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Review

Apaf1 and the apoptotic machinery

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Abstract

The molecular characterization of the *Caenorhabditis elegans* cell death genes has been crucial in revealing some of the biochemical mechanisms underlying apoptosis in all animals. Four *C. elegans* genes, *egl-1, ced-9, ced-4* and *ced-3* are required for all somatic programmed cell death to occur. This genetic network is highly conserved during evolution. The pro-death gene *egl-1* and the anti-death gene *ced-9* have structural and functional similarities to the vertebrate *Bcl2* gene family. The killer gene *ced-3* encodes a cystein-aspartate protease (caspase), which is the archetype of a family of conserved proteins known as effectors of apoptosis in mammals. Zou and collaborators[1] reported the biochemical identification of an apoptotic protease activating factor (Apaf1), a human homolog of *C. elegans* CED-4, providing important clues to how CED-4 and its potential relatives could work. A number of proteins have been shown to interact with Apaf1 or to be determinant for its activity as an apoptotic adapter. The aim of this review is to provide an overview of the recent progress made in the field of developmental apoptosis by means of the murine *Apaf1* targeted mutations. The central role of Apaf1 in the cell death machinery (apoptosome) and its involvement in different apoptotic pathways will also be discussed.

Keywords: Bax; BclX_L; Boo; caspases; CARD; Cytochrome *c*

Abbreviations: Apaf1, apoptotic protease activating factor 1; Boo, Bcl2 ovary homologue; CARD, caspase recruitment domain; Casp, caspase (cystein aspartate protease); *ced*, cell death abnormal; *ces*, cell death specification; *egl*, egg laying defective; ICE, interleukin (IL)-1β-converting enzyme; MEE, medial edge epithelial cells; MEF, mouse embryonic fibroblast; PARP, poly(ADP ribose) polymerase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase mediated nick end-labeling; VEGF, vascular endothelial growth factor

Introduction

Genetic studies in the nematode *C. elegans* have provided hints about a functional network involving four genes at its core: three of these, *egl-1* (*egl*, egg laying defective), *ced-3* and *ced-4* (*ced*, cell death abnormal), are required for programmed cell death, whereas the fourth, *ced-9*, can inhibit the action of *ced-3* and *ced-4* in surviving cells.[2–6] Mutations in these genes affect most if not all of the 131 somatic cell deaths that occur during *C. elegans* development and genetic experiments succeeded in ordering their functions.[2]

Several mammalian counterparts of the components of this genetic network have been identified and shown to be involved in programmed cell death. Interestingly, Bcl2, an integral membrane protein located mainly on the outer mitochondrial membrane, which represents the mammalian counterpart of CED-9,[7–9] could block programmed cell death in *C. elegans* and could substitute for the *C. elegans ced-9* gene in *ced-9* deficient mutants.[9,10] These findings strongly suggest that the molecular mechanism of programmed cell death is evolutionary highly conserved (Figure 1). Due to the higher degree of complexity in vertebrate development, it is not surprising that *Bcl2* belongs to a multigene family with several members in mammals.[11–14]

Likewise, *ced-3* encodes a protein homologue to human interleukin (IL)-1β-converting enzyme (ICE)[15] which belongs to a large family of related mammalian proteases termed caspases (cystein aspartate proteases). Slee and collaborators[16] recently reported that six caspases (Casp2, -3, -6, -7, -8 and -10) are processed in cell-free extracts in response to Cytochrome *c* and that three others (Casp1, -4 and -5) do not respond to this induction. In these assays Casp9 was required for all the downstream caspases activation events. In turn, Casp3 was required for the activation of four other caspases (Casp2, -6, -8 and -10). This proteolytic cascade will eventually lead to activation of nucleases and cleavage of nuclear structural proteins.[17]

The human CED-4 homolog has been recently identified in human cells by an *in vitro* reconstitution approach. This finding represented a key in apoptosis studies in vertebrates. The CED-4 homolog has been termed Apaf1 (apoptotic protease activating factor)[1] and it participates in the Cytochrome *c*/dATP-dependent activation of Casp3 through the auto-proteolytic activation of Casp9.[18]

The evolutionary conservation among *C. elegans* and vertebrates of the general apoptotic program at a biochemical and cellular level is thus evident, but the importance of apoptosis in animal development grows with the complexity of the organism. Apoptosis-deficient nematodes can have a normal life span, although they

have 15% more cells than normal animals and show a few functional deficiencies.[19,20] By contrast, mice in which *Casp3* or *Casp9* genes have been mutated by gene targeting, die perinatally showing a massive cell overgrowth in the central nervous system, as a result of apoptosis deficiency in the neuroepithelial cells.[21–23] *Casp8* knockout embryos died at embryonic day 11 (e11) with abnormal formation of the heart.[24] *BclX* deficient mice die later, at e13, exhibiting excess of apoptosis in brain, spinal cord and haematopoietic system.[25] *Bax*$^{-/-}$ mice show a milder phenotype, including hyperplasia of non-neuronal lineages, as lymphocytes and ovarian granulosa cells and testicular degeneration.[26] In the nervous system, Bax is required for neuronal death after deprivation of neurothropic factors.[27] *Apaf1* deficient embryos die between e16 and post-natal day 0 (pn0), exhibiting reduced apoptosis in the brain and dramatic craniofacial and eye alterations.[28,29] Apaf1 plays a crucial role in the common events of mitochondria-dependent apoptosis in most biochemical death pathways and its role is critical for development. However, analysis of the pathways by which cell death is regulated from outside the cell is complicated by the fact that the same pathways are also used to transmit other signals. Moreover, the means by which a cell monitors its internal environment are so far largely obscure. A number of proteins have been shown to interact directly or indirectly with Apaf1, which is a relatively large molecule, confirming its role as the heart of some of these pathways and the physical core of the apoptotic machinery, known as apoptosome.[30]

Knowledge gained about these interactions and their roles, as well as characterization of the molecular basis of the developmental alterations exhibited by the *Apaf1* and other apoptosis-related knockout lines, will be of great benefit in understanding the roles of physiological cell death pathways in disease.

Apaf1 in developmental apoptosis

According to Glücksmann,[31] apoptosis serves three functions in mammalian development: deleting unneeded structures (phylogenetic apoptosis), controlling cell number (histogenetic apoptosis) and sculpting structures (morphogenetic apoptosis). It remains to be determined when and to which extent each protein member of the apoptotic program is involved in each of these processes. Due to the centrality of Apaf1 in the apoptotic machinery, the targeted mutation of this adapter protein phenotypically reflects all these processes. Nevertheless, it is noticeable that the *Apaf1* knockout does not precisely mimic *Casp3* and *Casp9* knockouts which exhibit a predominantly neuronal phenotype. The number of developmental alterations observed in *Apaf1* deficient embryos is higher and they are more widespread, distributed all over the organism. Two different mouse lines carrying an *Apaf1* mutation have been independently generated. In one case *Apaf1* has been 'trapped' by a gene trap vector and the insertional mutation hampered the stability of the resulting truncated protein.[28] Yoshida *et al*.[29] disrupted the murine *Apaf1* using a targeting vector in which an exon encoding a portion of the nucleotide-binding loop was deleted. In both cases a null allele was generated. No alterations were observed in heterozygous embryos. The malformations observed in the homozygous embryos are summarized in Table 1 and shown in Figure 2.

Table 1 Apaf1$^{-/-}$ embryonic phenotype. The onset of the principal aspects of Apaf$^{-/-}$ embryonic phenotype and their descriptions are listed.[28,29] CNS, central nervous system

Onset	Organ	Description
e12.5	CNS	Open brain (and/or spina bifida)
e13.5	CNS	Forebrain overgrowth
e14.5	Eye	Retina overgrowth, lens reduction, lens mispolarization, lens fibres disorganization, accumulation of hyaloid endothelial cells
	Limbs	Poor development of digits, persistence of interdigital webs
	Palate	Lack of fusion of palatal shelves
	Skull	Absence of skull vault, delay in endochondral ossification, absence of the basisphenoid ossification centre
	Brain	Delay in cortical layering (absence of intermediate layer), differentiation into two layers of the choroid plexus, enlargement of the mantle layer, malformation of the infundibulum, thickening of the hindbrain
e15.5	Limbs	Lack of apoptosis of the hypertrophic chondrocytes

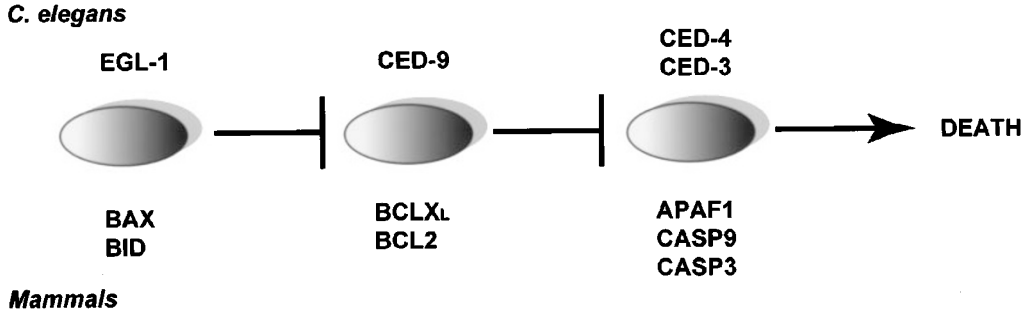

Figure 1 The order of action of programmed cell death genes is evolutionary highly conserved. The upper diagram shows the order of function of cell death proteins in *C. elegans*.[2] CED-9 activity protects cells from programmed cell death induced by CED-3. This protection occurs by negative regulation of CED-4. EGL-1 acts upstream of CED-9 to activate cell death. The lower diagram shows the conserved mechanisms in mammals. Due to the complexity of higher organisms, the counterpart roles of *C. elegans* genes are played in mammals by gene families

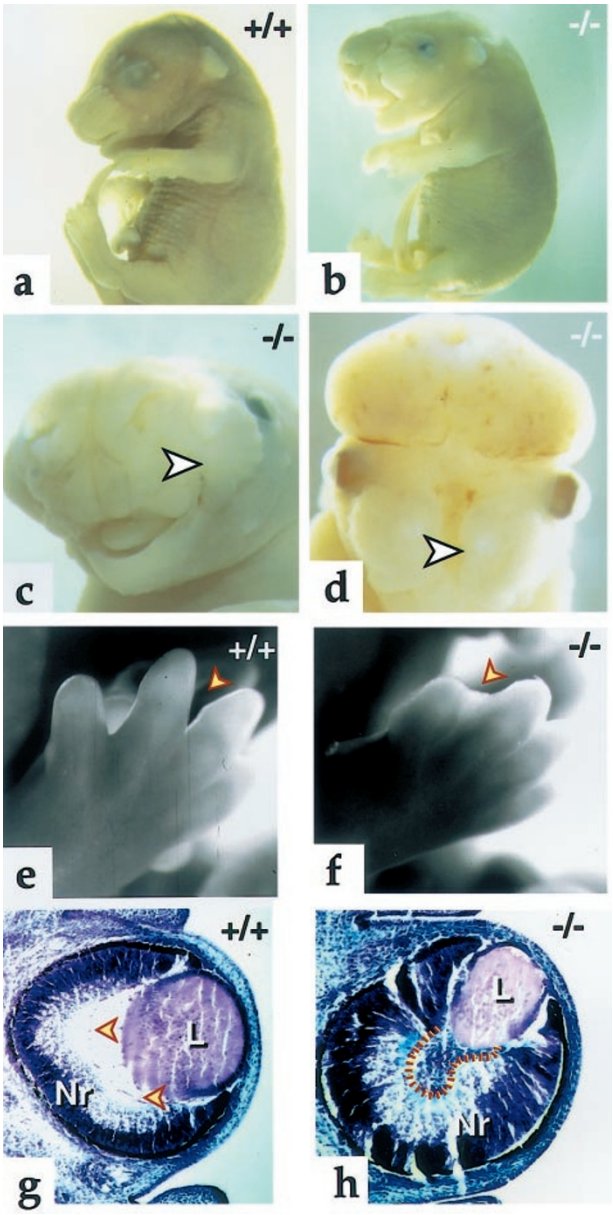

Figure 2 *Apaf1* deficient phenotype during mouse embryogenesis. Mouse embryos show brain overgrowth, cleft palate, persistence of the interdigital webs and eye malformation. (**a,b**) Rostral exencephaly in a homozygous e16.5 fetus (**b**) compared with a wild-type littermate. (**c**) Front view of the same embryo shown in (**b**). (**d**) An *Apaf1* deficient embryo showing a cone-shaped exencephalic brain mass. Red arrowheads: rostral borders of the whisker pads. (**e,f**) Comparison of the forelimbs of e15 mouse fetuses. The interdigital webs are still present in the $^{-/-}$ individual (**f**). Notice that this trait of the phenotype will be rescued later on development by an Apaf1-independent pathway.[29,38] The arrowhead points to the persistent web. (**g,h**) Sections through the eyes of *Apaf1* wild type (**g**) and $^{-/-}$ (**h**) e14.5 embryos. In (**h**) the neuroretina (Nr) is extremely enlarged and folded; the lens (L) is smaller, mispolarized and the onforming fibers are disorganized. The dashed line encircles the great number of endothelial hyaloid cells accumulated in the vitreous. The arrowheads in (**g**) point to the wild type scattered and rare hyaloid endothelial cells. See also Table 1

Apaf1 is involved in deleting unneeded structures (phylogenetic apoptosis)

Two main aspects of the phenotype of *Apaf1* deficient embryos fall into the category of phylogenetic programmed cell death: the persistence of the hyaloid vascular system of the lens and the lack of a proper palatal fusion.

Apaf1 is expressed in the endothelial cells of the transient vascular system of the eye. Already by e14.5 these cells obliterate entirely the vitreous of the *Apaf1*$^{-/-}$ embryos while they appear scattered and rare in the wild-type littermate (Figure 2g–h).[28] It was previously reported that the regression of the tunica vasculosa lentis, the capillary plexus that surrounds the posterior part of the lens, and the vasa hyaloidia propria, in the vitreous, begins in mouse at about pn5 and completes by about day pn21.[32] In a recent study performed by the terminal deoxynucleotidyl transferase mediated nick end-labelling technique (TUNEL) and electron microscopy, Mitchell *et al*.[33] observed widespread apoptosis of these endothelial cells much earlier in development, at e17.5. The authors proposed that VEGF (vascular endothelial growth factor) can act as a survival factor for the transient growth of this transient vascular system. The physical separation of the VEGF-producing cells (confined by e14.0 to the anterior and equatorial portion of the lens) and the endothelial cells expressing the VEGF receptor, could therefore activate the Apaf1 'executioner' pathway.

At e12.5 and thereafter, most *Apaf*$^{-/-}$ embryos showed dramatic craniofacial alterations whose major trait was a midline facial cleft (Figure 2a–d).[28,29] Morphogenesis of the mammalian secondary palate is characterized by the growth, orientation and union in the midline of bilateral palatal shelves to form a continuous structure. In order to obtain this continuity, the removal of the medial edge epithelial cells (MEE) is absolutely required. The completion of these processes occurs between e13 and e15.[34] At e16 in the mutant embryos, MEE cells that constitute the midline seam of the palatal shelves were still present. Conflicting views suggested epithelial-mesenchymal transformations and/or nasal and oral migrations of these cells in order to achieve their critical removal for palatal fusion.[34–36] In *Apaf1* deficient embryos, all the developmental processes enabling the palatal shelves to contact in the midline are preserved, while MEE cells are not removed. We conclude that the seam degeneration observed *in vivo* in normal embryos would then be largely due to programmed cell death. In contrast, the mandibular prominences properly fuse in the midline in *Apaf1*$^{-/-}$ embryos. Accordingly, Chai *et al*.[37] have concluded that medial epithelial cells at the fusion site of mandibular prominences migrate to the surface epithelium during the fusion process and neither transdifferentiate into mesenchyme nor die by apoptosis.

Apaf1 is involved in sculpting embryonic structures (morphogenetic apoptosis)

Apaf1$^{-/-}$ embryos show at e15.5 poorly shaped digits with persistence of the intertdigital webs, which should be

eliminated by cell death by e14.5 (Figure 2e,f);[28,29] however, at e16.5 a normal development of fore- and hind-limbs was observed in all mutant embryos. Most likely, alternative Apaf1-independent and caspase-independent pathways can be engaged later and rescue this aspect of the phenotype.[30,38]

It has been previously revealed that the programmed death of lens epithelium is a significant component of lens development.[39] In fact, dying cells were found within the anterior epithelium of the rat lens, and this was related to the availability of the survival factor aFGF.[40] By e10.5 several epithelial cells undergo apoptosis in the murine anterior lens epithelium, as revealed by the TUNEL technique, while in the *Apaf1*$^{-/-}$ lenses a few if any cells are dying (Figure 3d–f). Which is the consequence of this lack of apoptosis in the mutant embryos? The mutant lens is mis-polarized and smaller. This could be mainly due to an altered interaction with the underlying retinal environment which is severely malformed and overgrown. However, the disorganization of lens fibres shown in Figure 2h could be related to the presence of supernumerary cells in the anterior epithelium.

A better understanding of the molecular basis of the lens epithelial cells apoptosis would be extremely useful, since an enhanced cell death in the lens epithelium is a widespread cause of non-congenital cataract.[41]

Apaf1 is involved in controlling cell number (histogenetic apoptosis)

Apoptosis has been suggested as a mechanism to help match the numbers of different cell types in various organs.[42] In the most extreme form of this hypothesis, it was proposed that all cells in vertebrates, except for blastomers, are programmed to 'suicide' unless they are signaled by other cells not to do so.[39] Consistently, some of these cell death events, like in developing oligodendrocytes or lens anterior epithelial cells, can be prevented or delayed by experimentally increasing the level of survival factors in the animals.[39,43] Although the influence of cell proliferation in controlling cell numbers in animal development has been more studied than apoptosis, apoptosis can be the dominant mechanism.

According to this model, the *Apaf1*$^{-/-}$ embryos show an uncontrolled cell number in the retina and brain (Figure 2g,h).[28,29] The *Apaf1* gene is expressed predominantly in retinal ganglion cells already by e9.5 and these, which are the only differentiating cells at this developmental stage, are known to undergo extensive histogenetic apoptosis.[44] Interestingly, apoptotic loci were observed as well in the retinal pigmented epithelium and optic stalk by e10.5[45] and *Apaf1*$^{-/-}$ embryos do not display apoptosis in that area (Figure 3e,f). Thus the general thickening of the neuroretina observed in the mutants is a consequence of the lack of Apaf1-dependent cell death in all the retinal compartments, though a mis-regulated cellular over-proliferation could be an accompanying mechanism (see below).

In the immune system, apoptosis is known to control thymocytes selection.[46] However, the apoptotic pathway required for normal thymocyte development does not require Apaf1.[29] This evidence implies that other Apaf1-like molecules exist that play an equivalent role in the development of the immune system.

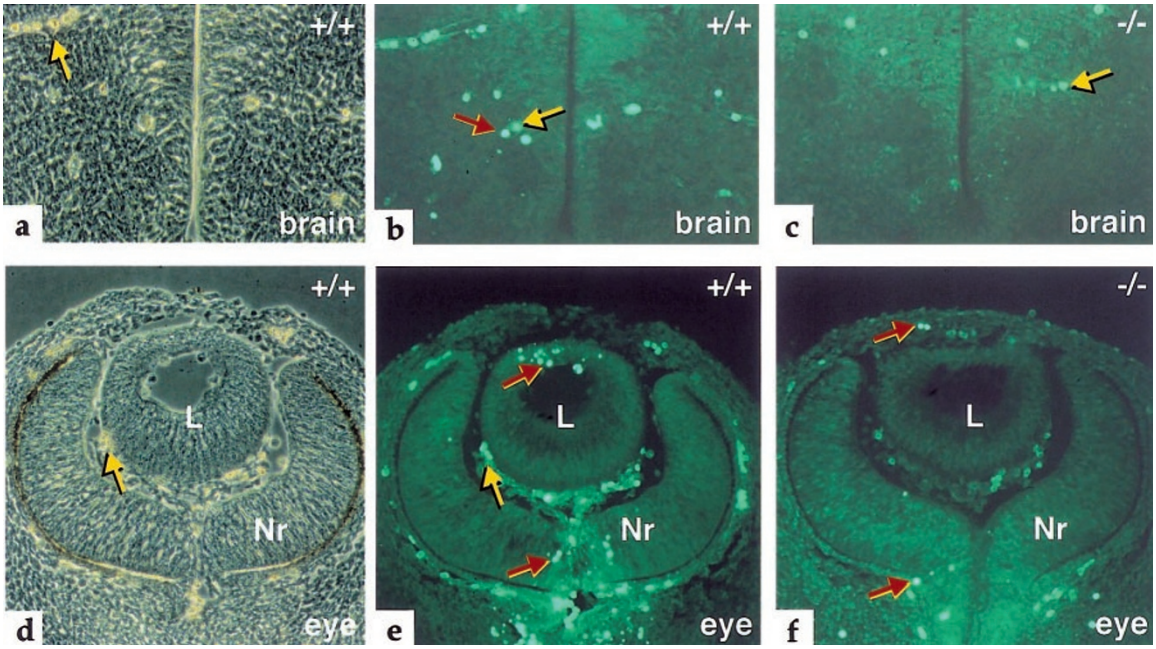

Figure 3 Decrease of apoptosis in *Apaf1* deficient embryos. (**a–c**) Several cells undergo apoptosis in the murine brain as revealed by the TUNEL technique (**b**), while in the *Apaf1*$^{-/-}$ tissues a few if any cells are dying (**c**). (**d–f**) Some of the anterior lens epithelium cells and several cells in the proximal central part of the retina and in the optic stalk undergo apoptosis (**e**) in the wild type e12.5 eye, while very few in the corresponding *Apaf1*$^{-/-}$ littermate (**f**). (**a,d**) bright fields and (**b,c,e,f**) UV light-filtered images of mouse e12.5 sections reacted with the TUNEL technique. The red arrows point to the specific fluorescent signal due to apoptosis detection; the yellow arrows indicate autofluorescent blood cells which represent unspecific background

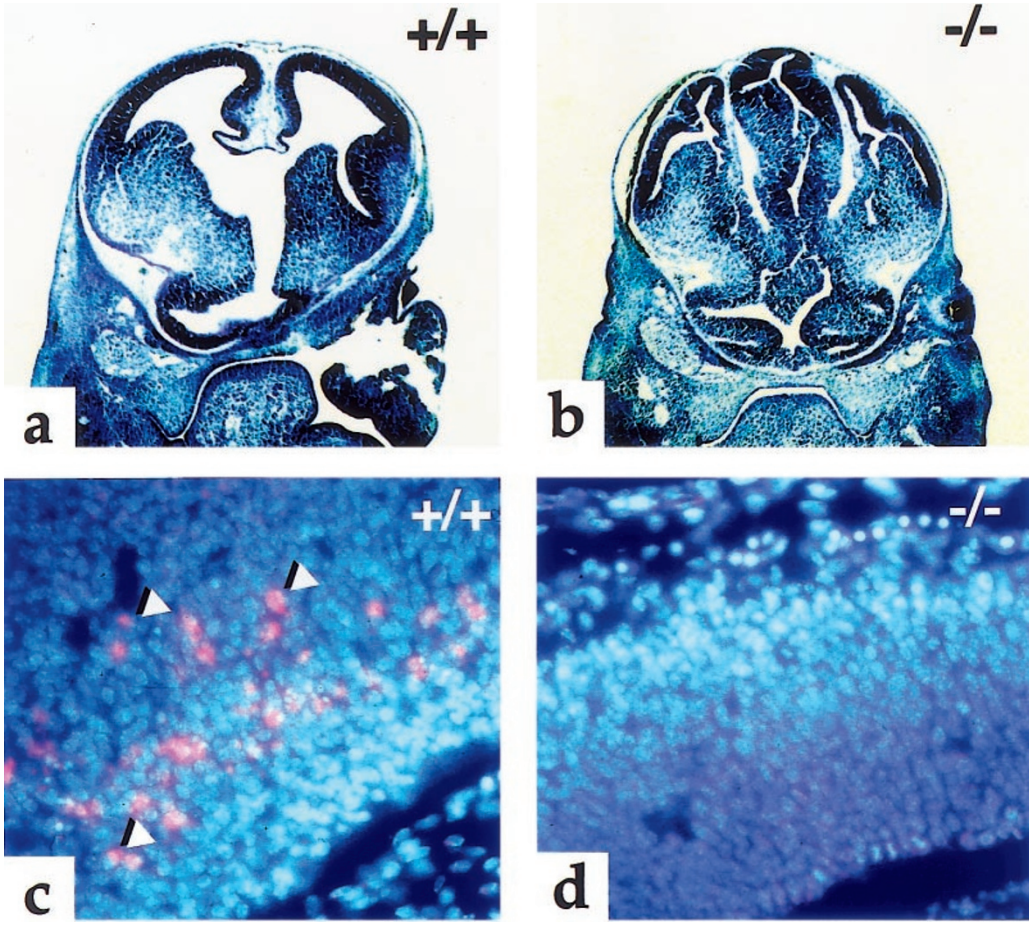

Figure 4 Brain hyperplasia and lack of Casp3 activation in *Apaf1* deficient embryos. (**a,b**) Cresyl violet-stained sections through the forebrain of an e12.5 wild type embryo (**a**) and an *Apaf1* deficient littermate (**b**). In the mutant, a convolute mass of neuroepithelium and mantle reduces dramatically the size of the telencephalic vesicles. (**c,d**) Activated Casp3 stained cells can be easily identified in the wild type nervous tissue (arrowheads in **c**) but not in the mutant (**d**). The signal is shown in the context of the tissue non-specifically stained with bisbenzimide. Apaf1 is absolutely required for Casp3 activation in brain tissues. Nervous tissue apoptosis in development could then be associated to the Apaf1-dependent pathway

The histogenetic cell death of early stem cells in the brain is a prominent developmental feature of the nervous system.[47] When this is prevented, like in the *Casp3*, *Casp9* or *Apaf1* knockouts, an enlargement of proliferative zones is observed later in development.[21,22,28,29] In Figure 2a–d, the brain overgrowth in *Apaf1*$^{-/-}$ embryos at e16.5 is clearly visible as a cauliflower-like protrusion of the forebrain or a cone-shaped exencephalic brain mass. Figure 3a–c show a nearly tenfold reduction of the average number of TUNEL-positive cells per sections in the e12.5 brain in the *Apaf1* knockout. Finally, Figure 4a,b show in section how proliferating cells can occupy the lumen of the cerebral ventricula at the same stage. Moreover, an extensive enlargement of the differentiating zones has been observed.[28] This evidence raises several questions about the fate of the cells rescued from 'suicide' by this targeted mutation. Will they undergo the normal differentiation program or will their proliferation be indefinitely protracted? The finding that in the hindbrain of *Apaf1*$^{-/-}$ embryos the morphology of all nuclei is preserved leads us to speculate that a certain degree of 'normal' differentiation is still present.[28]

It should be mentioned that *Apaf1* is expressed also in adult nervous tissue.[1] The study of its involvement in physiological or pathological apoptotic processes in adult life will be invaluable for a better understanding of human diseases.

Apaf1 biochemistry

Which is the role of Apaf1 in the apoptotic machinery and what is the molecular basis of the phenotype observed in the Apaf1 mutants? The following section is an excursion along the structure and function of the apoptosome, centered around Apaf1. Nevertheless, a complicated framework of signal transduction cascades regulates the apoptosome by targetting Apaf1. Apaf1 could therefore be considered as the *bona fide* core of the apoptosome.

Apaf1-interacting factors and their role in the apoptotic machinery

Apaf1 possesses an amino-terminal CED-3-like domain which includes a caspase recruitment domain (CARD),[48] a CED-4-like domain which contains a conserved P-loop and a long carboxy-terminal domain extremely rich in WD-40 repeats, involved in protein-protein interactions (Figure 5).[1] A number of pro- and anti-apoptotic factors have recently been shown to interact with some of these domains and mediate interactions of Apaf1 with other components of the apoptotic machinery. The topology of these interactions is shown in Figure 5 and described in Table 2. The most interesting Apaf1-involving network is the $BclX_L$/Apaf1/Casp9 complex,[49] which represents the structural and functional vertebrate counterpart of the CED-9/CED-4/CED-3 ternary complex observed in *C. elegans* as the primordial apoptosome.[50,51] However, Casp1 and Casp8 have also been shown to biochemically interact with CED-4 *in vitro*.[50] A small digression in the caspase world is therefore necessary.

Caspases are a family of cysteine proteases that cleave their substrate after aspartate residues.[52–54] They exist as inactive zymogens termed procaspases composed of a prodomain plus large and small catalytic subunits. Large prodomains function as signal integrators as they bind adapter molecules involved in signal transduction. An example is given from the CARD domain which establishes global homophilic interactions and is present in

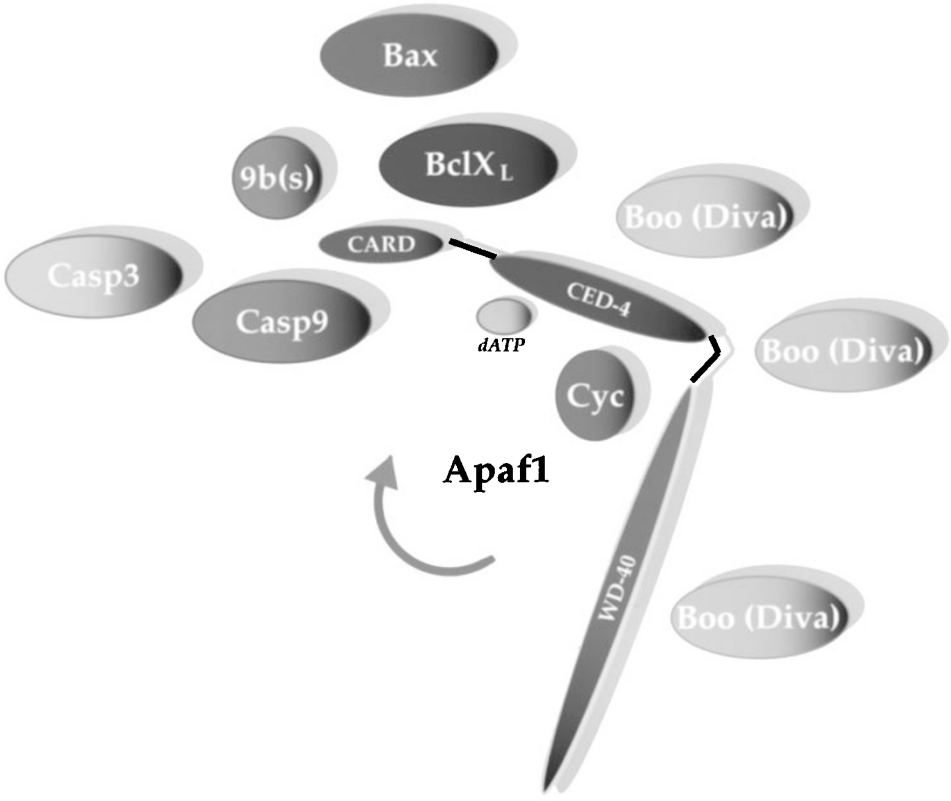

Figure 5 Topology of the Apaf1-interacting proteins. Schematic representation of the Apaf1-interacting proteins identified so far. The Apaf1 protein is composed of three domains, a caspase recruitment domain (CARD), an Ap-ATPase domain (CED-4-like) and a WD-40 repeats domain (WD-40) for a molecular weight of 135 kDa. All the apoptosome components are positioned in the close proximity of their potential site(s) of interaction along the Apaf1 domains. dATP is a cofactor essential for Apaf1 activity. The arrow indicates the potential of Apaf1 to get its amino- and carboxi-terminus into contact, moving from a 'closed' to an 'opened' conformational status and *vice-versa* with different functional implications. The short splicing isoform of Casp9 is termed as 9b(S). Bax and Casp3 do not directly interact with Apaf1, but they are functionally involved in the apoptosome through the mediation of $BclX_L$ and Casp9, respectively. See also Table 2

Table 2 Apaf1-interacting factors. Several factors have been shown to interact, directly or indirectly, with the adapter molecule Apaf1. The sites of interactions of these factors on Apaf1 or the proteins which mediate an indirect interaction are also listed. See Figure 5

Factor	Site of interaction on Apaf1	Role
Casp9	CARD	Executioner caspase
Casp9b(s)	CARD	Apaf1 inhibitor
Cytochrome c	CED-4/WD-40	Apaf1 activator
Casp3	Indirect (Casp9)	Executioner caspase
$BclX_L$	CARD (indirect?)	Anti-apoptotic factor
Bax	Indirect ($BclX_L$)	Pro-apoptotic factor
Boo/Diva	CED-4/unique joint/WD-40 (ind.?)	Anti-apoptotic factor

several prodomains (Casp2, Casp9).[48] In a recent elegant study, the structure of the CARD of the RAIDD adapter protein, that recruits Casp2, has been solved.[55] It consists of six tightly packed alpha-helices arranged in a topology homologous to the Fas death domain with a basic and an acidic patch on opposite sides of the surface. Modelling of other CARDs, including the Apaf1 CARD, showed that this basic/acid surface is highly conserved, suggesting a general model for CARD/CARD interactions. Apaf1 and Casp9 CARD surfaces lack hydrophobic patches observed in the Casp2/RAIDD interaction, indicating that (a) electrostatic interactions dominate the binding and (b) the unusual concentration of charges suggests a quite exclusive mutual interaction among Casp9 and Apaf1.[55,56] Cardone *et al*.[57] showed that two survival factors, the kinase Akt and p21-Ras (an Akt activator), induce phosphorylation of pro-Casp9, inhibiting its processing and activation; occupation of binding sites on Apaf1 by endogenously phosphorylated Casp9 molecules would then play a dominant negative effect in the apoptotic cascade. A similar role seems to be performed by a splicing isoform of Casp9, Casp9b/Casp9S, detectable in several cell lines.[58,59] The Casp9-Apaf1 interaction is thus finely regulated and Casp9 is most likely the only caspase directly interacting with Apaf1. Casp9 can in turn activate Casp3 which will trigger the proteolysis of several nuclear proteins, such as PARP or gelsolin and induce nuclear degeneration and death. Recently, an antibody able to recognize the active subunit of Casp3 was generated.[60] Immunocytochemistry experiments performed on *Casp9*$^{-/-}$ and *Apaf1*$^{-/-}$ embryonic brain sections, demonstrates *in vivo* the absolute requirement of Apaf1 and Casp9 for Casp3 activation (Figure 4c,d).[22,28]

Another putative component of the apoptosome core is the anti-apoptotic protein BclX_L. It has been shown that BclX_L co-immunoprecipitates with Casp9 and Apaf1 and that Apaf1 expression was enhanced by co-expression of BclX_L, suggesting that BclX_L may stabilize Apaf1 by stochiometric binding.[49] Moreover, the same authors demonstrated that epitope tagging BclX_L at its amino-terminus disrupts its ability to interact with Apaf1. As BclX_L targets the CED-4-like domain of Apaf1, it is not competing with Casp9 for the CARD interaction. However, it has been shown that binding of BclX_L to Apaf1 inhibits Casp9 self-activation.[61] The anti-apoptotic function of BclX_L is antagonized by pro-apoptotic members of the Bcl2 family, including Bax, Bak and Bik, which can heterodimerize with BclX_L.[62] Co-expression of Bax or Bak attenuated the interactions between BclX_L and Apaf1, suggesting a life-death balance among these factors at the level of Apaf1 binding.[49]

More recently Boo (also termed Diva)[63,64] a novel anti-apoptotic member of the Bcl2 family, whose expression in adult mice is restricted to the ovary and epididymis but detectable in several embryonic tissues, has been reported to be able to extensively interact with Apaf1. Boo/Diva has the ability to bind three distinct regions of Apaf1, the CED-4 homologous region (Ap-ATP-ase domain), the WD-40 repeats and the Apaf1 unique domain between the CED-4-like and WD-40 repeat regions. These interactions are probably needed to induce conformational changes in the Apaf1 molecule and thus inhibit the processing of Casp9. If expression of Boo in adult mouse is tissue-specific, the presence of other Boo-like proteins can not be excluded; thus the mechanism involving this kind of Boo-Apaf1 interaction could be more general. It must be mentioned that the existence of the Apaf1/BclX_L and Apaf1/Boo direct binding has been recently questioned by Moriishi *et al*.[65] This finding obviously does not exclude that BclX_L and Boo constrain their activity on Apaf1 indirectly.

Cytochrome *c* plays a surprising role in promoting apoptosis. This protein is required for the formation of a complex that is sufficient to activate caspases in cell-free extracts.[66,67] *In vitro* binding of Apaf1, pro-Casp9 and Cytochrome *c*, in the presence of dATP results in Casp9 activation.[18] However, the target site of Cytochrome *c* in Apaf1 has not yet been precisely defined. Apaf1 is currently considered to be substantially a distinct ATPase (Ap-ATPase), being a homolog of CED-4 which has ATPase-like functions.[68] Noteworthy, an alternatively spliced form of CED-4 that contains an insertion within the ATPase domain is a dominant negative inhibitor of caspase activation.[69] Conversely, a truncated form of Apaf1, lacking the WD-40 domain, is able to promote Casp9 activation in absence of Cytochrome *c* or dATP.[70] These observations lead to the conclusion that Cytochrome *c*, dATP and the WD-40 domain are involved in the conformational changes of Apaf1, which are necessary to achieve Casp9 activation.

Srinivasula *et al*,[70] Hu *et al*[71,72] and Zou *et al*[73] have shown that Apaf1 can form oligomers, upon Cytochrome *c* induction and dATP/ATP hydrolysis, and may facilitate pro-Casp9 autoactivation by oligomerizing its precursor molecules. However, this self-association can be inhibited by expression of the WD-40 domain. This finding and a series of elegant protein-dissection experiments implied that Apaf1 can adopt a close conformation in which the WD-40 domain interacts with its own amino-terminus, inhibiting Casp9 activation;[71] this 'closed' status could be held by Boo/Diva-like proteins, while Cytochrome *c* and dATP would lead Apaf1 to take on a more open conformation. This will allow Apaf1 self-association and consequent dimerization of Casp9, which in turn is a requirement for Casp9 autoactivation.

Apaf1 regulation and the apoptotic pathways

Apaf1 is regulated at a transcriptional level during early stages of development. The expression of this gene is initially restricted to the eye and the neural tube, becoming more widespread from e12.5 onwards and being almost ubiquitous in adult tissues.[28,29] A model in which transcriptional factors can trigger the cell's capability to be prone to undergo apoptosis through the apoptosome-mediated way is under investigation. In *C. elegans*, genetic analysis has identified two genes, *ces-1* and *ces-2* (*ces*, cell death specification) which control a subset of programmed cell death in the neural cell of nematodes.[74] *Ces-2* encodes a basic-leucin zipper transcriptional factor (bZIP). Mammalian members of the bZIP family could also play a role in the transcriptional regulation of

death genes. An interesting mechanism has been described for the forkhead-type transcription factor FKHRL1.[75] This transcriptional activator is kept in the cytosol in a phosphorylated inactive conformation by the presence of a survival factors-dependent serine/threonine kinase, Akt. Once the survival signal is abolished, FKHRL1 is dephosphorylated and can be translocated into the nucleus where it triggers the expression of apoptotic genes. An intensive study of the Apaf1 promoter region is one of the main goals to be achieved in the future.

For several years a huge effort has been made to understand the mechanisms behind the inter-cellular regulation of apoptosis and these mechanisms will be discussed in the following section.

Apaf1 and the signal transduction pathways

The signals by means of which a cell can induce the neighboring cells to undergo apoptosis are several and they are transduced through different pathways. We can recognize in a broad spectrum of cell lines two main signaling pathways: the mitochondrial and the death receptor signaling pathway. The mitochondrial signaling is still largely unknown while the death factors have been extensively studied. However, a death factor-dependent cross-talk with the mitochondria ensures in many cell types an enhancement of the apoptotic response.

The death receptors are characterized by an intracellular region, called the death domain, which is required for the transmission of the cytotoxic signal. Currently, five different receptors are known, tumor necrosis factor (TNF) receptor-1, CD95 (Fas), TNF-receptor-related apoptosis-mediated protein (TRAMP), and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and -2.[76] The pathways of transduction are highly similar. Ligand-receptor interaction induces receptor oligomerization, followed by recruitment of an adapter protein to the receptor death domain by homophilic interactions.[77] The adapter protein then binds a proximal caspase, as Casp8 (the caspase initiator) and connects receptor signaling to the apoptosome. Is this signaling communicating with the Apaf1-centred apoptosome? Some recent observations necessarily imply a cross-talk among these two pathways. Mouse embryonic fibroblast (MEFs) dissected from e14.5 *Apaf1*$^{-/-}$ embryos, exhibit a reduced apoptotic response to Fas-mediated and ceramide-mediated stimuli.[28] In particular, ceramide-in-

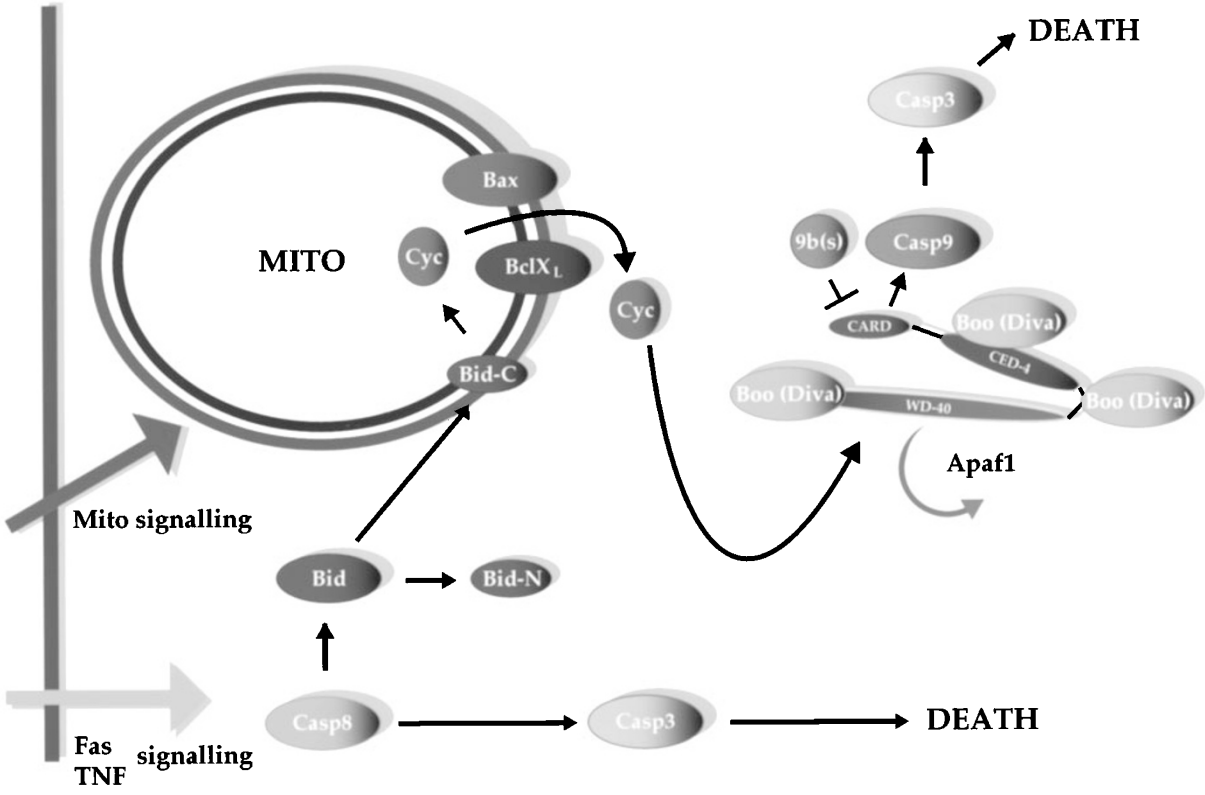

Figure 6 The apoptosome and its regulation. Two signaling cascades have been linked with the apoptosome functions: a Fas and TNF-mediated signal and an unknown mitochondrial signal. The two pathways are cross-talking. In particular, in response to Fas signal, Casp8 can cleave the pro-apoptotic protein Bid into two fragments. The carboxy-terminal fragment of Bid (Bid-C) would then translocate on the mitochondrial membrane in order to induce the efflux of Cytochrome *c* (indicated as Cyc) into the cytosol. This efflux is regulated by the balance between Bax, $BclX_L$ and other Bcl2-like proteins (For sake of simplicity only Bax and $BclX_L$, involved in the developmental regulation of Apaf1, have been depicted). Cytochrome *c* could then trigger conformational changes in Apaf1 structure, activating Apaf1. Apaf1 is held in an inactive 'closed' form by Boo/Diva. Once activated, Apaf1 can induce Casp9 self-processing through a CARD/CARD interaction. The direct interaction of Casp9 is inhibited by a Casp9 splicing isoform which plays a dominant-negative role by competing for the Apaf1 CARD binding. The last step of this cascade is the activation by the active form of Casp9, of Casp3 which rapidly leads to cell death. Alternatively, Casp3 could be activated directly by Casp8. The gray arrow emphasizes the potential of Apaf1 to get its amino- and carboxy-terminus into contact

duced apoptosis may occur via two independent mechanisms.[78] Ceramide signals, through the Jun kinase (JNK)-cascade mediate the transcriptional regulation of gene products such as Fas ligand or TNF, which mediate the death response. Alternatively, ceramide induces apoptosis directly through a ceramide-activated protein kinase/kinase suppressor of Ras (CAPK/KSR)-dependent mechanism which involves release of Cytochrome *c* from mitochondria. Accordingly, the response to the apoptosis induction in mouse *Apaf1*$^{-/-}$ MEFs is only partial to the Fas signal and almost completely abolished in response to the ceramide induction. This signaling cross-talk however, is cell-type specific as demonstrated by the absence of such a comprehensive low response to Fas apoptotic induction in *Apaf1*$^{-/-}$ thymocytes.[29] In a detailed analysis of *Apaf1*$^{-/-}$ thymocytes responsiveness to several apoptotic stimuli, the authors showed that Apaf1 is dispensable for the Fas-mediated apoptotic pathway in thymocytes but is required for cell death induced by dexamethasone, etoposide and γ-irradiation stimuli in this cell type.

The mitochondrial business

Cytochrome *c* release from mitochondria has been observed in cells undergoing apoptosis induced by signals including the activation of Fas and TNF, growth factor deprivation, excessive DNA damage and treatment with chemotherapeutical drugs.[79] However, the mechanism underlying this release and its regulation by the Bcl2 family of proteins remain unclear. Bid, a protein known to interact with Bcl2 and Bax,[80] has recently been reported to be a key factor in the cross-talk among the death factors and the mitochondrial pathways (named also Cif).[81–84] Bid is a specific proximal substrate of Casp8 in the Fas apoptotic signaling pathway. Full-length Bid is cytosolic, the Casp8-truncated Bid carboxy-terminus translocates to mitochondria transducing this apoptosis signal. Bid-c (termed also tBid) induces then the release of Cytochrome *c* into the cytosol and a consequent Apaf1/caspases-dependent apoptosis (Figure 6). But is the mitochondrial modification induced by a cellular stress or by any other unknown mitochondrial signaling (generating a general mitochondrial damage) able to induce a caspase-independent apoptosis? It is conceivable that the mitochondrial membrane permeability transition (PT) which accompanies apoptosis, the disruption of electron transport which follows Cytochrome *c* efflux and the generation of oxygen reactive species would induce a caspase- and therefore Apaf1-independent death. This responsiveness will as well vary from cell to cell according to the cell cycle timing or to the cell type, as revealed in *Casp3*$^{-/-}$ different cell lines using a series of elegant apoptotic paradigms.[85]

Does Apaf1 play a role in cell proliferation?

Are there other hypothetical roles for Apaf1 besides the pro-apoptotic function? At first, we could imagine that the presence of Apaf1 splicing-isoforms with opposite anti-apoptotic function could mimic the occurrence of such isoforms for Casp9 and for the Apaf1 *C. elegans* homolog CED-4. The functions of Apaf1 isoforms have not been detected yet,[73,86] but conversely an Apaf1 protein lacking the WD-40 domain would be a constitutive apoptotic activator (and it would be more evolutionary similar to its *C. elegans* counterpart), due to the inhibitor role of the WD-40 domain.[70] A truncated form of Apaf1 with these features could be generated by internal cleavage, in a sort of feedback regulatory loop driven by Casp3, using an almost perfect Casp3 cleavage site present in the Apaf1 unique CED-4-like/WD-40 joint domain.

As described above, the Apaf1 mutant embryos display an extended cell overgrowth in the brain (see Figure 2a–d) but the apoptosis detected in wild type littermates by the TUNEL method from day e9.5 onwards is limited to a relatively small number of cells (Figure 3a–c). How could this be explained? Does Apaf1 play a role in cell proliferation? Apoptosis *in vivo* is a rapid process with the dying cells immediately phagocytosed. However, the supernumerary cells observed in the ectopic masses shown in Figure 2b–d showed increased mitotic activity at e14.5 compared to the control as determined by BrdU incorporation.[29] An explanation could be that the absence of cell death in newly generated neurons within the ventricular zone fail to undergo normal apoptosis, but initially proliferate and then differentiate, doing this in a mis-regulated and accelerated way. Which kind of cell fate will characterize a 'survived' cell is not clear, but morphological evidence seems to confirm that the environmental cellular context is the leading factor in this survival and such cells are potentially able to properly differentiate.

This indication could certainly be useful in an attempt to rescue pathologically induced apoptosis in some diseases, where cell death is a consequence of a mis-signaling from the neighboring cells.

The Apaf1 gene family

Several authors have hypothesized, in the last 2 years, the existence of other Apaf1-like genes. Due to the evolutionary conservation of the general mitochondrial apoptotic pathway, with the Bcl2 family being the homolog of CED-9 and the caspases playing a CED-3 role in higher eukaryotes, it seems reasonable to think of the mammalian counterpart of CED-4 as a large gene family. However, only in the last few months two new Apaf1-like genes have been isolated.

The first one was the FLICE-associated huge protein (FLASH), identified by Imai *et al*.[87] FLASH has a domain similar to the Apaf1 CED-4-like domain and a DRD domain (DED-recruiting domain), which interacts with a death-effector domain in Casp8. As a consequence, FLASH is necessary for the activation of Casp8 in Fas-mediated apoptosis. It could actually represent the missing link in the immune system-specific apoptosis, where Apaf1 has been shown to not play a key role during development and in tissue homeostasis.[29] The second one was Nod1/CARD4, identified by Inohara *et al*[88] and Bertin *et al*.[89] Nod1/CARD4 associates with and regulates pro-Casp9. The sequence similarity among Apaf1 and Nod1/CARD4 is higher than among Apaf1 and FLASH, and the CARD and CED-4 like domains of Apaf1 and Nod1/CARD4 show strong homology regions. Unlike Apaf1, however, Nod1/

CARD4 contains leucine rich repeats (LRRs) instead of the WD-40 domain and is able to induce Nuclear factor kappa-B (NF-κB) activation. The difference in the carboxy-terminal domain indicates that different upstream signaling molecules can regulate the activation of Apaf1 and Nod1/CARD4. There are not yet experimental evidences about the roles of these proteins *in vivo*. The study of their activity in the apoptotic cascades will be a crucial issue in the future.

Concluding remarks

Studies investigating the genetic and molecular mechanisms of apoptosis in vertebrates and lower eukaryotes have provided insights into the complicated network that acts to inhibit or induce cell suicide in a broad variety of cell types. Targeted mutations of some of the proteins involved in this network helped to explain to which extent each one of these factors contributes to its functionality. In a comprehensive developmental and biochemical approach, Apaf1 has to be considered as the adapter molecule representing the core of the most studied apoptotic machinery. It may turn out to be an invaluable target for the engineering of therapeutic drugs aimed to block or induce the apoptotic cascade in a controlled way on numerous pathological conditions. As an example, Casp8 has been recently shown to be required for cell death induced by expanded polyglutamine repeats, suggesting a role for this caspase during the pathogenesis of Huntington's disease (HD).[90] Moreover, Casp3, by selective cleavage of the amyloid-β (Aβ) precursor protein, induces increased production of the pathogenetic Aβ peptides, playing a role in the onset of Alzheimer's disease (AD).[91] Since Casp8 is an upstream regulator of the Apaf1 pathway and Casp3 is an Apaf1 downstream death-effector, it is conceivable that Apaf1 also plays a role in HD and/or AD. Moreover, Apaf1 has been recently shown to play a role, together with Casp9, in controlling tumor development.[92] It is therefore evident why further characterization of Apaf1 biochemistry and its role in adult tissues will be essential.

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