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Human Genome and Diseases: Review

Apaf1 in developmental apoptosis and cancer: how many ways to die?

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Abstract. Apaf1 has been described as the core of the apoptosome. Deficiency in murine *Apaf1* leads to embryonic lethality with a phenotype affecting many aspects of developmental apoptosis. In the developing brain, Apaf1 is a death regulator of the neuronal founder cells. Combined intercrosses of mouse lines mutant for members of the mitochondrial death pathway are providing us with some clues about the relative regulation existing among neuronal cell populations. *Apaf1*-deficient embryos display an interesting phenotype in the inner ear and in limb development, which involves different caspase-dependent and -independent pathways. Moreover, *APAF1* is mutated in human melanomas, and its depletion contributes to malignant transformation in a mouse model of cancer. This review has a double aim: the analysis of the alternatives taken by the embryo to bring into the suicidal program different cells at different stages, and the relevance of APAF1 in the onset and progression of cancer.

Key words: *Bcl-X*; caspase; cell death; interdigital webs; neuronal founder cells; Nod; oncogenes.

Introduction

Neuronal apoptosis plays a major role in the construction, maintenance and repair of the developing brain and is a potentially crucial factor in neurodegenerative diseases. The apoptotic machinery or apoptosome, also activated during development and in the adult brain, is the armed executioner of the mitochondrial pathway of cell death. The apoptotic protease activating factor 1 (Apaf1) has been described as the core of the apoptosome [1, 2] (reviewed in [3]). Pro-apoptotic molecules such as Bax and Bid and anti-apoptotic molecules such as Bcl-X_L or Bcl-2 regulate, upon specific stimuli, the release of cytochrome c (Cyt-c) from mitochondria. Cyt-c has been shown to activate the Apaf1 protein, which in turns activates the cystein aspartate protease 9 [caspase-9, (Casp9)], resulting then in caspase-3 (Casp3)-dependent cell death (reviewed in [3–5]; see fig. 2). In fact, the disruption of murine *Apaf1* [6–8] leads to embryonic lethality with a phenotype affecting several aspects of developmental apoptosis, particularly prominent in the brain, inner ear and retinal tissues (table 2).

The analysis of Apaf1 activity in developmental apoptosis shed new light on the number of pathways involved in the cell death program at different stages in different embryonic organs. Induction of cell death by genotoxic activity was also studied in several mouse mutant lines, revealing the existence of at least two distinct pathways in neurodevelopment [9, 10]. We are now following the fate of *Apaf*1-deficient brain cells by stage-specific analysis of cell death, proliferation and differentiation markers in order to unravel the roles of Apaf1-dependent death pathways in developing neurons. We believe that Apaf1 is a death regulator of the neuronal founder cells, and we are

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presently investigating this hypothesis. Obviously, the elucidation of the cell death machinery in nervous tissues could provide the basis for therapeutic intervention in neurodegeneration.

Interestingly, apoptosis in the inner ear epithelium occurs upon regulation by Apaf1 and Bcl-X_L [M. Salminen and F. Cecconi, unpublished results]. Apaf1 and the apoptosome are therefore involved in a Bcl-X_L-dependent pathway of programmed cell death, which contributes to the morphogenetic remodelling of the developing inner ears in mice. Finally, in an *Apaf*1-deficient genetic background, a molecularly driven cell death program is maintained in the developing limb bud with a non-apoptotic or paraptotic cellular morphotype [11].

Altogether, this evidence implies the presence of several death pathways in a developing mammalian embryo. The choice of death, which could be intended as an extreme and irreversible process of differentiation, has to be taken in a short time, within different cell types and in a stringent cellular context. Although Apaf1 and the apoptosome represent an extremely efficient death machinery, this pathway is obviously not unique in development.

In a recent study, malignant melanoma cells were shown to have functional *P53* genes. Malignant melanoma is highly metastatic and resistant to chemotherapy. In melanoma cells, survival is guaranteed by inactivation of *APAF1*, indeed a gene downstream of *P53* in the death cascade [12]. We will also discuss the role played by APAF1 and the apoptosome in cancer onset and progression.

Apaf1 is a *single* no more

Lower organisms seem to manage with a limited repertoire of 'death' genes. Genetic studies in the nematode *Caenorhabditis elegans* have provided hints about a functional network involving four genes at its core: the pro-apoptotic factors *egl-1* (*egl,* egg-laying defective), *ced-3* and *ced-4* (*ced*, cell death abnormal), and *ced-9*, which can inhibit the action of *ced-3* and *ced-4* in surviving cells (reviewed in [13]). *Ced-3* is one of the four known caspases in *C. elegans* [14]. By contrast, there are currently 14 members of the mammalian *caspase* family, several of which display overlapping specificities and apparent redundancy (for a review see [15]). Nevertheless, the *Bcl-2* gene family plays *ced-9* functions in vertebrates. The *Bcl-2* family is characterized by specific regions of homology termed Bcl-2 homology domains (BH1–4), which are critical to the function of these proteins. The family can be divided into two classes: genes which suppress cell death such as *Bcl-2* and *Bcl-X_L*, and genes which seem to promote apoptosis, such as *Bak* and *Bax*. The BH3-only proteins such as Bid and Bik may be part of a regulatory network integrating cell survival and death signals. This schematic view is supported by the identification of a single BH3-only protein, egl-1, as part of the central core of cell death signalling in *C. elegans*. Another central player in apoptosis signalling in vertebrates is the Cyt-c, a molecule which is essential for mitochondrial respiration and energy production, but which can also be released from mitochondria and function as a cofactor in the activation of caspases through the apoptosome complex [1, 2, 16].

Apaf1 is the vertebrate *ced-4* structural homolog, and it has been identified in human cells by an in vitro reconstitution approach [1, 2]. This finding represented a key in apoptosis studies in vertebrates. Three groups recently reported the identification of a fly *ced-4*/*Apaf1* homolog [17–20]. These groups have named the gene *dapaf-1*, *dark* and *hac-1*, respectively; in the online *Drosophila* database the gene is called *ark* (apaf1-related killer). These findings demonstrate that the mechanisms of apoptosome functions in flies are very likely to be the same as those occurring in worms and mammals.

In the last 2 years, several authors have hypothesized the existence of other *Apaf1*-like genes in vertebrates. Due to the evolutionary conservation of the general mitochondrial apoptotic pathway, with the *Bcl-2* family being the homolog of *ced-9* and the caspases playing *ced-3* role in higher eukaryotes, it seems reasonable to consider of the mammalian counterpart of *ced-4* as a large gene family. Only very recently, some gene products have been identi-

Table 1. The *Apaf1*-like gene family in vertebrates.

Gene	Domains	Functions	Expression	Refs.
Apaf1	CARD, NBD, WD-40	activation of Casp9, physical component of the apoptosome	broad expression	1
FLASH (?)	*CED-4-like or NBD (?), DRD*	*activation of Casp8*	*broad expression*	21
Nod1/CARD4	CARD, NBD, LRRs	activation of NF-κB through RICK	broad expression	23, 24
Nod2	CARDs (2), NBD, LRRs	activation of NF-κB through RICK	monocytes	25
Nac/Defcap	PLM, PR, CARD, NBD, LRRs	Cyt-c-inducible interaction with Apaf1 and Casp9; interaction with Casp2 and Casp9;	epithelia, periph. blood leukocytes, spleen, heart and thymus	29

CARD, caspase recruitment domain; NBD, nucleotide binding domain; DRD, DED (death-effector domain) recruitment domain; LRRs, multiple leucine-rich repeats; PLM, pyrin-like motif; PR, proline-rich sequence (see also fig. 1).

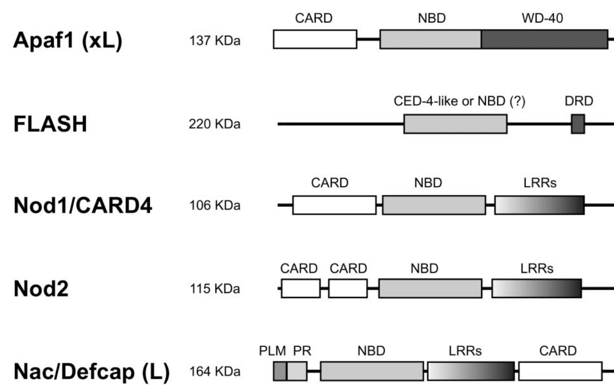

Figure 1. The *Apaf1* gene family in vertebrates. The domain structures of Apaf1 (xL isoform), FLASH, Nod1/CARD4, Nod2 and Nac/Defcap (L isoform). FLASH similarity to Apaf1 has been argued [22]. The molecular weight is also indicated. The reciprocal proportions of domains are not preserved. CARD, caspase recruitment domain; NBD, nucleotide binding domain; LRRs, multiple leucine-rich repeats; PLM, pyrin-like motif; PR, proline-rich sequence (see also table 1).

Table 2. The *Apaf1*$^{-/-}$ phenotype.

Onset	Organ	*Description*
e11.5	CNS	open brain (and/or spina bifida)
	inner ear	malformation of the otic vesicle
	blood	erythropenia
e13.5	CNS	forebrain overgrowth
e14.5	eye	retina overgrowth, lens reduction, lens mispolarization, accumulation of hyaloid endothelial cells
	limbs	persistence of interdigital webs
	palate	lack of fusion of palatal shelves
	skull	absence of skull vault, absence of the basisphenoid ossification centre
	brain	differentiation into two layers of the choroid plexus, expansion of the neural progenitor cell population, thickening of the hindbrain
Adult*	germ cells	degeneration of spermatogonia resulting in virtual absence of sperm
	behavior	hyperactivity

* 5% of homozygotes survived to adult life [8]. CNS, central nervous system.

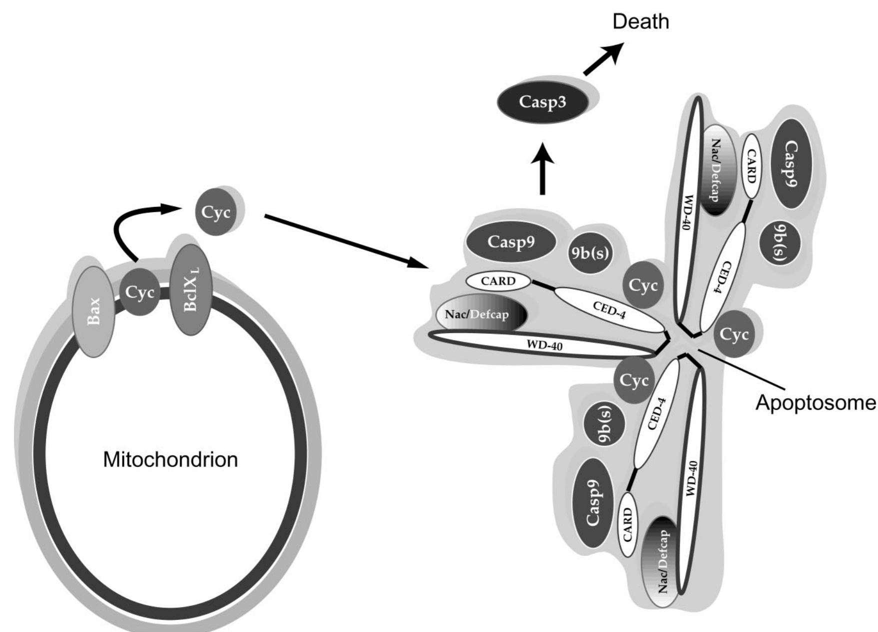

Figure 2. The apoptosome pathway. The balance between Bax, Bcl-X$_L$ and other Bcl-2-like proteins regulates the efflux of Cyt-c (Cyc) from mitochondria. Cyc could then trigger conformational changes in Apaf1 structure, activating the apoptosome, whose stoichiometry is not known. Once activated, Apaf1 can induce Casp9 self-processing through a CARD/CARD interaction. The direct interaction of Casp9 is inhibited by a Casp9 splicing isoform [9b(s)] which plays a dominant-negative role by competing for the Apaf1 CARD binding. In addition, Nac/Defcap binding to Apaf1 can increase its potential to activate Casp9. Cytosolic-Cyc increase, in a sort of feedback loop, can enhance Nac/Defcap affinity for Apaf1 binding. The last step of this cascade is the activation, by the active form of Casp9, of Casp3, which rapidly leads to cell death. The stimuli which can induce Cyc release from mitochondria (Fas or TNF receptor activation by their ligands, and Bid cleavage by Casp8, or p53 induction) are not represented in this scheme.

fied and related to *Apaf1* in terms of function and sequence (fig. 1, table 1).

The first was the FLICE-associated huge protein (FLASH), identified by Imai and colleagues [21]. FLASH was shown to possess a domain similar to the Apaf1 ced-4-like domain and a DED-recruiting domain (DRD), which interacts with a death-effector domain in caspase-8 (Casp8). As a consequence, FLASH is necessary for the activation of Casp8 in Fas-mediated apoptosis [21]. However, Koonin and colleagues have brought strong arguments against the structural similarity between Apaf1 and FLASH, supporting them with phylogenetic evidence [22]. The P-loop signature of FLASH putative CED-4-like domain was not conserved between mouse and humans, arguing against its functional importance. As a result of these findings, FLASH cannot be considered an Apaf1-like protein [22].

The second was the nucleotide oligomerization domain 1 (Nod1)/CARD4 protein, identified by Inohara and colleagues [23] and Bertin and colleagues [24]. Nod1/CARD4 is an Apaf1-like molecule composed of a caspase-recruitment domain (CARD), a nucleotide-binding domain (NBD), and, instead of the Apaf1 WD-40 domain, several leucine-rich repeats (LRRs) that associates with the CARD-containing kinase RICK and activates nuclear factor kappaB (NF-κB). Nod2, another member of the family more recently identified [25], is composed of two NH_2-terminal CARDs, an NBD and multiple COOH-terminal LRRs. Whereas Nod1 and Apaf1 were broadly expressed in tissues, the expression of Nod2 was highly restricted to monocytes. As already reported for plant disease resistant R proteins (reviewed in [26]), the LRRs of Nod1 and Nod2 were required for lipopolysaccharides (LPS)-induced NF-κB activation [27]. These observations suggested that Nod1 and Nod2 are the mammalian counterparts of plant R gene products that may function as cytosolic receptors for the pathogen components derived from invading bacteria. The identification of Nod2 defines a subfamily of Apaf1-like proteins that function through RICK to activate a NF-κB signalling pathway.

Over the last year, a novel member of the Apaf1-like family appeared on the apoptosome scenario. Hlaing and colleagues [28] and Chu and colleagues [29] independently reported the deduced amino acid sequence of a protein named Defcap (death effector filament forming ced-4-like apoptosis protein) and Nac (NBD and CARD), respectively. Similar to Apaf1 and Nod1, Nac/Defcap contains a CARD domain and a putative NBD domain, marked by a consensus Walker's A Box (P-loop) and B Box Mg^{2+} binding site. Like Nod1, but different from Apaf1, Defcap contains a putative regulatory domain containing multiple LRRs. However, a distinguishing feature of Nac/Defcap's primary sequence is that Nac/Defcap contains a pyrin-like motif (PLM) and a proline-rich sequence (PR) at its NH(2)-terminus. Unlike Apaf1/ced-4 family proteins, which also contain CARD and NBD domains, the CARD domain of Nac/Defcap is placed at its carboxyl- rather than amino-terminus. Nac/Defcap is strongly expressed in peripheral blood leukocytes, spleen, heart and thymus. The authors demonstrated that both splice variants of the protein, long and short, were capable of interacting with Casp2 and exhibited a weaker interaction with Casp9. The CARD of Nac/Defcap interacts selectively with the CARD domain of Apaf1, and this interaction plays a role in Cyt-c-mediated activation of caspases in cytosolic extracts and in cells [29]. Furthermore, in a sort of Cyt-c-dependent feedback loop, the association of Nac/Defcap with Apaf1 is Cyt-c-inducible. This results in a mega-complex (>1 MDa) containing both Nac/Defcap and Apaf1, and correlates with enhanced recruitment and proteolytic processing of pro-Casp9 (fig. 2). Nac/Defcap is therefore a new component of the apoptosome, and in our opinion it represents the first member of a novel Apaf1-like subfamily which seems to be related to the Apaf1 apoptosomal functions more strictly than the Nod factors (see figs. 1, 2).

Due to the probable deficiency of a Cyt-c-mediated apoptotic pathway in *C. elegans* and to the unclear correlation of the apoptosome with the Bcl-2-like proteins, it cannot be excluded that the true ced-4 homolog in vertebrates is not Apaf1 and has yet to be discovered. There is still no experimental evidence about the role of the novel Apaf1-like proteins in vivo and during development. The study of their activity in the apoptotic cascades will be one of the crucial issues to be investigated in the future.

The *Apaf1*-deficient phenotype

In the context of a large-scale gene trap project, we have cloned the full-length mouse *Apaf1* complementary DNA (cDNA) and analyzed the embryonic phenotype in mice homozygous for the insertional mutation [6]. Abnormal *Apaf1*$^{-/-}$ embryos were found at embryonic day 12.5 (e12.5) and later. The insertional mutation is lethal around e16.5. *Apaf1*$^{-/-}$ fetuses show a characteristic craniofacial phenotype whose major traits are midline facial cleft, absence of skull vault and of all vomer and ethmoidal elements, rostral exencephaly and cleft palate. Since this phenotype is the only apoptosis-deficient phenotype showing a midline fusion defect, Apaf1 could be part of a specific apoptotic pathway involved in midline fusion. *Apaf1* mutants show alterations of the retina, lens and eye vascular system. At e14.5, a hyperplasic retina occupies most of the optic cup and is folded. Apoptosis has been described as a regulator of cell number during normal development of the retina. *Apaf1* is also expressed in the endothelial cells of the transient vascular system of the eye (hyaloid capillary system). By e14.5, vascular en-

Table 3. Glücksmann's scheme [35] is designed to explain the biological functions of developmental cell death. Apaf1 and the apoptosome are involved in all aspects of apoptosis in embryogenesis and, particularly, in brain development.

Type of death	Embryo	Brain
Histogenetic	Control of cell number in the developing retina and brain	Control of cell number of postmitotic neurons to match the size of their peripheral targets
Morphogenetic	In neural tube, lens, skull, face, limbs, inner ear	Early brain-region-specific apoptosis for normal neural tube closure
Phylogenetic	Elimination of the hyaloid vascular system in the developing eye; Elimination of the interdigital webs	Death within the proliferative ventricular zones to regulate the size of the progenitor pool

dothelial cells seem to obliterate completely the optic cup of the *Apaf1*$^{-/-}$ embryos. This phenomenon suggests that apoptosis regulates the number of hyaloid capillaries already in the prenatal period. In wild-type (wt) and heterozygous embryos, the interdigital cells of the limbs, which undergo apoptosis as a mean of sculpting the digits in many vertebrates, disappeared by e15.5. In the homozygotes at this stage, however, these cells can still be seen. These findings not only confirm in vivo the need for programmed cell death in the sculpting of the normal limb but also support the hypothesis that Apaf1 is a key component in apoptosis in multiple cell types. From e12.5 onwards, the brain of the *Apaf1* mutants shows important morphological distortions. The telencephalic vesicles seem abnormally folded and reduced in size. Anatomically, the brain hyperplasia found in the mutants is particularly intense in the diencephalon and midbrain. The similarity of brain phenotypes in *Apaf1*, *Casp3* and *Casp9* null mutations [6–8, 30, 31] strongly indicates that these proteins are components of the same apoptotic pathway during brain development; this is consistent with their functional interactions shown in vitro [1, 2].

In summary (Table 3), Apaf1 is involved in histogenetic cell death (control of cell number in the developing retina and brain), morphogenetic cell death (in the neural tube, lens, skull, face, inner ear and limbs) and phylogenetic cell death (elimination of the hyaloid artery system in the developing eye).

Apaf1 and the apoptosome in brain development

As described above, *Apaf1*$^{-/-}$ phenotype is particularly prominent in brain development. The ectopic brain structure (emerging as cone-shaped exencephaly or cauliflower-like mass or bilateral protrusion) from the embryonic skull is accounted for by a lack of cell death and an excessive mitotic activity between e11.5 and e15.5. The exhibited BrdU-labelling index is twofold higher in the mutant phenotype versus the wt [7]. Which cells, characterizing the mutant ectopic brain, are the supernumerary cells? Are they functional neurons? Do they retain the potential to differentiate? In order to answer these questions, we studied the expression of several marker genes in neural differentiation. The *Apaf1*$^{-/-}$ supernumerary cells express proneural markers. Moreover, not only are these markers detectable in the mutant brain, but they also map to the regions that presumably correspond to the expression domains in the wt [F. Cecconi and P. Gruss, unpublished results]. Our conclusion, supported by the absolute requirement of Apaf1 in the activation of Casp3-dependent developmental death of neural cells [6, 7], is that Apaf1, Casp9 and Casp3 are epistatically related in the apoptotic pathway of neural progenitor cells. This view is coherent with the cascade of epistatic or nonepistatic relationships demonstrated in a series of elegant double-crossing experiments with some apoptosome-mutant mouse lines by Zaidi and colleagues [10] and Roth and colleagues [32]. The first double cross performed was between *Bcl-X*$^{+/-}$ and *Casp3*$^{+/-}$ mice [32]. In double homozygotes, *Casp3*-deficiency abrogated the increased apoptosis of postmitotic neurons, but not the increased hematopoietic cell death and embryonic lethality caused by the *Bcl-X* mutation. In contrast, only the *Casp3*-deficiency was able to modify the normal incidence of neuronal progenitor cell apoptosis, consistent with the lack of expression of *Bcl-X* in the dividing cell population of the embryonic cortex. The distinguishing characteristic of Casp3 emerging from this work is its dual functions in both postmitotic and neuronal progenitor apoptosis (table 3). Although *Casp3*-deficiency results in decreased apoptosis of postmitotic neurons in the developing cortex, this effect is unlikely to be sufficient to cause the extensive neuronal overgrowth in the mutants. Likewise, in *Bax*-deficient brain, the increased number of neurons caused by reduced apoptosis in selective cell populations, does not correspond to gross or microscopic abnormalities [33]. Therefore, the unique effect of caspases on progenitor cell apoptosis is critical in establishing the size of the neuronal founder pool [33].

In the double mutant *Casp9*$^{-/-}$/*Bcl-X*$^{-/-}$ embryos, the increased apoptosis of immature neurons observed in *Bcl-X*-deficient embryos was completely prevented by concomitant *Casp9* deficiency. Instead, the double mutant embryos exhibited the same neuronal malformations observed in *Casp9*$^{-/-}$ embryos [10]. As for Casp3, Casp9 is epistatically and independently related to Bcl-X regulation in different cell populations, immature postmitotic neurons and progenitor cells, respectively. It is widely accepted that trophic factor deprivation activates a cellular suicide program in vertebrate neurons. However, Bcl-X has already been shown to be critical for the survival of

immature neurons before they establish synaptic connections with their targets. The identification of the apoptosome, by single or double mutations of *Casp9* and *Casp3* or *Apaf1* genes, as the regulator of founder cell number suggests that programmed cell death might participate in determining the production of specific progenitor populations, sparing the others. It is conceivable that this selective progenitor cell apoptosis plays a role in cortical expansion, where changes in proliferation kinetics and in death rate increase the number of radial columnar units without changing the number of neurons within each unit significantly. This coordinated action would enhance the capacity of the cortex to establish new patterns of connectivity [34].

The apoptosome seems to be regulated by Bcl-X in immature postmitotic neuron, and apoptosome deficiency alters cell death in many spots of the developing brain (the edge of neural plate and within the optic stalk). Therefore, the Apaf1/Casp9/Casp3 pathway may also be related to the morphogenetic process of brain formation. Table 3 summarizes a parallelism between the roles of the apoptosome in embryonic development and its importance in brain development. Apaf1 is substantially involved in all aspects of developmental apoptosis of the brain, histogenetic, morphogenetic and phylogenetic [35].

It should be mentioned that since apoptotic pathways are stimulus specific, Zaidi and colleagues analyzed telencephalic neuron apoptosis in vivo upon induction by a DNA-toxic agent such as AraC [10]. As expected, both *p53*-deficient neurons as well as $Bax^{-/-}$ neurons showed remarkable protection against death. Whereas, *Casp9*-deficient neurons were only mildly protected against AraC-induced death. In neural progenitor cells, p53 and Casp9 are required, although Casp3 and Bax are not when executing genotoxic apoptosis [9]. The scheme of death which seems to emerge depends therefore on neuronal differentiation state and death stimulus.

Apaf1 in the inner ear development

The developing inner ear in vertebrates acquires a three-dimensional arrangement of its constituent epithelial cells to form three semicircular canals, the endolymphatic duct, the vestibule and the cochlea. Epithelial cells disappear from several areas of the developing structures. This phenomenon is, however, widely debated. For example, processes such as epithelial-mesenchymal transdifferentiation, cell migration and cell death have been implicated at several stages in canal formation [36, 37]. In general, four main apoptotic events were observed: i) adsorption of the central plaque of the prospective semicircular canals, ii) folding between the utriculus and the endolimphatic duct, iii) constriction of the junctions of the saccule with the cochlea and iv) folding of the vestibular portion to form the semicircular ducts (see fig. 3 and [38]). However, a detailed molecular analysis has never been performed to link these events to specific apoptotic pathways. Neither *Bax* nor *Bcl-X* messenger RNAs (mRNAs) are expressed in the ear sensory epithelium from embryonic stage e13 to e19. By contrast, *Bcl-2* is expressed by e15 to e19, being later reduced in the early postnatal period [39]. No data are available on the expression of other pro- and anti-apoptotic factors in epithelial cells during inner ear development. We determined by immunohistochemistry and X-gal staining that Bcl-X and Apaf1 are strongly expressed in the epithelium of the inner ear between e12 and e13 and are presently investigating the occurrence of a phenotype in $Apaf1^{-/-}$ and

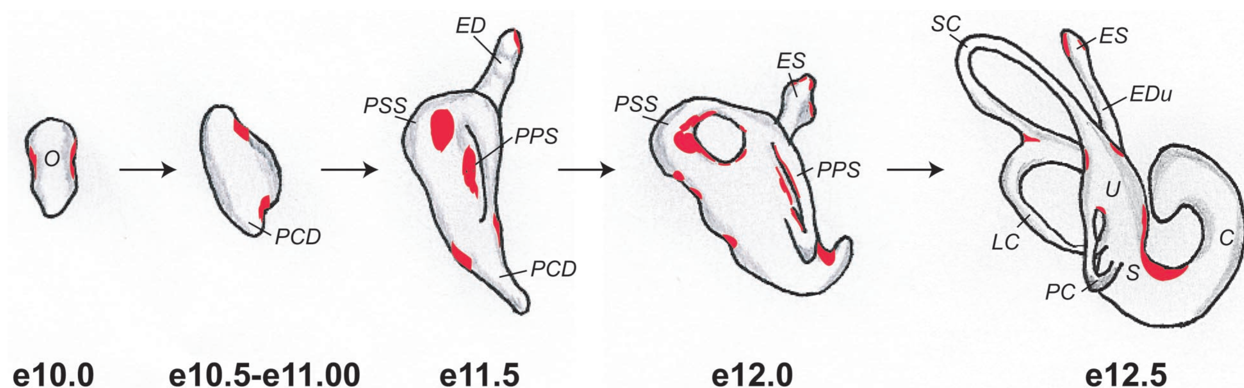

Figure 3. Apoptosis in developing inner ear. The development of the murine left inner ear from 3-D reconstructions of serially sectioned mouse embryos. At e10.5–11.0 the early otocyst (O) has elongated and flattened. By e11.5 onwards, the endolymphatic diverticulum (ED) evaginates rostrally from the otocyst and the primordium of cochlear duct (PCD) elongated caudally. Posterior and superior primordia of the semicircular canals (PPS and PSS, respectively) are already visible at this stage. Extensive adsorption of the central area of PPS and PSS will take place from e11.5 onwards. At e12.0, the extremity of ED has given rise to the endolymphatic sac (ES). At e12.5, the three semicircular canals (SC, superior; LC, lateral; PC, posterior) and the endolymphatic duct (Edu) are formed, and utricle (U), saccule (S) and cochlea (C) are connected by constrictions of the otocyst. Areas of apoptosis are labeled in red. *Apaf1* and *Bcl-X* mutants show aberrant formation of all the structures (not shown). All panels have the same orientation and magnification.

Bcl-X$^{-/-}$ inner ear structures [M. Salminen, F. Cecconi and P. Gruss, unpublished results]. At e9.5 the *Apaf1*$^{-/-}$ neuroepithelium lining the otic vesicles is thickened and poorly organized, and in the few surviving adult mice a substantial hyperactivity was observed, compatible with a pronounced inner ear defect [8]. These results suggest that Apaf1 and the apoptosome are involved in a Bcl-X-dependent pathway of programmed cell death which contributes to the morphogenetic remodelling of the developing inner ear in mice. However, it should be mentioned that several papers have recently shown that Bcl-X_L and Bcl-2 do not directly interact with Apaf1 and that Bcl-2 has cytoprotective functions independent of Apaf1, preserving mitochondrial function through a caspase-independent mechanism [3, 40–42]. We are presently trying to elucidate the functional correlation of Apaf1 and Bcl-X in inner ear development.

Death of an interdigital web: more than one killer

When apoptosis was prevented genetically, as in *Apaf1*$^{-/-}$ embryos, interdigital death still occurred [7]. The cell death was negative for the terminal-deoxynucleotidyl-mediated dUTP nick end-labeling (TUNEL) assay, and there was no overall cell condensation [11]. Although caspase activity confers cell death characterized by an apoptotic morphotype, in the absence of apoptosome and caspase activity an underlying mechanism independent from known caspases can also confer cell death, but with a non-apoptotic morphotype (fig. 4). These results imply that a novel caspase-independent pathway, leading to non-apoptotic death, could be molecularly regulated. More recently, a similar non-apoptotic cellular morphotype was observed by Sperandio and colleagues [43] in different cell types and named *paraptosis* (from 'para' for 'next to' or 'related to', and apoptosis). The authors also showed that this form of cell death is programmatic, because it requires gene expression. It should be mentioned that despite its lack of response to caspase inhibitors and Bcl-X_L, this alternative form of cell death was surprisingly inhibited by a catalytic mutant of caspase-9 zymogen. This kind of morphotype, characterized by mottled chromatine condensation, nuclear membrane detachment and rupture, dilated mitochondria and cytoplasmic vacuoles and sometimes external membrane rupture, is common to the death morphotype induced by activity of the apoptosis inducing factor (AIF), isolated by Susin and colleagues [44]. Therefore, a hypothetical AIF-dependent pathway could represent a potential alternative to the Apaf1-dependent death, enabling the cells to perform suicide in a slower but ineluctable way.

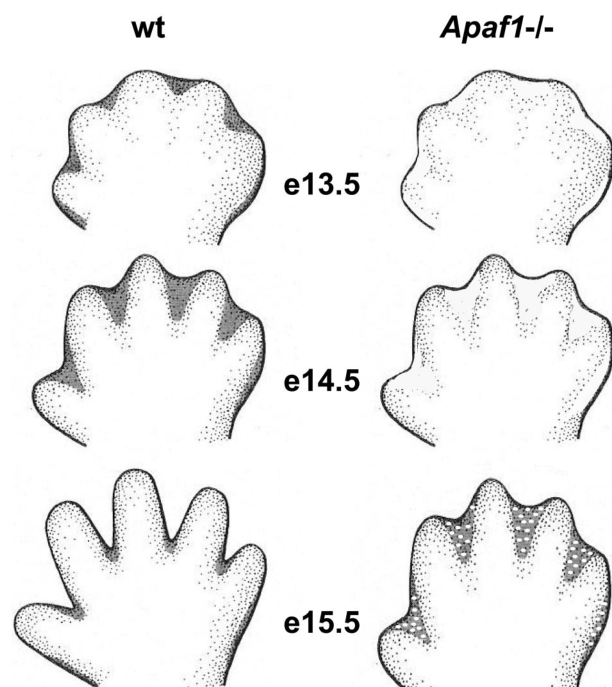

Figure 4. Apoptosis and non-apoptotic death in the interdigital webs. Among the many processes involved in the development of the limb bud, cell death is the most prominent. It first appears in the forelimb in the region of the armpit (not shown). Then patches of TUNEL-positive cells are seen around the developing digits (e13.5, upper panels, in grey). In *Apaf1* mutant limbs, the persistence of webs of soft tissues between the digits is associated with reduced amounts of cell death (upper and middle panels). Webbed digits represent a developmental anomaly (e15.5, lower panel). The web cells, white-dotted in the lower right panel, die later with a non-apoptotic or *paraptotic* phenotype. Modified from Patten's Foundations of Embryology [54].

Roles of Apaf1 in cancer

Fearnhead and colleagues [45] have demonstrated that in cells sensitized by E1A, an adenoviral oncogene, oncogene-dependent apoptosis was inhibited by preventing Casp9 activity. This pathway of cell death required the activity of the apoptosome, consisting of at least Casp9, Apaf1 and Cyt-c. Six months later, experiments on p53 activity as an effector of mitogenic apoptosis were critical in defining the apoptosome-dependent pathway as the core of Myc-induced apoptosis [46]. The authors mimicked in mutant fibroblasts for *p53*, *Apaf1* and *Casp3* the pro-apoptotic conditions of developing tumors and showed that inactivation of *Apaf1* or *Casp9* substituted for *p53* loss in promoting the oncogenic transformation of Myc-expressing cells. At the beginning of 2000, Bala and colleagues [47] found that the region containing *APAF1* in human chromosome 12q23 [6, 48] was deleted or aberrant in a few male germ cell tumors (GCT) cell lines. However, the authors were unable to support a direct role of APAF1 as tumor suppressor gene in GCT. The same chromosomal region was linked with pancreatic ductal adenocarcinoma at a frequency of 60% of tested samples [49, 50]. The results were obtained by micro-

satellite analyses and verified by fluorescence in situ hybridization. Further analysis of 19 pancreatic cancer cell lines showed that 16 of them displayed allelic loss within the same region. In addition, the selected region was completely covered by a yeast artificial chromosome clone [49]. Finally, frame-shift mutations in *APAF1* were observed in gastrointestinal cancer of the microsatellite mutator phenotype [51]. But only recently a possible link of APAF1 allelic loss with cancer onset and progression has been indicated by in vitro and in vivo experiments. Soengas and colleagues [12] showed that metastatic melanomas often lose APAF1 and are chemoresistant and invariably unable to execute a typical apoptotic program in response to P53 activation. Interestingly, the allelic loss of *APAF1* observed in metastatic melanomas could be recovered in melanoma cell lines by treatment with a methylation inhibitor. This suggests that a transcriptional silencing by methylation may contribute to *APAF1* inactivation in melanoma [12]. Since extensive methylation is not a feature of the proximal *APAF1* promoter region, it cannot be excluded that a trans-activating modulator of *APAF1* may be regulated by methylation. Altogether these results imply that the APAF1/CASP9 pathway is a key network in tumor control and that the apoptosome plays a rate-limiting effect in cell death. Obviously, restoring the APAF1 physiological concentration in melanomas retains the potential to restore chemosensitivity, since the suicide pathway can potentially be reactivated.

Furthermore, the implication of a transcriptional regulation of *APAF1* promoter in allelic loss points for the first time to a possible dosage effect of the APAF1 molecule on the stoichiometry of the apoptosome. In other words, APAF1 may not be present at standard high levels in the cytosol, being, in contrast, finely regulated at a transcriptional or posttranscriptional level. For this reason, it should be mentioned that the APAF1 5′ untranslated region contains an internal ribosome entry site (IRES; [52]). Some mRNAs, translated under a variety of stress conditions including hypoxia, serum deprivation, irradiation and apoptosis, are translated by a cap-independent mechanism, mediated by an IRES element located in the 5′ untranslated region (reviewed in [53]). The *APAF1* IRES was found to be active in several cancer cell types, from cervical carcinoma (HeLa), to liver carcinoma (HepG2) and breast carcinoma (MCF7). The *APAF1* IRES may thus ensure a constant cellular level of APAF1 protein, even under conditions where cap-dependent translation is compromised.

Consistent with this dosage-effect hypothesis, we observed, in an inbred weak genetic background such as 129Sv/Pas, a mild developmental phenotype (ectopic brain masses) in *Apaf1* heterozygotes [F. Cecconi et al., unpublished results].

Concluding remarks

In a comprehensive developmental and biochemical approach, Apaf1 is the adaptor molecule representing the core of the most studied apoptotic machinery, the apoptosome. We believe that the elucidation of the apoptosome in developmental and adult tissues could provide the basis for intervention in neurodegeneration and cancer. Indeed, beside an extension of our basic knowledge, research focused on the roles of the apoptosome might contribute to provide better tools for genetic diagnosis and identify novel targets for pharmaceutical approaches. For example, *Apaf1* mutations could influence the onset and progression of neurodegenerative diseases; pharmacological tools, devised to modulate the apoptosome functionality, might retain the potential to slow down those processes. Since the degenerative changes observed in most neurodegenerations occur over a long period of time, a decrease in the rate of toxic peptide formation and/or neuronal loss may significantly prolong patients' lifespan and improve their quality of life. Moreover, modulating apoptosis in mouse models for cancer, disrupting or overexpressing *Apaf1* specifically in transformed tissues will tell us whether the apoptosome could be a target for drug action in oncogenesis. Obviously the newly identified *Apaf1*-family members, such as the *Nod* genes or *Nac/Defcap*, are good candidates to play crucial roles in the cell cycle and in apoptosis and can therefore be considered as new frontiers in pharmacological modulation of cell proliferation and death.

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