The Role of Caspases in Development, Review Immunity, and Apoptotic Signal Transduction: Lessons from Knockout Mice

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Introduction

Apoptosis is the innate mechanism by which the organism eliminates unwanted cells. It is the most common form of cell death and occurs during development, tissue remodeling, cell homeostasis, defense processes, and immune responses. Dysregulation of apoptosis may be directly involved in several human pathologies including degenerative and autoimmune diseases, neoplasia, AIDS, and other viral or bacterial infections (Fisher, 1994; Thompson, 1995; Los et al., 1997). Significant progress in our understanding of apoptosis came from genetic studies of developmental cell death in the nematode Caenorhabditis elegans, in which a set of a few genes, termed CED for cell death-defective, regulates the apoptotic machinery (Ellis et al., 1991). Two of them, ced-3 and ced-4, are required for the execution of cell death. Another gene, ced-9, protects cells from undergoing programmed cell death. Loss-of-function mutations in ced-9 cause cells that normally live to undergo apoptosis (Hengartner et al., 1992). This cell death requires the activity of ced-3 and ced-4, indicating that ced-9 acts by preventing ced-3 and ced-4 from causing cell death. ced-9 is the structural and functional homolog of Bcl2, one of a large family of genes intimately involved in prevention of vertebrate apoptosis. CED-4 is highly related to an apoptosis regulator in mammals, designated Apaf1 for apoptotic protease-activating factor-1 (Zou et al., 1997). The mammalian counterpart of CED-3 has been identified as a member of a family of intracellular proteases that form the core of the apoptotic machinery (Yuan et al., 1993). Since these are cysteine proteases that cleave cellular substrates at specific aspartate residues, they are termed caspases (Alnemri et al., 1996).

Mammalian caspases comprise a group of at least fourteen members that can promote apoptosis (Cohen, 1997; Nicholson and Thornberry, 1997; Cryns and Yuan, 1998; Stroh and Schulze-Osthoff, 1998). Some caspases, in addition, generate mature proinflammatory cytokines and thereby regulate immune responses. Based on phylogenetic analysis and positional scanning studies of their peptide substrates, caspases are divided into three subfamilies: The ICE-like protease family includes Casp1, -4, -5, -13, and -14 as well as murine Casp11 and -12. The CED-3 subfamily includes Casp3, -6, -7, -8, -9, and -10, whereas the third subfamily consists of only one member, Casp2. Within each subfamily, the peptide sequence preferences in the substrates are similar (Thornberry et al., 1997; Garcia-Calvo et al., 1998). Because at least in some cases different caspases can cleave the same substrates, this illustrates some degree of functional redundancy within the caspase family.

Caspases exist as latent zymogens that contain an N-terminal prodomain followed by the region that forms the two subunits with the catalytic domain. The core of the catalytic center is formed by the conserved pentapeptide sequence QACXG. The proforms of caspases are activated by proteolytic cleavage at specific aspartate residues. Generally, an initial cleavage event separates the C-terminal short subunit from the rest of the molecule, allowing assembly of an active protease that autocatalytically cleaves off its prodomain. As deduced from the crystal structure of Casp1 and Casp3, the mature enzyme of all caspases is a heterotetrameric complex composed of two large subunits and two small subunits (Walker et al., 1994; Wilson et al., 1994; Rondona et al., 1996; Mittl et al., 1997). Once activated, some caspases can propagate activation of other family members and thus initiate a proteolytic cascade.

Based on their structure and order in cell death pathways, caspases can be divided into initiator and effector caspases. Effector caspases generally contain only a small prodomain and cleave diverse cellular substrates, whereas initiator caspases have a long prodomain and exert regulatory roles by activating downstream effector caspases. Activation of initiator caspases is mediated by binding of adaptor molecules to protein interaction motifs in their prodomains. Two general types of interaction have been identified (Martin et al., 1998; Muzio et al., 1998; Srinivasula et al., 1998). Pro-Casp8 and -10 each contain two tandem death effector domains (DEDs), while pro-Casp1, -2, -4, and -9 contain a caspase-recruitment domain (CARD). In each case, the procaspases bind to adaptor molecules containing similar domains, and they either directly aggregate or interact with other molecules (Figure 1). Although not related, CARDs and DEDs have a similar structure composed of amphipathic, antiparallel α helices, which are also present in the death domain of death receptors. Upon ligation, death receptors such as CD95 and TNF-R1 bind to and oligomerize adaptor proteins and procaspases. FADD, which contains a DED, is recruited directly to CD95 and indirectly to TNF-R1, resulting in the autoactivation of Casp8. In an analogous manner, the adaptor protein RAIDD/CRADD, which contains a CARD, can associate with TNF-R1 and promote activation of pro-Casp2 (Ahmad et al., 1997; Duan and Dixit, 1997; Li et al., 1997a). Besides death receptor-mediated apoptosis, a related but different way of caspase activation exists that is triggered for instance by cytotoxic drugs or p53 and essentially controlled by mitochondria. In early phases of apoptosis, mitochondria release cytochrome c, which, together with dATP, activates the adaptor protein Apaf1 (Liet al., 1997c). Apaf1 binds to the prodomain...
Figure 1. Two Principal Signaling Pathways of Apoptosis

One pathway (left) involves ligation of death receptors, resulting in the recruitment of the adaptor protein FADD through interaction between the death domains (DD) of both molecules. The death effector domain (DED) of FADD in turn recruits pro-Casp8, which is cleaved and activated at the receptor complex. Another pathway (right), which is triggered by many apoptotic stimuli, is initiated at the mitochondrion. An early, not well-understood step is the mitochondrial release of cytochrome c into the cytosol, which, together with dATP, binds to the CED-4 homolog Apaf1. This event unmasks the CARD motif in Apaf1 and allows binding of procaspase-9 through CARD/CARD interaction. The mitochondrial but not the death receptor pathway is inhibited by Bcl2. Antiapoptotic members of the Bcl2 family may interfere with the relocalization of cytochrome c or with the binding of cytochrome c to Apaf1. Following activation of the initiator caspase Casp8 or Casp9, the two pathways converge on the activation of effector Casp3, -6, and -7, which finally cleave various death substrates. Because Casp8 cleaves Bid and generates a truncated, proapoptotic BH3-containing fragment (tBid) that induces cytochrome c release, both pathways cross-communicate. Casp8, in turn, can be also activated by Casp6 following Casp9 cleavage, thereby amplifying the apoptotic signal.

of pro-Casp9 via CARD-CARD interaction, while a different region of Apaf1 self-associates resulting in Casp9 activation (Figure 1).

Although caspases are the essential components of many, if not all apoptotic pathways, their precise physiological role remains controversial. The large number of members of the caspase family, their overlapping tissue distribution and similar cleavage specificities present a challenge for identifying the function of individual caspases in vivo. A number of questions must be raised: First, are caspases redundant or do individual caspases play a dominant apoptotic role in a tissue- or cell-type-specific manner? Second, is any caspase indispensable for the activation of other caspases? Third, does an individual caspase participate in some forms of cell death, but not in others? Finally, does the inhibition of a certain caspase exert global effects on apoptosis or only prevent specific forms of death in certain tissues? Very recent studies using gene targeting and transgene technologies have provided some answers to these questions and have shed new light on the distinctive role of individual caspases in cell death as well as in other unexpected biological processes.

Caspase-1 and Caspase-11 Knockout Mice

Casp1 was originally identified as interleukin 1β-converting enzyme (ICE), the protease that cleaves the precursor of IL-1β into the active cytokine (Cerretti et al., 1992). More recently, Casp1 has been shown to further process the cytokine precursors of IL-16 and IL-18 (Ghayur et al., 1997; Gu et al., 1997; Fantuzzi et al., 1998). Since the demonstration that ced-3 of C. elegans encodes a protein similar to mammalian Casp1 (Yuan et al., 1993), and that overexpression of both genes promotes apoptosis (Miura et al., 1993; Los et al., 1995), Casp1 has been a subject of intensive research. Point mutations in a region homologous between CED-3 and Casp1 eliminated the ability of both proteins to induce cell death. Apoptosis caused by overexpression could be also suppressed by Bcl2 and CrmA, a viral inhibitor of caspases.

Gene targeting revealed that Casp1 plays an important role in the regulation of the immune response, but is presumably dispensable for most apoptotic pathways (Li et al., 1995; Kuida et al., 1995). Casp1−/− mice had a major defect in the production of mature IL-1β and impaired IL-1α synthesis (Table 1). Secretion of TNF-α and IL-6 in response to LPS stimulation was also diminished. In addition, macrophages from Casp1−/− mice were defective in LPS-induced IFN-γ production (Fantuzzi et al., 1998). Casp1−/− mice were highly resistant to the lethal effects of endotoxin. At a dose of LPS that killed wild-type mice within 30 hr, all Casp1−/− mice survived (Li et al., 1997b). The proinflammatory role of Casp1 was strengthened by the finding that Casp1-deficient mice revealed decreased necrosis, edema formation, and serum levels of amylase and lipase during experimentally induced pancreatitis (Norman et al., 1997). Pharmacological blockade or genetic deletion of Casp1 also inhibited pancreatitis-linked secretion of...
Table 1. Phenotypes of Caspase-, FADD- and Apaf1-Deficient Mice

<table>
<thead>
<tr>
<th>Phenotypes of Caspase-, FADD- and Apaf1-Deficient Mice</th>
<th>Apoptosis</th>
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<tr>
<td><strong>Development and Cytokine Expression</strong></td>
<td><strong>Apoptosis</strong></td>
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<tr>
<td>Caspase 1</td>
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<tr>
<td>No developmental defects;</td>
<td>Sensitive to most apoptotic inducers;</td>
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<tr>
<td>Resistant to LPS-induced septic shock;</td>
<td>Reduced ischemic brain injury;</td>
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<tr>
<td>Increased survival in experimental pancreatitis;</td>
<td>CD95-induced apoptosis attenuated in thymocytes;</td>
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<tr>
<td>No IL-1β and IL-18 processing, impaired production</td>
<td>Neurons resistant to trophic factor withdrawal</td>
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<td>of IL-1α, IL-6, TNF-α and IFN-γ</td>
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<tr>
<td>Caspase 2</td>
<td>Oocytes resistant to drug-induced death;</td>
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<tr>
<td>Viable, no gross abnormalities;</td>
<td>Defective B cell death in response to granzyme B;</td>
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<tr>
<td>Excess numbers of female germ cells</td>
<td>Lymphocytes sensitive to drugs and anti-CD95;</td>
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<td></td>
<td>Increased susceptibility of sympathetic neurons to</td>
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<td>trophic factor withdrawal</td>
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<tr>
<td>Caspase 3</td>
<td>Fibroblasts resistant to TNF-R1, CD95, and DR3, but</td>
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<tr>
<td>Mice born at lower frequency and with smaller size;</td>
<td>sensitive to drug-induced apoptosis;</td>
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<tr>
<td>Death at 1-3 weeks of age;</td>
<td>Normal JNK and NF-κB activation</td>
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<tr>
<td>Disturbed brain development with excessive</td>
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<td>numbers of postmitotic cells</td>
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<tr>
<td>Caspase 8</td>
<td>Decreased number of hematopoietic stem cells</td>
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<tr>
<td>Lethal in utero; embryos of smaller size</td>
<td>Embryonic stem cells and fibroblasts resistant to</td>
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<tr>
<td>Impaired heart muscle development;</td>
<td>several apoptotic stimuli;</td>
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<tr>
<td>Congested accumulation of erythrocytes and</td>
<td>Thymocytes resistant to dexamethasone- and</td>
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<tr>
<td>massive hemorrhage</td>
<td>γ-irradiation-induced apoptosis, but sensitive</td>
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<tr>
<td>Decreased number of hematopoietic stem cells</td>
<td>to UV irradiation and CD95;</td>
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<td></td>
<td>Splenocytes not protected against drug-induced</td>
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<td></td>
<td>apoptosis</td>
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<tr>
<td>Caspase 9</td>
<td>Embryonic stem cells and fibroblasts resistant to</td>
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<tr>
<td>Perinatal lethality;</td>
<td>several apoptotic stimuli;</td>
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<tr>
<td>Enlarged and malformed cerebrum due to reduced</td>
<td>Thymocytes resistant to dexamethasone- and</td>
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<tr>
<td>apoptosis during brain development;</td>
<td>γ-irradiation-induced apoptosis, but sensitive</td>
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<tr>
<td>Lack of Casp3 activation in embryonic brains</td>
<td>to UV irradiation and CD95;</td>
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<td></td>
<td>Splenocytes not protected against drug-induced</td>
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<td></td>
<td>apoptosis</td>
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<td>Caspase 11</td>
<td>Cells resistant to apoptosis induced by</td>
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<tr>
<td>No developmental defects, similar to Casp1</td>
<td>overexpression of Casp1</td>
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<td>deficiency</td>
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<tr>
<td>Resistant to endotoxic shock;</td>
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<tr>
<td>Lack of IL-1α and IL-1β production due to blocked</td>
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<tr>
<td>Casp1 activation</td>
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<tr>
<td>FADD</td>
<td>Fibroblasts resistant to death receptor, but sensitive</td>
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<tr>
<td>Cardiac failure and massive hemorrhage;</td>
<td>to drug-, E1A-, and c-Myc-induced apoptosis;</td>
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<td>Phenotype similar to Casp8-/- mice;</td>
<td>Thymocytes of RAG1 chimeras show impaired</td>
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<tr>
<td>IL-2 expression of thymocytes intact</td>
<td>survival and proliferation</td>
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<tr>
<td>Apaf1</td>
<td>Embryonic fibroblasts exhibit reduced response to</td>
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<tr>
<td>Lethal at E16.5;</td>
<td>various apoptotic stimuli;</td>
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<tr>
<td>Brain overgrowth, exencephaly;</td>
<td>Thymocytes sensitive to CD95-, but resistant to</td>
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<tr>
<td>Severe craniofacial and ossification defects;</td>
<td>drug- and irradiation-induced apoptosis</td>
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<tr>
<td>Strong alterations of the lens and retina;</td>
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<td>Persistence of interdigital webs</td>
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Proinflammatory cytokines and was associated with dramatic survival benefits.

In contrast to inflammatory responses, Casp1 null mice did not show any gross abnormalities in development or profound defects in apoptosis. For instance, ATP-induced apoptosis of peritoneal macrophages was not affected (Li et al., 1995). Thymocytes from young Casp1-/- mice equally underwent apoptosis, when triggered by dexamethasone or γ-irradiation. However, these thymocytes revealed a partial resistance toward CD95-induced apoptosis (Kuida et al., 1995). Studies from knockout (KO) mice indicated that Casp1 mediates partially apoptosis induced by IFN-γ. In this scenario, a positive amplification loop between IFN-γ and Casp1 seems to exist. IFN-γ is able to induce Casp1 expression through activation of the STAT (signal transducer and activator of transcription) signaling pathway (Chin et al., 1997). IFN-γ did not induce Casp1 expression and apoptosis in STAT1-deficient cells. Therefore, activation of the STAT pathway may induce apoptosis in some systems through the induction of Casp1 expression.

An involvement of Casp1 in some forms of apoptosis was shown in experimental models of neuronal cell death. Mutant Casp1, in which the active site cysteine was changed into a glycine, inhibited trophic factor withdrawal-induced apoptosis in dorsal root ganglial cells (Friedlander et al., 1997a). Transgenic mice expressing this mutant Casp1 under control of a neuron-specific promoter appeared normal. However, brain injury induced by middle cerebral artery occlusion, a mouse model of stroke, was significantly reduced in Casp1 mutant and deficient mice (Friedlander et al., 1997a; Schielke et al., 1998). In addition, expression of mutant Casp1 in neurons of superoxide dismutase-deficient mice, a model of amyotrophic lateral sclerosis, was able to delay disease progression (Friedlander et al., 1997b). Recently, it has been proposed that Casp1 is activated by a direct physical interaction with murine Casp11 (Ich-3), which is most homologous to human Casp4 and Casp5 (Wang et al., 1998). Casp11 transcription and translation is strongly induced upon stimulation with LPS. Overexpression of Casp11 induced apoptosis,
which could be inhibited by CrmA and Bcl2 (Wang et al., 1996). Casp11-deficient cells were resistant to apoptosis induced by Casp1 overexpression, suggesting that activation of Casp1 requires Casp11. Analogously to Casp1−/− mice, Casp11-deficient animals developed normally and were resistant to endotoxic shock. Production of both IL-1α and IL-1β after LPS stimulation, a critical event during septic shock, was blocked in Casp11−/− mice. Thus, Casp1 and Casp11 seem to play an important role in inflammation by activating cytokines, but may not mediate apoptosis in development and most circumstances.

Targeted Disruption of Caspase-2

Casp2 (Ichi-1, Nedd2) was initially identified by a subtractive cloning approach as a gene highly expressed in embryonic brain and downregulated in adult brain (Kumar et al., 1992). Overexpression of Casp2 in mammalian cells induced apoptosis that could be blocked by Bcl2 (Kumar et al., 1994; Wang et al., 1994). Using antisense technology, decreasing Casp2 levels have been shown to delay cell death induced by trophic factor deprivation in hematopoietic and neuronal cell lines.

Gene targeting revealed that Casp2−/− mice reached adulthood and were devoid of severe phenotypic abnormalities (Bergeron et al., 1998). However, Casp2 appeared to be required for female germ cell death. During development, over one-half of the ovarian germ cells undergo apoptosis. This massive germ cell death occurs at later stages of fetal life and persists through day 3 postpartum, at which time remaining oocytes are enclosed by granulosa cells to form primordial follicles. Casp2-deficient mice contained significantly higher numbers of newly formed primordial follicles than wild-type mice. Oocytes of Casp2 KO mice were also resistant to cell death following exposure to chemotherapeutic drugs.

Unlike apoptosis of germ cells, cell death of facial motor neurons was not suppressed but even accelerated in Casp2-deficient mice (Bergeron et al., 1998). Similarly, Casp2-deficient sympathetic neurons underwent apoptosis more effectively than wild-type neurons when deprived of nerve growth factor. Thus, Casp2 not only acts as a positive effector of apoptosis, but in some cells it may also delay cell death. The pro- versus anti-apoptotic effect of Casp2 gene targeting may be caused by the existence of splice variants that are expressed to variant degrees in different tissues and developmental stages. Alternative splicing generates two Casp2 messages that encode Casp2α, which induces cell death, and Casp2β, a truncated protein that antagonizes cell death (Wang et al., 1994).

Unlike Casp1, Casp2 is not involved in ischemic brain injury caused experimentally by middle artery occlusion, as the extent of ischemic injury was unaffected in mutant mice. There were also no differences in the rate of motor neuron death after denervation in vivo. Furthermore, Casp2 does not obviously contribute to cell death of sympathetic neurons induced by trophic factor withdrawal, although it has previously been reported that Casp2 antisense constructs inhibited this form of cell death (Kumar et al., 1994).

B lymphoblasts from Casp2 KO mice were deficient for granzyme B−, but not CD95-mediated apoptosis. Thymocytes and T lymphocytes were also equally sensitive to apoptosis caused by doxorubicin, etoposide, staurosporine, or γ-irradiation. Interestingly, no differences in TNF-induced apoptosis were observed in Casp2-deficient embryonic fibroblasts. Casp2 has been shown to interact physically with RAIDD, an adaptor protein containing a region homologous to the death domain and prodomain of Casp2 (Ahmad et al., 1997; Duan and Dixit, 1997). The death domain of RAIDD interacts with RIP, a death domain containing serine/threonine kinase that is part of the TNF death pathway. The results with Casp2 mutant mice therefore suggest that Casp2 and RAIDD may be redundant in TNF signaling.

In conclusion, Casp2 may be essential for apoptosis in female germ cells, whereas in other situations it may function to delay cell death. The ultimate action of Casp2 appears to be dependent on the tissue type, cell lineage, developmental stage, differential splicing of its mRNA, and the presence and absence of other caspases.

Caspase-3-Deficient Mice

Casp3 (CPP32, YAMA, Mch2, apopain) is regarded as a prototype effector caspase able to degrade the vast majority of “death substrates.” The three-dimensional structure of a complex of Casp3 with a tetrapeptide inhibitor has been determined (Rotonda et al., 1996; Milti et al., 1997). Its architecture resembles Casp1 in overall structure, but its P4 substrate residue (four amino acids N-terminal of the cleavage site) is different. These differences account for the distinct specificity of the two proteases and may enable the design of selective inhibitors.

Gene targeting of Casp3 generated the first mice with profound defects in apoptosis (Kuida et al., 1996; Woo et al., 1998). The mice, born at a frequency lower than expected by Mendelian genetics, were smaller than their littermates and died at 1-3 weeks of age. Their phenotype was remarkably restricted. The most pronounced effect was seen in the central nervous system, while no discernible abnormalities were found in embryonic heart, lung, liver, or kidney. Casp3-deficient animals showed massive hyperplasia and ectopic cell masses of the brain with a variety of disorganized cellular structures. Also protrusions of the neuroepithelium in the retina, which caused compression of the lens, could be seen. Pyknotic clusters, which are normally detected at sites of major morphogenetic change during normal brain development, were not observed in the mutant embryos. In contrast, the excessive cells were postmitotic and terminally differentiated. Hence, Casp3 plays a critical role during morphogenetic cell death in the mammalian brain.

The central role of Casp3 for induction of apoptosis has been shown also in hepatocytes and thymocytes derived from KO mice (Zheng et al., 1998). Although Casp3−/− hepatocytes or thymocytes were killed at a similar rate as control cells, when cocultured with CD95 ligand expressing 3T3 cells, wild-type cells displayed typical apoptotic features such as cytoplasmic blebbing and nuclear fragmentation within 6 hr, but neither event was observed for Casp3−/− cells. The cleavage of various caspase substrates implicated in apoptotic events,
including gelsolin, fodrin, lamin B, and DFF/ICAD, an endonuclease inhibitor, was delayed or absent in the Casp3 −/− cells. Thus, the altered cleavage of these key substrates is likely to be responsible for the aberrant apoptotic phenotype in Casp3-deficient cells.

Although Casp3 is highly expressed in cells of hematopoietic origin, lack of Casp3 did not affect immature T and B cell development (Kuida et al., 1996). Genetically targeted mice contained fewer thymocytes, related to their overall smaller size. Surprisingly, thymocytes from Casp3 null and wild-type mice were equally sensitive to induction of apoptosis by anti-CD95, dexamethasone, ceramide, staurosporin, and γ-irradiation. Using Casp3 −/− lymphocytes from Rag1 chimeric mice, it was shown that peripheral T cells were resistant to activation-induced cell death and apoptosis triggered by anti-CD3 and anti-CD95 (Woo et al., 1998). The requirement for intact Casp3 appeared to depend on the apoptotic stimulus. In embryonic stem cells, Casp3 was necessary for efficient apoptosis following UV irradiation, but not γ-irradiation or cytotoxic killing of target cells. Conversely, the same stimulus can show a tissue specificity: TNF-α treatment induced normal levels of apoptosis in Casp3 −/− thymocytes, but defective apoptosis in oncogene-transformed fibroblasts (Woo et al., 1998). Hence, the consequences of Casp3 deficiency appear to be remarkably context-dependent with apoptotic defects being both cell type- and stimulus-specific.

Gene Targeting of Death Receptor Pathways

Casp8 (FLICE, Mach, Mch5) was originally identified as the proximal caspase in the death-inducing signaling complex of CD95 (Boldin et al., 1996; Muzio et al., 1996; Srinivasula et al., 1996). The prodomain of Casp8 as well as that of Casp10 contain duplicates of a death effector domain, which enable Casp8 to interact with FADD, an adaptor molecule associating with the CD95 receptor (Figure 1). It is assumed that besides CD95 a similar pathway is activated by other death receptors including TNF-R1, DR3, and TRAIL receptors. Unlike most other caspases, Casp8 is believed to autoproteolytically activate itself upon recruitment and oligomerization at the vicinity of death receptors (Martin et al., 1998; Muzio et al., 1998). Very recently, a novel molecule called FLASH has been identified to associate with Casp8 in the CD95 death-inducing signaling complex through a death effector domain related motif (Imai et al., 1999). FLASH, in addition, contains a region with homology to Apaf1, which binds ATP and which is required for self-association of FLASH molecules. However, although this would suggest similarities between FLASH and Apaf1, it is currently unknown whether FLASH is essentially required for or just facilitates death receptor-mediated apoptosis.

While Casp8 plays a central role in propagating death receptor-mediated signals, proteolytic processing of Casp8 has been found also in other apoptosis settings, such as chemotherapeutic drug-induced apoptosis (Ferrari et al., 1998; Wesselborg et al., 1999). In this pathway, Casp8 activation is presumably independent of death receptors and mediated by prior cytochrome c release and Apaf1 activation. It has been shown that Casp8 cleaves Casp6, which in turn can directly activate Casp8 (Slee et al., 1999). A Jurkat T cell line deficient in Casp8 was not only completely resistant to death receptor-induced apoptosis, but also partially resistant to cell death induced by UV irradiation, adriamycin, and etoposide (Juo et al., 1998). Complementation of these cells with Casp8 restored apoptosis sensitivity. In addition, adenoviral E1A induced pro-Casp8 processing and apoptosis in cells deleted of FADD, indicating the existence of alternative activation pathways (Nguyen et al., 1998). A possible candidate in this context could be an apoptosome-like complex in the endoplasmic reticulum (ER). It is composed of the integral ER membrane protein p28 Bap31, Casp8, and possibly Apaf1 or a related molecule (Ng and Shore, 1998). Activation of this complex might be induced upon viral infection or in other situations associated with abnormal ER function. Hence, Bap31 could play a similar function in the ER as the CD95 death-inducing signaling complex at the surface membrane.

While Casp8 can be activated indirectly in the mitochondrial pathway, it has also been found that, conversely, CD95-mediated activation of Casp8 can deliver a signal to mitochondria (Scaffidi et al., 1998). This event is initiated by Casp8-mediated cleavage of the Bcl2 protein Bid, which generates a proapoptotic fragment that triggers cytochrome c release (Li et al., 1998; Luo et al., 1998). Thus, both the death receptor and mitochondrial pathway of caspase activation may be interconnected leading to the amplification of an apoptotic signal.

Homozygous disruption of the mouse Casp8 gene was found to be lethal in utero (Varfolomeev et al., 1998). However, whereas Casp3 −/− mice exhibited profound brain defects, Casp8 −/− embryos presumably died from cardiac failure. Two salient features of Casp8 null mice were impaired heart muscle development and abdominal hemorrhage. Extensive erythrocytosis was present also in other organs such as liver and lung. The reason for this is not understood but may be caused by a defect in angiogenesis, disturbed hematopoiesis, or heart failure. Although the heart of Casp3 −/− mice was not appreciably larger than normal, the trabeculae and ventricular musculature were thin and resembled early mesenchyme. The fact that the heart was hypotrophic rather than enlarged strongly suggests that Casp8 may be involved in the transmission of survival rather than death signals. This is supported by the fact that hematopoietic precursor cells from KO mice revealed a strongly impaired colony-forming activity. Disruption of Casp8 thus appears to result in a primary or secondary depletion of the hematopoietic precursor pool (Varfolomeev et al., 1998).

Embryonic fibroblasts deficient in Casp8 were completely resistant to apoptosis mediated by death receptors, such as CD95, TNF-R1 and DR3, whereas they retained sensitivity to a wide range of apoptotic stimuli including UV irradiation, ceramide, chemotherapeutic drugs, and infection with the cytopathic vesicular stomatitis virus. Yet in contrast, embryonic fibroblasts responded normally to nonapoptotic signals emanating from death receptors and activated Jun N-terminal kinases as well as transcription factor NF-κB equally as wild-type cells. Thus, these findings indicate that Casp8 plays a necessary and nonredundant role in apoptosis induction by death receptors.
Like Casp8 KO mice, gene targeting of the adaptor protein FADD resulted in a lethal phenotype with profound signs of cardiac failure and hemorrhage (Yeh et al., 1998). Overexpression of CD95, TNF-R1 and DR-3 could not induce apoptosis in embryonic fibroblasts from FADD−/− mice, whereas DR4 and chemotherapeutic drugs, as well as oncogene expression of c-Myc and E-1A, did. Since FADD−/− mice die in utero, T cell maturation has been analyzed in chimeric mice deficient for the recombination activating gene product RAG1, which activates rearrangement of immunoglobulin and T cell receptor genes (Zhang et al., 1998). T lymphocytes from FADD−/− chimera were completely resistant to CD95-induced apoptosis. Thymocyte populations were apparently normal in newborn chimeras. As these mice age, their thymocytes, however, decreased to undetectable levels, although peripheral T cells were present. Thus, FADD−/− mice seem to be inefficient in maintaining thymic cellularity possibly due to an intrinsic survival defect.

Whereas mice lacking a functional CD95 system develop lymphadenopathy and splenomegaly as a result of the accumulation of an abnormal T cell population in the periphery, this population was not detected in FADD KO mice 5 months old. The absence of lymphoproliferative disease and the T cell population characteristic of CD95 deficiency in FADD−/− may result from a defect in the proliferation of T cells. Indeed, activation-induced proliferation was impaired in FADD−/− T cells, despite normal production of IL-2. Thus, this suggests a rather unexpected connection between cell proliferation and apoptosis (Zhang et al., 1998).

A growth-promoting activity of FADD is supported by the phenotype of transgenic mice expressing a dominant-negative FADD mutant (FADD-DN) under control of a T cell-specific promoter (Newton et al., 1998). Expression of FADD-DN enhanced negative selection of self-reactive thymic lymphocytes. FADD-DN mice displayed increased apoptosis and reduced proliferation and clonogenic growth of mitogen-activated T cells. Interestingly, this impaired T cell proliferation was not observed in CD95-deficient mice or animals overexpressing the viral caspase inhibitor CrmA (Smith et al., 1997). Thus, signaling through FADD does not lead exclusively to cell death, but under certain circumstances can promote cell survival and proliferation. Since T lymphocytes from CrmA transgenic mice responded normally to mitogens, it may be speculated that, unlike the pathway leading to apoptosis, the growth-promoting signal induced by FADD in lymphocytes does not require activation of Casp8. Furthermore, because CD95-deficient mice do not show any cardiovascular or hematopoietic abnormalities, another receptor may exist that employs the FADD/Casp8 pathway in order to induce proliferative or morphogenetic signals during the development. The molecular nature of the putative growth signals generated by FADD/Casp8 action, however, is entirely unknown.

Knocking Out Mitochondria-Controlled Caspase Activation

Besides death receptors that couple to FADD and Casp8, another pathway is essentially controlled by mitochondria (Figure 1). Induction of cell death in response to a variety of apoptotic stimuli is associated with the early mitochondrial release of cytochrome c, an event that is blocked by antiapoptotic members of the Bcl2 family. In the cytosol, cytochrome c, together with dATP, forms a complex with Apaf1 that results in the cleavage of pro-Casp9 and subsequent activation of downstream caspases (Li et al., 1997). It has been further demonstrated that, upon ligation of death receptors, activation of Casp8 can result in the proapoptotic cleavage of Bid, which induces cytochrome c translocation (Li et al., 1998; Luo et al., 1998). Thus, the death receptor and the mitochondrial pathway can cross-communicate in order to increase the speed and efficiency of the death process.

Recently both Casp9- and Apaf1-deficient mice have been generated (Cecconi et al., 1998; Hakem et al., 1998; Kuida et al., 1998; Yoshida et al., 1998). The phenotypes of these mice resembled Casp3-deficient animals in that they primarily developed brain malformations and overgrowth with excess cells in the central nervous system. The ectopic cell masses consisted of differentiated postmitotic cells that had escaped apoptosis. A nearly 10-fold reduction of TUNEL-positive cells was found in the brain of Casp9−/− mice at 12.5. In situ immunostaining showed the absence of Casp3 activation in vivo. Both Casp9- and Apaf1-deficient mice died at about day 16.5 of development. Unlike the brain, other nonneural organs such as heart, lung, and liver or spinal cord were unaffected and appeared remarkably normal. The similar phenotypes of Casp3−/−, Casp9−/−, and Apaf1-deficient mice therefore indicate that the molecules act in line in a common apoptotic pathway.

It is interesting to note that the phenotypes of the three KO mice are similar, but do not accurately mimic each other. Although disruption of either Casp9 or Casp3 caused brain malformations, the abnormalities were more severe in mice lacking Casp9. Casp3-deficient cells rarely exhibited an overt exencephaly abnormality. One possibility to account for this difference in severity of the malformation is that Casp9 may also activate other effector caspases such as Casp6 or Casp7.

While Casp9−/− mice exhibited a more pronounced phenotype than mice with a Casp3 null mutation, the most severe morphogenetic distortions were found in Apaf1 KO mice (Cecconi et al., 1998; Yoshida et al., 1998). Apaf1−/− mice showed strong craniofacial abnormalities such as ossification defects, deficient midline fusion of the palatal shelves, as well as dramatic alterations of the lens and retina. Also cauliflower-like masses on the face present in Apaf1−/− mice were not observed in either Casp3 or Casp9 gene-targeted mice. Another obvious difference was the formation of interdigital webs. Whereas removal of interdigital webs was normal, although slightly delayed, in Casp9−/− mice, interdigital webs persisted in Apaf1−/− mice. Although it has not formerly been excluded that this cell death occurs independently of caspases, the intact retraction of interdigital webs in Casp9−/− mice indicates that Apaf1 may either interact with another caspase or play additional roles beyond that of caspase activation. It has been suggested that Casp8 can physically interact with
Apaf1 in overexpression systems. A more detailed analysis, however, showed that Casp9 is the only Apaf1-interacting protease in living cells (Slee et al., 1999). Thus, the idea that another caspase may substitute Casp9 is appealing, but currently none of the other known caspases appears to be activated by Apaf1.

Despite a normal development of the thymus, thymocytes from Casp9- and Apaf1-deficient mice were resistant to a number of apoptotic stimuli. Nevertheless, both mice contained a comparable number of double-positive thymocytes, indicating that Apaf1 and Casp9 are not involved in negative selection. In thymocytes and embryonic stem cells from KO mice, cytochrome c was translocated into the cytosol, confirming that Apaf1 and Casp9 act downstream of cytochrome c. Thymocytes from Apaf1−/− mice were also resistant to a loss of mitochondrial transmembrane potential (Yoshida et al., 1998). It has been suggested that opening of the mitochondrial permeability pore and loss of transmembrane potential may be an initial event for cytochrome c release and subsequent caspase activation (Kroemer, 1997). The results observed in KO mice, however, strongly suggest that the decrease of the transmembrane potential is downstream of Apaf1-mediated caspase activation. This assumption is supported by the fact that in several apoptotic systems cytochrome c release preceded mitochondrial membrane depolarization by many hours.

A comparison of the so far established KO mice allows the separation of distinct in vivo apoptotic pathways that are strikingly cell type-specific and differentially utilized in response to certain stimuli (Figure 2). First, the classical death receptor pathway that is Casp3-dependent but independent of Casp9: The existence of this pathway is exemplified in activated T cells. Casp3 deficiency protects activated T cells from apoptosis-induced anti-CD95, TRAIL, and anti-CD3, whereas Casp9 deficiency has no obvious effect. Second, the classical mitochondrial pathway that is dependent on Apaf1 and Casp9: For example, embryonic stem cells from both Apaf1−/− and Casp9−/− mice are resistant to several apoptotic signals including UV and γ-irradiation or chemotherapeutic drugs. The mitochondrial pathway may be further distinguished based on the requirement of certain effector caspases, such as Casp3. Mutation of Casp9 or Casp3 produces similar effects on brain development, suggesting that both caspases in concert are required for apoptosis in brain development. In contrast, there also exists a Casp9-dependent pathway that works independently of Casp3: Whereas Casp9−/− thymocytes are resistant to several apoptotic stimuli including dexamethasone and γ-irradiation, Casp3−/− thymocytes remain sensitive to these inducers, implying that in thymocytes these stimuli trigger the activation of an apoptotic pathway that is Casp3-independent but Casp9-dependent. Presumably, in this pathway other effector caspases such as Casp6 or Casp7 may be dominant.

Third, an apoptotic pathway independent of Casp9 and Casp3: Although Casp9−/− embryonic stem cells are resistant to a broad range of apoptotic stimuli, Casp9 deficiency does not protect other cell types from apoptosis induced by the same inducers. Unlike embryonic stem cells, however, Casp9−/− thymocytes and splenocytes undergo apoptosis in response to UV irradiation. Thus, these finding suggest a functional diversification of caspase cascades in vivo that may be highly dependent on the cell type and apoptotic stimulus. Certainly, Apaf1 and Casp9 play a central role in events of mitochondria-dependent pathways of apoptosis that are critical for several histogenetic and morphogenetic cell deaths during development. The difference in the degree of resistance between different cell types, however, illustrates a distinct requirement of Apaf1 and Casp9 in the apoptotic machinery. Therefore, it is highly conceivable that other hitherto unidentified pathways exist which may be relevant for certain apoptotic stimuli in particular tissues.

What Do We Learn from Knockout Mice?

Previously, most of the information regarding the different roles of caspases and their signaling cascades has been obtained from overexpression of proteins and non-functional mutants in transfected cells or from yeast two-hybrid assays. Because supraphysiological overexpression of proteins may not reflect the in vivo situation, our knowledge about the functional significance of apoptotic cascades is still very limited. An apparent example of a discrepancy between data obtained in vitro with cell lines and with gene-targeted mice is apoptotic pathways that have been suggested to involve CD95 or other death receptors. Some reports demonstrated that antitumor drug-induced cell death may involve the CD95 system (Friesen et al., 1996; Müller et al., 1997).
Different drugs used in chemotherapy have been shown to induce CD95 ligand expression in certain tumor cells. Because drug-induced cell death was inhibited by CD95 neutralizing reagents, ligation of CD95 has been proposed to trigger the apoptosis cascade in chemosensitive tumor cells. Experiments from FADD- and Casp8-deficient mice, however, convincingly demonstrate that a death receptor-mediated pathway may not be the principal mechanism of drug-induced apoptosis (Yeh et al., 1998; Varfolomeev et al., 1998). Although embryonic fibroblasts from both mutant mice were resistant to apoptosis mediated by CD95 and other death receptors, they retained sensitivity to anticancer drugs. Like drug-mediated apoptosis, FADD or Casp8 gene-targeted cells did not exhibit an altered apoptosis sensitivity following irradiation or overexpression of c-Myc. Thus, despite the fact that cell type-specific variations may account for these discrepancies, FADD and Casp8, although strictly required for death receptor-mediated pathways, are not a prerequisite in other apoptosis settings.

The finding that fibroblasts from Casp8 KO mice are resistant to death receptor-mediated apoptosis further indicates that other receptor-associated pathways, including activation of Daxx or RAIDD/Casp2, have little importance (Varfolomeev et al., 1998). This is supported by the observation that cells from Casp2 null mice were still sensitive to death receptor-mediated apoptosis (Bergeron et al., 1998). Several previous reports have emphasized a role of stress-activated protein kinases in apoptosis mediated by CD95 (Goillot et al., 1997; Lenczowski et al., 1997). In contrast, fibroblasts from Casp8<sup>-/-</sup> mice retained kinase activation, indicating that at least in fibroblasts these protein kinases have no considerable impact for the propagation of the apoptotic signal (Varfolomeev et al., 1998).

Disruption of the CD95 system results in pronounced effects on immune functions (Nagata 1997; Schule-Osthoff et al., 1998; Krammer, 1999). lpr (for lymphoproliferation) mice that lack a functional CD95 receptor, as well as gld (for generalized lymphoproliferative disease) mice that bear a mutant CD95 ligand, exhibit various autoimmune phenomena resembling systemic lupus erythematosus in men. Both mouse strains produce autoantibodies and accumulate abnormal CD4<sup>+</sup> CD8<sup>-</sup> T cells leading to lymphadenopathy, splenomegaly, and other autoimmune symptoms. It is interesting to note that autoimmune disorders have not been detected in KO embryos of either FADD, Casp8, or other caspases nor in deficient lymphocytes of RAG1 chimeric mice. In addition, neither T cell hyperplasia nor serum autoantibodies were observed in transgenic animals overexpressing the viral caspase inhibitor CcmA or dominant-negative FADD in T lymphocytes (Smith et al., 1996; Newton et al., 1998). Therefore, the lpr phenotype may not be simply due to a disturbed apoptotic pathway, but rather may involve other signaling events of CD95. All mutant mice studied so far contained normal populations of thymocytes indicating also that negative selection involves neither Apaf1/Casp9- nor death receptor/Casp8-linked pathways. Interestingly, transgenic mice overexpressing the baculovirus protein p35, a powerful, broad caspase inhibitor, revealed disturbed negative selection (Izquierdo et al., 1999). This suggests either that a caspase other than Casp1, -2, -3, -8, or -9 is involved in negative selection or that, due to a redundant role of certain caspases, gene targeting of a single caspase may not be sufficient to block this process.

A very remarkable feature of the currently studied KO mice is their rather restricted phenotype. Whereas Casp3, Casp9, and Apaf1 null mice revealed massive malformations of neural tissues, FADD and Casp8 KO animals died primarily from defective heart development. Both types of mutant mice, however, had a normal development of other organs including lung, liver, and thymus. Interestingly, several cell types of these mice were still sensitive to a variety of apoptotic stimuli, suggesting that presumably still other unidentified apoptotic cascades exist. Whether these are mediated by caspases or by other apoptotic signal transducers, such as the recently identified apoptosis-inducing factor (AIF), a mitochondrial flavoprotein (Susin et al., 1999), remains to be determined. Unlike in mice, targeted disruption of the caspase DCP-1 in Drosophila resulted in multiple developmental defects associated with widespread melanotic tumors and larval death (Song et al., 1997).

Are There Nonapoptotic Functions of Caspases?

There are some evidences that both receptor- and mitochondria-controlled caspase cascades may not only control apoptosis but play additional roles beyond that of cell death. It is noteworthy that for instance Casp3 and Casp8 mutant mice have a smaller size, although the opposite phenotype may be expected. Why Casp3 and Casp8 deficiency primarily leads to neuronal hyperplasia is not clear. In contrast, mice deficient for p27/Kip1, an inhibitor of cyclin-dependent kinases, reveal hyperplasia of all organs, and not only neuronal tissues (Nakayama et al., 1996).

Thymic lymphocytes from FADD null chimeric mice or from animals expressing a dominant-negative mutant display increased apoptosis and reduced proliferation in response to mitogens (Newton et al., 1998; Woo et al., 1998). This suggests that the FADD/Casp8 cascade may be linked to an intrinsic survival pathway. A growth-promoting effect of caspases is also evident from the observation that hematopoietic precursors of Casp8 null mice show a reduced colony-forming activity (Varfolomeev et al., 1998). In addition, caspases may not necessarily trigger cell death as transient activation of Casp3 was observed during T cell stimulation and alloergic mixed lymphocyte reaction, which is not linked to apoptosis (Miossec et al., 1997).

The impaired heart muscle development and massive hemorrhage caused by Casp8 deficiency provides further evidence for a morphogenetic role of caspases, which may not only be associated with apoptotic events but also involve other biological processes including proliferation or differentiation of cells. Gene targeting of Braf, a member of the Raf kinase family, results in a phenotype with alterations similar to Casp8-deficient mice (Wojnowski et al., 1997). It will be interesting to investigate whether erythrocytosis observed in both mice is due to disturbed blood vessel formation. A failure
to activate Casp8 may be linked to a disturbed differentiation of angioblasts, a decreased proliferation of endothelial cells, or dysregulation of extracellular matrix formation. In this respect, it has been found in vitro that spreading of cells on extracellular matrix may be regulated by caspase activation (Watanabe and Akaike, 1999). Certainly, future investigations are needed to elucidate whether, apart from execution of apoptosis, caspases participate in other cellular pathways.

Concluding Remarks
The rapid discovery of a great number of caspases, together with multiple control points of their activation, proceeds well ahead of our knowledge of their physiological roles within the organism. There are still major gaps, but recent gene targeting of caspases provides us with several new and fundamental aspects of their physiological functions. The fact that different lines of KO mice exhibit preferential apoptosis defects rather than a global suppression of apoptosis indicates that caspases play a largely nonredundant apoptotic role in a tissue- and stimulus-dependent manner. Furthermore, despite compelling evidence for a key role of Casp8 and Casp9, the restricted phenotype of both Casp8- and Casp9-deficient mice suggests that other apical caspases must exist regulating apoptotic processes. Casp10, for instance, which is very similar in its structure to Casp8, has been shown to be recruited to death receptors (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997). Although fibroblasts from Casp8 null mice are almost completely resistant to death receptor-mediated apoptosis, it cannot be excluded that Casp10, having little importance in fibroblasts, exerts crucial functions in other cell types. In addition, since certain cell types such as embryonic fibroblasts from Apaf1 KO mice are still considerably sensitive to a variety of apoptosis inducers, it is very likely that other yet undiscovered key regulators exist. We also have to be aware that the restricted phenotype of most KO mice may underestimate the role of the targeted caspases, because single caspases may substitute other family members. A major obstacle of most KO mice is their prenatal lethality, which precludes manifestations of caspase functions in the adult organism. Therefore, in future research, conditional disruption in a cell type-specific manner or in certain developmental stages will be required to elucidate more precisely the in vivo functional significance of individual caspases in development, immune functions, and pathological forms of apoptosis.

References


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