I., Daugas, E., Ravagnan, L., Larochette, N., Zamzami, N. and Kroemer, G. (2002). Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie* **84**, 215-222.

Cecconi, F., Alvarez-Bolado, G., Meyer, B. I., Roth, K. A. and Gruss, P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. *Cell* **94**, 727-737.

Chautan, M., Chazal, G., Cecconi, F., Gruss, P. and Golstein, P. (1999). Interdigital cell death can occur through a necrotic and caspase-independent pathway. *Curr. Biol.* **9**, 967-970.

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

Daugas, E., Susin, S. A., Zamzami, N., Ferri, K., Irinopoulos, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J. et al. (2000). Mitochondrio-nuclear redistribution of AIF in apoptosis and necrosis. *FASEB J.* **14**, 729-739.

Ferri, K. F. and Kroemer, G. K. (2001). Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* **3**, E255-E263.

Ferri, K. F., Jacotot, E., Blanco, J., Esté, J. A., Zamzami, A., Susin, S. A., Brothers, G., Reed, J. C., Penninger, J. M. and Kroemer, G. (2000). Apoptosis control in syncytia induced by the HIV-1-envelope glycoprotein complex. Role of mitochondria and caspases. *J. Exp. Med.* **192**, 1081-1092.

Green, D. R. and Kroemer, G. (1998). The central executioner of apoptosis: mitochondria or caspases? *Trends Cell Biol.* **8**, 267-271.

Hakem, R., Hakem, A., Dunca, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., delaPompa, J. L., Kagi, D., Khoo, W. et al. (1998). Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell* **94**, 339-352.

Hara, H., Takeda, A., Takeuchi, M., Wakeham, A. C., Itie, A., Sasaki, M., Mak, T. W., Yoshimura, A., Nomoto, K. and Yoshida, H. (2002). The apoptotic protease-activating factor 1-mediated pathway of apoptosis is dispensable for negative selection of thymocytes. *J. Immunol.* **168**, 2288-2295.

Hisatomi, T., Sakamoto, T., Goto, Y., Yamanaka, I., Oshima, Y., Hata, Y., Ishibashi, T., Inomata, H., Susin, S. and Kroemer, G. (2002). Critical role of photoreceptor apoptosis in functional damage after retinal detachment. *Curr. Eye Res.* **24**, 161-172.

Hisatomi, T., Sakamoto, T., Murata, T., Yamanaka, I., Oshima, Y., Hata, Y., Ishibashi, T., Inomata, J., Susin, S. A. and Kroemer, G. (2001). Relocalization of apoptosis-inducing factor in photoreceptor apoptosis induced by retinal detachment in vivo. *Am. J. Pathol.* **158**, 1271-1278.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y. J., Sasaki, T., Elia, A. J., Cheng, H.-Y. M., Ravagnan, L. et al. (2001). Essential role of the mitochondrial apoptosis inducing factor in programmed cell death. *Nature* **410**, 549-554.

Kaufmann, S. H. and Hengartner, M. O. (2001). Programmed cell death: alive and well in the new millennium. *Trends Cell Biol.* **11**, 526-534.

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

Kroemer, G. and Reed, J. C. (2000). Mitochondrial control of cell death. *Nat. Med.* **6**, 513-519.

Kuida, K., Zheng, T. S., Na, S., Kyan, C.-Y., Yang, D., Karasuyama, H., Rakic, P. and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384**, 368-372.

Kuida, K., Haydar, T. F., Kuan, C. V., Gu, Y., Taya, C., Karasuyama, H., Su, M. S. S., Rakic, P. and Plavell, R. A. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell* **94**, 325-337.

Lassus, P., Opitz-Araya, X. and Lazebnik, Y. (2002). Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* **297**, 1352-1354.

Li, K., Li, Y., Shelton, J. M., Richardson, J. A., Spencer, E., Chen, Z. J., Wang, X. and Williams, R. S. (2000). Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell* **12**, 389-399.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K. et al. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol. Cell* **6**, 1389-1399.

Liu, X. S., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* **86**, 147-157.

Loeffler, M., Daugas, E., Susin, S. A., Zamzami, N., Métivier, D., Nieminen, A.-L., Brothers, G., Penninger, J. M. and Kroemer, G. (2001). Dominant cell death induction by extramitochondrially targeted apoptosis inducing factor. *FASEB J.* **15**, 758-767.

Mate, M. J., Ortiz-Lombardia, M., Boitel, B., Haouz, A., Tello, D., Susin, S. A., Penninger, J., Kroemer, G. and Alzari, P. M. (2002). The crystal structure of the mouse apoptosis-inducing factor AIF. *Nat. Struct. Biol.* **9**, 442-446.

Miramar, M. D., Costantini, P., Ravagnan, L., Saraiva, L. M., Haouzi, D., Brothers, G., Penninger, J. M., Peleato, M. L., Kroemer, G. and Susin, S. A. (2001). NADH-oxidase activity of mitochondrial apoptosis inducing factor (AIF). *J. Biol. Chem.* **276**, 16391-16398.

Ohiro, Y., Garkavtsev, I., Kobayashi, S., Sreekumar, K. R., Nantz, R., Higashikubo, B. T., Duffy, S. L., Higashikubo, R., Usheva, A., Gius, D. et al. (2002). A novel p53-inducible apoptogenic gene, PRG3, encodes a homologue of the apoptosis-inducing factor (AIF). *FEBS Lett.* **524**, 163-171.

Okada, H., Suh, W. K., Jin, J., Woo, M., Du, C., Elia, A., Duncan, G. S., Wakeham, A., Itie, A., Lowe, S. W. et al. (2002). Generation and characterization of Smac/DIABLO-deficient mice. *Mol. Cell. Biol.* **22**, 3509-3517.

Olie, R. A., Durrieu, F., Cornillan, S., Loughran, G., Gross, J., Earnshaw, W. C. and Golstein, P. (1998). Apparent caspase independence of programmed cell death in Dictyostelium. *Curr. Biol.* **8**, 955-958.

Ravagnan, L., Gurbuxani, S., Susin, S. A., Maisse, C., Daugas, E., Zamzami, N., Mak, T., Jaattela, M., Penninger, J. M., Garrido, C. et al. (2001). Heat shock protein 70 antagonizes apoptosis-inducing factor. *Nat. Cell Biol.* **3**, 839-843.

Ravagnan, L., Roumier, T. and Kroemer, G. (2002). Mitochondria – the killer organelles and their weapons. *J. Cell. Physiol.* **192**, 131-137.

Robertson, J. D., Enoksson, M., Suomela, M., Zhivotovsky, B. and Orrenius, S. (2002). Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. *J. Biol. Chem.* **277**, 29803-29809.

Susin, S. A., Zamzami, N., Castedo, M., Daugas, E., Wang, H.-G., Geley, S., Fassy, F., Reed, J. and Kroemer, G. (1997). The central executioner of apoptosis. Multiple links between protease activation and mitochondria in Fas/Apo-1/CD95- and ceramide-induced apoptosis. *J. Exp. Med.* **186**, 25-37.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M. et al. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446.

Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prévost, M.-C. et al. (2000). Two distinct pathways leading to nuclear apoptosis. *J. Exp. Med.* **192**, 571-579.

Wei, M. C., Zong, W.-X., Cheng, E. H.-Y., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B. and Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727-730.

Woo, M., Hakem, R., Soengas, M. S., Ducan, G. S., Shahinian, A., Kägi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S. A. et al. (1998). Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev.* **12**, 806-819.

Wu, M., Xu, L. G., Li, X., Zhai, Z. and Shu, H. B. (2002). AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. *J. Biol. Chem.* **277**, 25617-25623.

Ye, H., Cande, C., Stephanou, N. C., Jiang, S., Gurbuxani, S., Larochette, N., Daugas, E., Garrido, C., Kroemer, G. and Wu, H. (2002). DNA

binding as a structural requirement for the apoptogenic action of AIF. *Nat. Struct. Biol.* **9**, 680-684.

Yoshida, H., Kong, Y.-Y., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Penninger, J. M. and Mak, T. W. (1998). Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* **94**, 739-750.

Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M. and Dawson, V. L. (2002). Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science* **297**, 259-263.

Zamzami, N., El Hamel, C., Muñoz, C., Brenner, C., Belzacq, A.-S., Costantini, P., Molle, G. and Kroemer, G. (2000). Bid acts on the permeability transition pore complex to induce apoptosis. *Oncogene* **19**, 6342-6350.

Zhang, X., Chen, J., Graham, S. H., Du, L., Kochanek, P. M., Draviam, R., Guo, F., Nathaniel, P. D., Szabo, C., Watkins, S. C. et al. (2002). Intranuclear localization of apoptosis-inducing factor (AIF) and large scale dna fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. *J. Neurochem.* **82**, 181-191.