

Review

Caspases in cell survival, proliferation and differentiation

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Caspases, a family of evolutionarily conserved cysteinyl proteases, mediate both apoptosis and inflammation through aspartate-specific cleavage of a wide number of cellular substrates. Most substrates of apoptotic caspases have been conotated with cellular dismantling, while inflammatory caspases mediate the proteolytic activation of inflammatory cytokines. Through detailed functional analysis of conditional caspase-deficient mice or derived cells, caspase biology has been extended to cellular responses such as cell differentiation, proliferation and NF- κ B activation. Here, we discuss recent data indicating that non-apoptotic functions of caspases involve proteolysis exerted by their catalytic domains as well as non-proteolytic functions exerted by their prodomains. Homotypic oligomerization motifs in the latter mediate the recruitment of adaptors and effectors that modulate NF- κ B activation. The non-apoptotic functions of caspases suggest that they may become activated independently of – or without – inducing an apoptotic cascade. Moreover, the existence of non-catalytic caspase-like molecules such as human caspase-12, c-FLIP and CARD-only proteins further supports the non-proteolytic functions of caspases in the regulation of cell survival, proliferation, differentiation and inflammation.

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Caspases are an evolutionarily conserved family of aspartate-specific cystein-dependent proteases with central functions in apoptotic and inflammatory signalling pathways.¹ Certain caspases have large prodomains that contain related homotypic oligomerization motifs such as the caspase recruitment domain (CARD, caspase-1, -2, -4, -5, -9, -11, -12) and the death effector domain (DED, caspase-8 and -10). Proximity-induced caspase auto-activation occurs in large multimeric complexes that consist of a platform protein that specifically recruits large prodomain caspases, either directly or by means of adaptors.² Well-studied caspase complexes include the apoptosome,³ the death inducing signalling complex (DISC),^{4,5} the PIDDosome⁶ and the caspase-1-containing inflammasomes.⁷ The short prodomain caspases (caspase-3, -6, -7, -14) are activated by proteolytic maturation by large prodomain caspases or other proteases. The final outcome of these proteolytic cascades is the specific cleavage of a wide variety of substrates that are implicated in apoptosis.

The observation of caspase activity and the identification of caspase substrates in the absence of cell death have recently sparked a strong interest in caspase functions in cellular

responses beyond apoptosis. Here, we discuss recent data indicating that non-apoptotic functions of caspases involve both proteolysis exerted by their catalytic domains and non-proteolytic functions exerted by their prodomains. Detailed functional analysis of conditional caspase-deficient mice or derived cells confirmed the pleiotropic roles of these proteases in cell differentiation, proliferation and NF- κ B activation (Table 1). The non-apoptotic functions of caspases suggest that they may become activated independently of – or without – inducing an apoptotic cascade, thus leading to the cleavage of a specific subset of substrates. A list of reported caspase substrates under non-apoptotic conditions is provided in Table 2. These substrates include members of diverse protein families such as cytokines, kinases, transcription factors and polymerases (Table 2). Finally, the regulation of these non-proteolytic functions by the caspase-like decoy molecules c-FLIP (cellular caspase-8 (FLICE)-like inhibitory protein), human caspase-12, COP, INCA and ICEBERG provides additional support for the involvement of caspases in the regulation of cell survival, proliferation, differentiation and inflammation.

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Abbreviations: BcR, B-cell receptor; CDK, cyclin-dependent kinase; CMB, CARMA-Bcl10-MALT1; CARD, caspase recruitment domain; caspase, cystein-dependent aspartate-specific protease; DED, death effector domain; DISC, death inducing signalling complex; FLASH, FLICE-associated huge protein; IAP, inhibitor of apoptosis protein; I κ B, inhibitor of κ B; IKK, I κ B kinase; IL, interleukin; LPS, lipopolysaccharide; LRR, leucine rich repeats; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor κ B; PARP, poly(ADP-ribose) polymerase; PIDD, p53-induced death domain protein; RIP, receptor interacting protein; TcR, T-cell receptor; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor-receptor associated factor

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Table 1 Overview of non-apoptotic caspase functions

Caspase	Phenotype	References
Caspase-1	Cytokine maturation	97,98
	Induction of pyroptosis	99,100
	Prodomain-mediated NF- κ B activation	27
Caspase-2	Switch between stress-induced NF- κ B activation and induction of apoptosis	6,31,32
	Prodomain-mediated NF- κ B activation	24
Caspase-3	Inhibition of B-cell proliferation	59
	Inhibition of MHC II expression and dendritic cell maturation	79
	Proliferation of forebrain cells and keratinocytes	57,71
	Differentiation of lens epithelial cells, erythroblasts, platelets, myoblasts, osteoblasts and neural stem cells	56,66,68,75–77
Caspase-4	Not determined	
Caspase-5	Maturation of pro-inflammatory cytokines in the inflammasome	101
Caspase-6	Not determined	
Caspase-7	Not determined	
Caspase-8	Proliferation of T, B and NK cells	14,39,40
	NF- κ B activation following TcR-, TNF-, TLR-4- and Apo2/TRAIL-stimulation	5,12,16
	dsRNA-induced NF- κ B activation via its prodomain	25,26
	Differentiation of monocytes into macrophages	72,73
	Differentiation of placental villous trophoblasts	74
Caspase-9	Not determined	
Caspase-10	dsRNA-induced NF- κ B activation via its prodomain	25,26
Caspase-11	Maturation of pro-inflammatory cytokines	102
Caspase-12	Attenuation of inflammation and susceptibility to sepsis	94,95
Caspase-14	Terminal differentiation of keratinocytes?	70,103

Table 2 Caspase substrates in non-apoptotic conditions

Cellular process	Substrates	Function	References
Cell-cycle regulation	Wee1 kinase	Inactivated	104
	P21 ^{Waf1}	Inactivated	59
	p27 ^{KIP1}	Inactivated	58,105,106
	NF-AT	Inactivated	62
	SATB1 (special AT-rich sequence-binding protein 1)	Inactivated	107
Cytokine maturation	IL-1 β	Activated	98
	IL-18	Activated	97
NF- κ B activation	PARP-1	Activated	18
T and B cell Activation	c-FLIP	Activated	38,48
	Lamin B	Unknown	104
	PARP-1	Activated	104
	Wee1 kinase	Inactivated	104
	PAK-2	Activated	108
	α -Tubulin	Inactivated	108
	Vinculin	Unknown	108
Macrophage differentiation	Nucleophosmin	Unknown	108
	PAI-2	Inactivated	108
	hnRNP-H	Unknown	108
	hnRNP-C1/C2	Unknown	108
	β 1-adaptin	Inactivated	79
	γ -Adaptin	Inactivated	79
	PARP-1	Inactivated	68
Erythrocyte differentiation	Lamin B	Inactivated	68
	Acinus	Activated	68
Myoblast differentiation	MST1 kinase	Activated	75
	Calpastatin	Inactivated	109
Lens fiber formation	PARP-1	Inactivated	110
Keratinocyte differentiation	PKC δ	Activated	71

Caspase-Mediated NF- κ B Activation

From an evolutionary perspective, the link between caspases and NF- κ B activation predates the emergence of mammals. The *Drosophila* innate immune system discriminates between Gram-positive and Gram-negative pathogens and responds by inducing the expression of specific antimicrobial peptides.⁸

Loss-of-function mutations in the gene encoding the *Drosophila* caspase-8 ortholog DREDD renders flies highly susceptible to Gram-negative bacteria.⁹ As the phenotype of *Dredd* knock-out flies resembles that of mutants lacking the NF- κ B-like factor Relish, it is likely that DREDD functions upstream of Relish to control the production of antibacterial peptides such as drosomycin and metchnikowin.^{9,10} Activation of Relish was

recently reported to require cleavage at the canonical caspase site LQHD₅₄₅-G₅₄₆.¹¹ With the notable exception of DREDD, RNAi-mediated knockdown of the other fly caspases did not affect Relish processing in cells.¹¹ However, *in vitro* reconstitution experiments to confirm direct processing of Relish by DREDD failed so far,¹¹ possibly because cleavage occurs in a protein complex in cells. Nevertheless, these results suggest that DREDD may mediate the production of antibacterial peptides through the direct cleavage and activation of this NF- κ B-like transcription factor. In recent years, it has become clear that mammalian caspases fulfil roles in NF- κ B activation, attesting to the evolutionarily conserved role of DREDD in *Drosophila* innate immunity. At least two different molecular mechanisms for caspase-mediated NF- κ B activation in mammals have been suggested.

A first molecular mechanism of caspase-mediated NF- κ B activation involves the catalytic activity of large prodomain caspases. Upon T-cell Receptor (TcR) stimulation, caspase-8 and its adaptor protein FADD (Fas-associated death domain-containing protein) are recruited to the CARMA-Bcl10-MALT1 (CBM) complex.¹² The paracaspase MALT1 harbours a death domain and a caspase-like domain and is required for TcR-induced NF- κ B activation.¹³ As demonstrated by means of reconstitution experiments in patient-derived caspase-8 deficient T cells, this TcR-induced NF- κ B activation apparently depends on enzymatically active, although unprocessed, caspase-8 for the recruitment of the IKK complex.¹² Studies with biotinylated-zVAD-fmk revealed that only a minor fraction of the total caspase-8 pool in Jurkat T cells became enzymatically active after TcR stimulation.¹² In contrast, Salmena *et al.*¹⁴ could not observe differences in TcR-stimulated NF- κ B activation in caspase-8^{-/-} mouse T cells. This discrepancy might be due to the fact that NF- κ B activation in the caspase-8-deficient mouse T cells was determined quite late after TcR stimulation (6 h). The importance of kinetics in NF- κ B signalling pathways is underscored by observations in TRAF2-deficient cells. Indeed, the absence of TRAF2 only delays the kinetics of NF- κ B activation in response to TNF, while at a later time point (90 min), the extent of activated NF- κ B in TRAF2 deficient cells is equivalent to that in control cells.¹⁵ Remarkably and in agreement with the observations in fibroblasts and epithelial cells,¹⁶ the enzymatic activity of caspase-8 is dispensable for TNF-induced NF- κ B activation in T cells.¹² These observations suggest that in T cells, the enzymatic activity of caspase-8 may only be required for NF- κ B activation in response to a specific subset of NF- κ B-activating agents.

In certain cell types (HT1080 human fibrosarcoma, Jurkat T cells, SK-MES-1 human lung squamous carcinoma and A549 human lung carcinoma), RNAi-mediated knockdown of caspase-8 was shown to impede or delay Apo2L/TRAIL-induced NF- κ B activation.⁵ The release of FADD from the primary TRAIL-R-associated DISC complex would trigger the formation of a secondary signalling complex, which lacks TRAIL-R but contains FADD, caspase-8, RIP1, TRAF2 and/or NEMO.⁵ The latter cytosolic protein complex would mediate the activation of JNK, p38 and NF- κ B.⁵ As the pan-caspase inhibitor z-VAD-fmk significantly delayed Apo2/TRAIL-induced I κ B phosphorylation and secondary complex formation, the authors proposed that the enzymatic activity of

caspase-8 is required for the dissociation of FADD from the TRAIL-R and for subsequent secondary complex formation.⁵ However, it cannot be excluded that the identified signalling complex is formed in response to general apoptotic stress, independently from the TRAIL-R-bound DISC complex, as NF- κ B activation upon TRAIL-receptor stimulation is observed rather late (hours) as compared to TNF-induced NF- κ B activation (minutes). Undoubtedly, the precise role of caspase-8 in these NF- κ B signalling cascades and the identity of its substrates under NF- κ B-activating conditions warrant further investigation.

Besides the role of caspase-8 in Apo2L/TRAIL- and TcR-mediated activation of NF- κ B,^{5,12} also short prodomain caspases may be involved in NF- κ B activation. Cleavage of poly-(ADP-ribose) polymerase-1 (PARP-1) has been used for years as a hallmark of caspase-3/-7 activation and apoptosis. PARP-1 cleavage during apoptosis impairs the DNA repair capacity of the cell.¹⁷ Recently, however, caspase-generated PARP-1 fragments were implicated in an inflammatory response. Macrophages from knock-in mice expressing caspase-resistant PARP-1 show an impaired LPS-induced NF- κ B-mediated gene activation despite normal binding of NF- κ B to DNA.¹⁸ Thus, caspase-3 and -7 would apparently propagate the transactivation, rather than the nuclear translocation of NF- κ B through a PARP-1-mediated mechanism.¹⁸ This hypothesis is underscored by the observation that caspase-generated PARP-1 fragments can interact with both the p50 and p65 subunit of NF- κ B and with the transcriptional co-activator p300.^{19,20} The observed resistance to endotoxic shock and to intestinal and renal ischemia-reperfusion of caspase-resistant PARP-1 knock-in mice further supports the *in vivo* contribution of executioner caspases to inflammatory responses.¹⁸ These results demonstrate that caspase-mediated PARP-1 cleavage under non-apoptotic conditions can contribute to the level of NF- κ B transcriptional activity. However, it is still unresolved whether this contribution to NF- κ B transcriptional activity depends on a structural role and/or the poly(ADP-ribose) polymerase activity of PARP-1. On the one hand, it is shown that co-activation of NF- κ B by p300 in TNF- or LPS-stimulated primary fibroblasts or macrophages does not depend on the enzymatic activity of PARP-1, whereas on the other hand, studies using PARP-1 inhibitors do indicate a role for PARP-1 enzymatic activity in the enhancement of NF- κ B transcriptional activity.^{21,22} Further analysis is required to clarify this intriguing issue.

A second mechanism to propagate the activation of NF- κ B makes use of the CARD and DED motifs present in the prodomains of specific caspases.²³⁻²⁵ This is underscored by the observation that transient expression of caspase-1, -2, -8 and -10 are able to activate NF- κ B whereas murine caspase-9, -11 and -12 are not.²⁴⁻²⁷ Caspase-1 CARD specifically recruits the CARD-containing kinase RIP2, which is involved in Toll-like receptor (TLR)- and TcR-mediated NF- κ B activation.²⁸ The involvement of RIP2 in caspase-1 CARD-induced NF- κ B activation is demonstrated by the inhibition of the latter by expression of a RIP2 dominant-negative mutant.²⁷ Recently, this caspase-1/RIP2 interaction was shown to be modulated by the adaptor molecule ASC, which also interacts with caspase-1 via its CARD.²⁹ ASC interferes with the caspase-1/RIP2 interaction and inhibits the subsequent

NF- κ B activation in a dose-dependent fashion, while stimulating caspase-1-dependent pro-IL-1 β maturation.²⁹ Consistent with a role for ASC as an inhibitor for caspase-1/RIP2-induced NF- κ B activation, siRNA-induced knockdown of ASC in THP-1 cells leads to higher NF- κ B activation levels, while caspase-1 activation and release of mature IL-1 β are diminished.²⁹ Altogether, these results suggest that caspase-1 may contribute to pro-IL-1 β processing through its enzymatic activity, while its interaction with RIP2 induces NF- κ B activation. Furthermore, ASC may function as a switch that directs caspase-1 away from RIP2-mediated NF- κ B activation towards the maturation of pro-IL-1 β in the inflammasome complexes.

The CARD motif in the prodomain of caspase-2 can mediate the recruitment of TNF receptor-associated factor 2 (TRAF2) and the death domain-containing receptor-interacting protein 1 (RIP1) in an endogenous caspase-2 complex, capable of inducing the activation of NF- κ B.²⁴ Recently, the caspase-2 containing PIDDosome⁶ has been extended with the proteins NEMO and RIP1, explaining the activation of NF- κ B following genotoxic stress.^{30,31} It is claimed that the active NF- κ B pathway blocks or delays the caspase-2-mediated cell death pathway as caspase-2 activation is highly accelerated in RIP1^{-/-} cells. The PIDDosome may thus act as an integrator or molecular switch between inflammatory/anti-apoptotic and apoptotic signalling pathways following DNA damage.³² However, a crucial physiological role of caspase-2 in this pathway, remains to be established and may not be likely or highly redundant, as caspase-2^{-/-} mice do not display an overt phenotype.³³

Following TNF-R1 triggering, caspase-8 reportedly forms a complex with TRAF2 and FLASH (FLICE-associated huge protein) to initiate the NF- κ B activation pathway.¹⁶ In agreement with these findings, TNF-induced NF- κ B activation in fibroblast and epithelial cell lines depends on caspase-8, as underscored by the abrogation of NF- κ B activation by antisense-mediated depletion of caspase-8.¹⁶ However, as discussed above, the enzymatic activity of caspase-8 seems to be dispensable for TNF-induced NF- κ B activation in these cells. In this respect, caspase-8 is known to associate with TRAF2 and FLASH through the DED motifs present in its N terminal prodomain.¹⁶ Furthermore, the caspase-8 prodomain is reportedly capable of inducing NF- κ B activation independent of caspase-8 enzymatic activity.²³⁻²⁵ Altogether, these results suggest a structural caspase-8 requirement for TNF-induced NF- κ B activation through its DED-dependent interaction with TRAF2 and FLASH, independent of caspase-8 catalytic activity.

It is conceivable that prodomain fragments generated through limited caspase autoactivation are implicated in NF- κ B signalling. Indeed, such a mechanism would provide an elegant framework to explain the available data on caspase catalytic activity-dependent and -independent mechanisms and may suggest that the release of CARD- and DED-containing prodomain fragments (from caspases and caspase-like molecules) is the principal target of caspase activation during NF- κ B signalling (Figure 1). In this respect, dsRNA-induced NF- κ B activation was recently suggested to require the released prodomains of caspase-8 and -10 downstream of the cytoplasmic RNA helicases retinoic acid-

inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) as these caspases become processed upon dsRNA stimulation.²⁶ This model is further supported by the observation that overexpression of small amounts of the corresponding prodomain-encoding constructs strongly activated NF- κ B, while full-length caspases-encoding constructs failed to do so.²⁶ Finally, the induction of inflammatory cytokines in response to dsRNA was significantly reduced in caspase-8- and -10-deficient cells.²⁶ In addition to the possible role of autocatalytically released caspase-8 and -10 prodomain fragments in antiviral signalling, other lines of evidence implicate caspase-generated DED-containing cFLIP fragments in NF- κ B signalling. Indeed, caspase-8-mediated cleavage of c-FLIP_L to p43^{FLIP} has been shown to induce NF- κ B activation via a p43^{FLIP}/caspase-8/TRAF2 complex (Figure 2).³⁴⁻³⁷ Alternatively, Golks *et al.*³⁸ discovered that a shorter caspase-8-generated N-terminal fragment of c-FLIP_L, so called p22-FLIP, corresponding to the prodomain region of c-FLIP by cleavage at Asp198, is a key mediator of activation-induced NF- κ B activation in lymphocytes and dendritic cells by binding directly the IKK complex (Figure 2). The physiological and molecular contexts that control caspase-8-mediated cleavage of c-FLIP to either p43^{FLIP} or p22-FLIP warrant further study.

Caspases in Cell-Cycle Control and Proliferation

Several studies demonstrate an essential role for caspase-8 in the proliferation of immune cells.^{12,14,39,40} Patients bearing inactivating mutations in caspase-8 endure impaired proliferation of T, B and natural killer (NK) cells.³⁹ Similarly, peripheral murine T cells in which caspase-8 is conditionally deleted are unable to proliferate following TcR activation.¹⁴ Interestingly, this mouse model recapitulates certain aspects of the disorder described in humans with *caspase-8* mutations.^{14,39} Indeed, caspase-8 deficient T cells of both murine and human origin display decreased IL-2 production following CD3/CD28 stimulation.^{14,39} The function of caspase-8 upstream of IL-2 production and human T-cell proliferation is confirmed by the requirement of caspase-8 catalytic activity in TcR-induced activation of NF- κ B,¹² as discussed above. Based on these findings, T-cell-specific deletion of *caspase-8* in mice was concluded to recapitulate the immunodeficiency identified in humans with *caspase-8* mutations.^{14,39} These patients succumb frequently to microbial infections as a result of their inability to activate T, B, and NK cells.³⁹ Somewhat paradoxically, however, is that humans with a germline point mutation in the *caspase-8* gene reportedly manifest lymphadenopathy and splenomegaly, conditions associated with autoimmunity instead of immunodeficiency.³⁹ A recent report on the long-term effects of T-cell-specific deletion of the caspase-8 gene in mice describes a similar age-dependent lethal lymphoproliferative and lymphoinfiltrative phenotype characterized by lymphadenopathy, splenomegaly, and accumulation of T-cell infiltrates in the lungs, liver and kidneys.⁴¹ Moreover, T cells isolated from old animals were found to be constitutively active, possibly explaining the observed pathological phenotypes.⁴¹ Thus, loss of caspase-8 leads to a complex immune condition manifesting features of immunodeficiency and autoimmunity. Noteworthy is that the

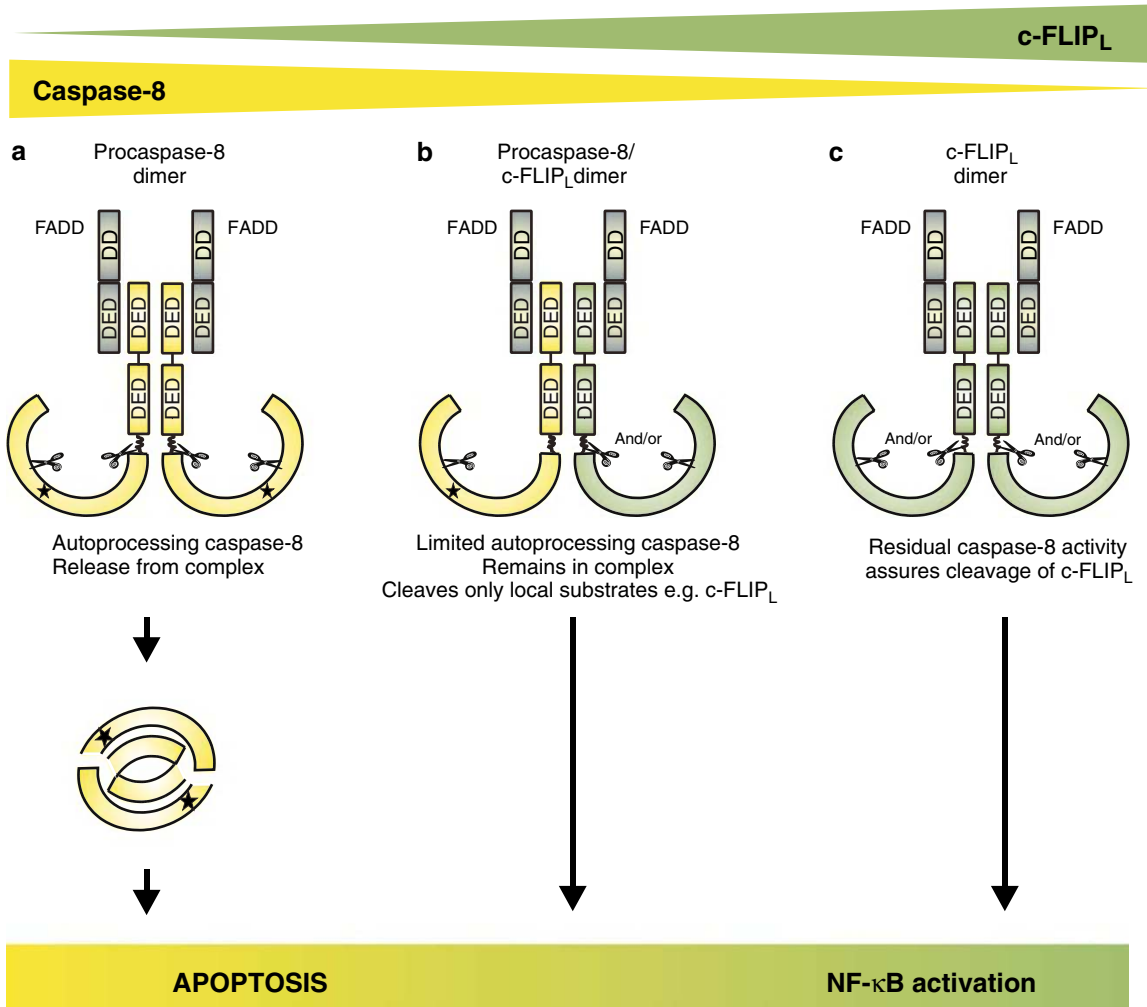


Figure 1 c-FLIP_L levels modulate caspase-8 activation and downstream signalling. (a) In the absence of c-FLIP_L, procaspase-8 dimerization leads to full processing and activation of procaspase-8. Active caspase-8 released from the DISC initiate apoptotic signalling pathways through the cleavage of cytosolic substrates. (b) At low concentrations of c-FLIP_L, procaspase-8 preferably forms heterodimers with c-FLIP_L. In these conditions, limited procaspase-8 autoprocessing occurs and active heterodimers remain associated with the DISC complex. Autoprocessing occurs either between the prodomain and the caspase domain or between the p20 and p10 subunits of the caspase domain. Depending on the specific subset of substrates that is cleaved, either apoptosis or NF-κB activation can ensue. (c) High concentrations of c-FLIP_L prevent procaspase-8 recruitment and autoprocessing in the DISC. Basal caspase-8 activity ensures c-FLIP_L cleavage and subsequent NF-κB activation

immune defects observed in patients with defective caspase-8 are clearly different from those observed in patients with caspase-10 deficiency, suggesting that these two gene products may also have non-redundant functions in T-cell proliferation.^{39,42} Indeed, humans harbouring caspase-10 point mutations display defective FasL- and TRAIL-induced apoptosis, resulting in the accumulation of lymphoid cells, which is characteristic for type II autoimmune lymphoproliferative syndrome (ALPS type II).⁴² Interestingly, the homozygous V410I mutation that correlates with the ALPS type II phenotype in one kindred of this study⁴² was subsequently found to be present as a heterozygous polymorphism in approximately 6.8% of the Danish population.⁴³ Screening of a larger number of healthy individuals is required to resolve whether homozygosity in this polymorphism contributes to the ALPS type II phenotype. Nevertheless, the fact that caspase-10 has no ortholog in mice, but may compensate for specific

caspase-8 functions in human cells, may explain the moderate phenotype of caspase-8 mutations in humans as compared to the embryonic mortality of mice with a targeted deletion of caspase-8.⁴⁴

There are some contrasting findings on the role of caspase-8 in NF-κB activation and proliferation in B cells, depending on the species. In mice, LPS or dsRNA-induced proliferation of caspase-8-deficient B cells is impaired, although TLR-4-induced activation of NF-κB is not impeded in these cells.⁴⁰ In human B cells harbouring homozygous caspase-8 mutations, both NF-κB activation and proliferation are abrogated.¹² Whether human and mouse caspase-8-deficient B cells respond differently because of the different nature of caspase-8 deficiency, the initiation of different signalling pathways or of different experimental set-ups to analyse the activation of NF-κB is not clear to date. If this discrepancy resists scrutiny, it would suggest a role for caspase-8 in

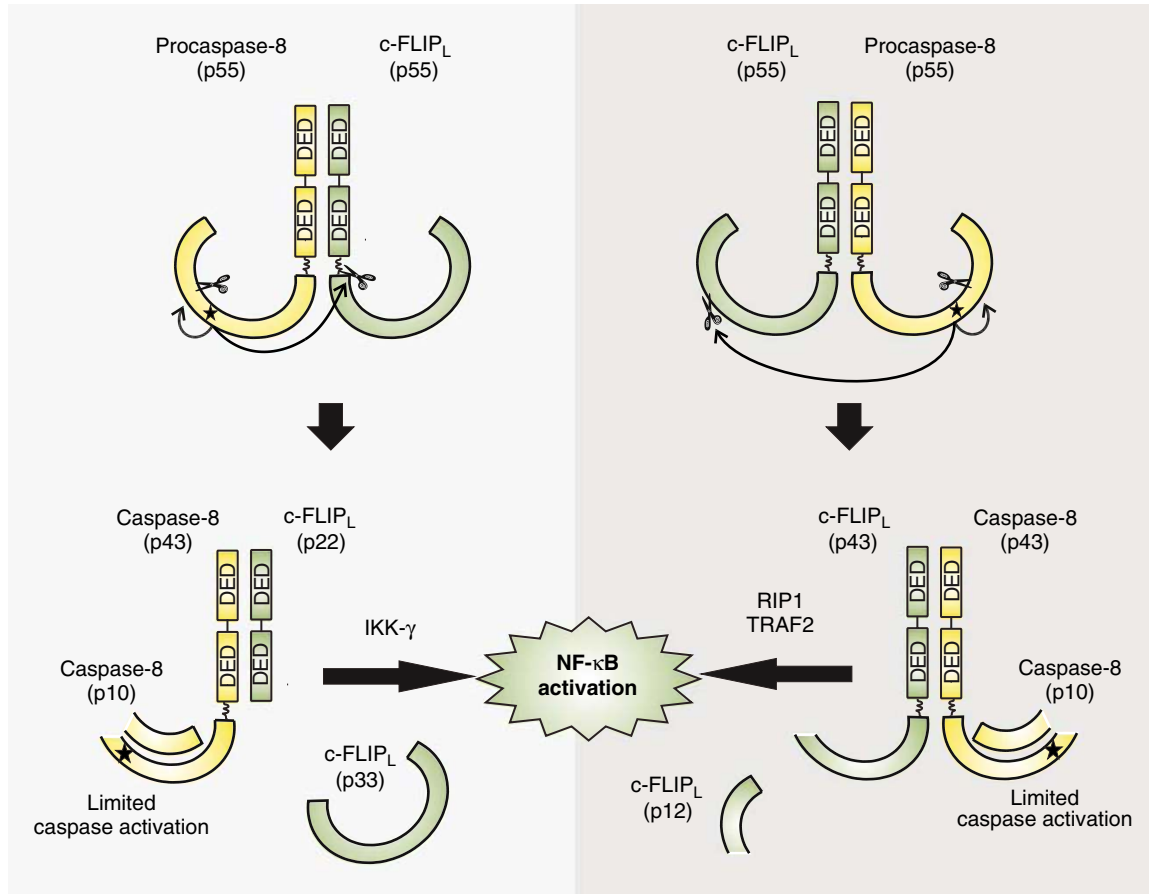


Figure 2 Molecular mechanisms of c-FLIP_L-mediated NF-κB activation. Two models of c-FLIP_L-induced NF-κB activation have been proposed: (left) Caspase-8-mediated cleavage of c-FLIP_L at Asp198 in lymphocytes and dendritic cells releases a 22 kDa fragment (p22-FLIP), essentially comprising the two N terminal DED motifs. P22-FLIP interacts directly with NEMO/IKK-γ in the IKK complex, leading to NF-κB activation. (right) Caspase-8 cleaves c-FLIP_L at Asp376 to generate an N terminal p43^{FLIP} fragment, which subsequently recruits TRAF2 and RIP1 to induce NF-κB activation. ★, catalytic cysteine in the conserved QACQG motif of caspase-8

mouse B-cell proliferation independent of NF-κB signalling and may reveal additional caspase-8 functions that may contribute to the phenotype of caspase-8-deficient human B cells.

Caspase-8 thus can initiate apoptosis, but is also implicated in the activation of NF-κB and regulation of lymphocyte proliferation. Whether the outcome of caspase-8 activation is apoptotic or non-apoptotic is most probably determined by the extent of its activation (Figure 1). In proliferating cells, caspase-8 remains unprocessed and becomes only weakly activated.¹² In contrast, caspase-8 processing and strong activation is seen during FasL-induced apoptosis (Figure 1a).⁴ An important player in the regulation of caspase-8 activation at the level of the DISC is c-FLIP_L, a caspase-8-like molecule that lacks caspase activity.^{45,46} At low concentrations of c-FLIP_L, procaspase-8 activation is enhanced due to the ability of c-FLIP_L to form a heterodimeric chimeric enzyme with caspase-8^{34,46,47} even more efficiently than caspase-8 homodimers.⁴⁷ In these conditions, limited caspase-8 autoprocessing occurs, but the presence of c-FLIP_L ensures that caspase activity remains in the complex (Figure 1b). Therefore, only DISC proximal substrates such as caspase-8 itself, c-FLIP_L and RIP1 are cleaved, whereas

cytosolic substrates such as Bid are not.⁴⁶ At this point, either apoptosis or apoptosis-independent functions of caspase-8 such as NF-κB activation and cell proliferation can ensue (Figure 1b).^{37,48} At high concentrations of c-FLIP_L, recruitment of caspase-8 to the DISC is strongly prevented and caspase-8 processing is impeded, thus blocking the induction of cell death while promoting FLIP_L-mediated NF-κB activation (Figure 1c).^{34,49}

Viral FLIP molecules resemble c-FLIP in that they also bear two DEDs.⁵⁰ Unlike the v-FLIP molecules E8 (from the Equine Herpes Virus 2) and MC159L (from the Molluscum Contagiosum virus), the Human Herpes Virus 8 (HHV-8)-encoded v-FLIP K13 can also activate NF-κB next to its capacity to block cell death.⁵¹ This different behaviour of v-FLIP molecules towards the activation of NF-κB is comparable to the observation that some prodomains of CARD-containing caspases can induce the activation of NF-κB whereas others cannot.^{24,27} Transgenic overexpression of HHV-8 v-FLIP in the lymphoid compartment leads to constitutive NF-κB activation and increased incidence of lymphomas, but does not affect Fas-induced apoptosis or the development and maturation of lymphocytes.⁵² Similar to HHV-8 v-FLIP-overexpressing splenocytes and thymocytes,

c-FLIP_L expression in transgenic (tg) mice causes the T cells to hyperproliferate upon activation.^{48,52,53} The proliferative response of c-FLIP_L tg CD8⁺ T cells is dependent on caspase activity as proliferation is largely inhibited by the pan-caspase inhibitors zVAD-fmk and QVD-Oph. Additionally, an increase in NF- κ B activity was observed 2 days after activation of the c-FLIP_L tg CD8⁺ T cells.⁴⁸ Active caspase-8 cleaves c-FLIP_L to generate a p43^{FLIP} fragment.^{34–36} p43^{FLIP} was shown to interact with TRAF2 and induce NF- κ B activation via a p43^{FLIP}/caspase-8/TRAF2 complex (Figure 2).³⁷ The activation of NF- κ B by this ternary complex could be mediated by RIP1, since the latter is also able to bind to p43^{FLIP}.⁴⁸ Moreover, a dominant-negative form of RIP1 that contains only the death domain was able to prevent p43^{FLIP}-induced NF- κ B activity.⁴⁸ These results demonstrate that the NF- κ B-activating and concomitant proliferation-enhancing function of c-FLIP involves RIP1.

Mice conditionally lacking c-FLIP in T lymphocytes display severe defects in the development and proliferation of mature T cells,^{52,54} although TcR-induced activation of NF- κ B is not affected.⁵⁴ On the contrary, in caspase-8 deficient T cells both TcR-induced NF- κ B activation and T-cell proliferation are abolished.^{12,14,39} These results suggest that besides common mechanisms of NF- κ B activation involving RIP1, caspase-8 possesses additional ways for TcR-induced NF- κ B activation, which most probably involve its catalytic activity. If we compare the phenotypes related to TcR-induced proliferation and NF- κ B activation in different human and mouse models, the following conclusions can be formulated. TcR-induced proliferation is reduced in the absence of c-FLIP, caspase-8 or FADD, indicative of their functions in a common pathway initiated by stimulation of the TcR. The precise roles of these molecules in the NF- κ B activation pathway(s) remain to be determined. Analysis of the NF- κ B signalling cascades in transgenic mice overexpressing the caspase-8 inhibitor CrmA (cytokine response modifier A)⁵⁵ could help to elucidate the contribution of the protease activity of caspase-8. As mentioned above, TcR-induced NF- κ B activation is required⁵⁶ but not sufficient for T-cell proliferation and activation to occur.⁵⁴ The crucial factor or cellular condition determining the switch between FADD/caspase-8-mediated proliferation and FADD/caspase-8-mediated apoptosis is still unresolved. It is conceivable that accessibility and specific proteolysis of particular caspase-8 substrates may influence this switch.

Short prodomain caspases, in particular caspase-3, were recently shown to exert several non-apoptotic functions as well. The subcellular localization and the proteolysis of substrates associated with cell cycle control suggest a function of caspase-3 in the modulation of cell growth. For example, nuclei of dividing cells in the proliferative regions of rat forebrain display the presence of active caspase-3.⁵⁷ In proliferating lymphoid cells, caspase-mediated cleavage of the cyclin-dependent kinase (CDK) inhibitor p27^{KIP1} contributes to the induction of cell cycle progression.⁵⁸ In contrast, hyperproliferation of B cells is observed in caspase-3 knockout mice, indicating that caspase-3 may act as a negative regulator of B-cell cycling.⁵⁹ Although the CDK inhibitor p21 is a known inhibitor of cell cycle progression, p21 can also promote cell proliferation when associated with PCNA, a processivity factor which promotes entry into mitosis.⁶⁰ In line

with these observations, caspase-3-mediated cleavage of p21 at the C terminal PCNA-binding site specifically abolishes interaction of p21 with PCNA, thus explaining the anti-proliferative effect of this cleavage event in B cells.^{59,61} Analogous studies in conditional knockout mice may reveal additional roles for caspases in cell cycle regulation. Indeed, the transcription factor nuclear factor for activated T cells (NF-AT) was recently reported to be a caspase-3 substrate in cycling T cells.⁶² Interestingly, NF-AT protein levels and NF-AT transcriptional activity are significantly reduced in non-apoptotic T cells from c-FLIP_L tg mice, which manifest elevated caspase activity.⁶²

The pleiotropic functions of mammalian caspases have recently also been reported to occur in *Drosophila*. For example, Hay *et al.*⁶³ demonstrated in an elegant setting that caspases can mediate from the same cell a paradoxical combination of pro-apoptotic and proliferative signals. When a large number of cells in the *Drosophila* wing disc are lost through injury, these cells are known to be compensated for by the proliferation of adjacent cells.⁶⁴ Apparently, the enzymatic activity of the *Drosophila* caspase Dronc in the dying cells drives the proliferation of neighbouring cells to restore the wings.⁶³ The molecular signalling cascade that connects this pro-apoptotic caspase to the compensatory proliferation of adjacent cells remains to be uncovered, but one could envision cleavage of a substrate that leads to the secretion of the growth-stimulating factors *wingless* and *decapentaplegic*, hence inducing proliferation of nearby non-affected cells in a Dronc-dependent manner. This mechanism is reminiscent of apoptotic cells inducing tissue repair mechanisms in mammalian tissues. Indeed, phagocytosis of apoptotic Jurkat T cells and peritoneal macrophages leads to the secretion of growth and survival factors such as vascular endothelial growth factor (VEGF), which subsequently promote growth of endothelial and epithelial cells in a paracrine fashion.⁶⁵ These processes may in fact reflect one of the primary functions of caspases, that is the restoration of homeostasis after tissue damage by forming the link between cell death through apoptosis and the induction of tissue repair.

Caspases in Cell Differentiation

Several reports have demonstrated that caspases are involved in the terminal differentiation of a variety of cell types involving enucleation processes such as lens cell differentiation, erythrocyte and platelet formation and the terminal differentiation of keratinocytes. Caspase-3 activity is apparently required for the maintenance of lens transparency, since caspase-3^{-/-} mice exhibit marked cataracts at the anterior lens pole.⁶⁶ These authors also presented genetic evidence that neither loss of any single executioner caspase (-3, -6, -7) nor simultaneous loss of caspase-3 and -6 prevents organelle loss during lens development. These results appear to contradict studies using synthetic inhibitors,⁶⁷ which claimed a role for executioner caspases in terminal differentiation of lens epithelial cells into anucleate lens fibers. The terminal differentiation of human and murine erythroblasts also requires caspases. Caspase inhibitors interfere with the maturation of erythroid progenitors at an early stage of differentiation.⁶⁸ Caspases-2, -3 and -9 are transiently

activated during erythroblast differentiation and are involved in the cleavage of the nuclear proteins Lamin B, PARP and Acinus.⁶⁸ These caspase substrates are implicated in chromatin condensation and enucleation, features characteristic of erythrocyte differentiation. Platelets are a third type of enucleate cells, and their formation from mature megakaryocytes coincides with caspase-3 and -9 activation.⁵⁶ Furthermore, synthetic caspase inhibitors block platelet formation *in vitro*, underscoring the role of caspases in their differentiation.⁵⁶ In addition, it was suggested that caspases are involved in platelet activation events, such as phosphatidylserine exposure and release of microparticles.⁶⁹ In the epidermis, the terminal differentiation of keratinocytes into enucleated corneocytes coincides with the expression and processing of caspase-14.⁷⁰ No specific inhibitors of this caspase or mice deficient in it have been reported yet. Therefore, the involvement of this caspase in the terminal differentiation of the skin remains to be established. Interestingly, it was recently shown that caspase-3 is activated in the embryonic epidermis, but not in newborn epidermis.⁷¹ Lack of caspase-3 resulted in increased proliferation and reduced differentiation of embryonic keratinocytes.

The differentiation processes discussed above all result in enucleated cells, namely lens cells, erythrocytes, megakaryocytes and keratinocytes. As enucleated cells are a biological dead end, these terminal differentiation processes could be regarded as specialized forms of programmed cell death in which the body corpses are not removed by phagocytosis, but remain and are functional. However, caspases do also promote differentiation of cells without inducing any form of programmed cell death. One example is the differentiation of human blood monocytes into macrophages, a process that is blocked by synthetic caspase inhibitors.⁷² In addition, genetic deletion of caspase-8 in cells of the myelomonocytic lineage prevented them from differentiating into macrophages upon treatment with M-CSF.⁷³ Interestingly, the activation of caspases seems to be specifically associated with differentiation into macrophages, and not with monocytes undergoing dendritic cell differentiation.⁷² Using antisense oligonucleotides and caspase inhibitors, it was shown that activation of caspase-8 is essential during differentiation of human placental villous trophoblasts.⁷⁴ Another report established a role for caspase-3 in skeletal muscle differentiation: primary myoblasts from caspase-3 knockout mice displayed a severe lack of myotube and myofiber formation and a reduced expression of muscle-specific genes.⁷⁵ Furthermore, the authors identified the serine/threonine Mammalian Sterile Twenty-like kinase (MST1) as a crucial effector of caspase-3, since a proteolytic fragment of MST1 rescued the differentiation defect of caspase-3 deficient myoblasts.⁷⁵ In line with these results, caspase-3 null mice that survive to early perinatal life are significantly smaller than heterozygous littermates and reportedly have a lower total skeletal muscle mass.⁷⁵ Moreover, ossification is delayed and bone mineral density is decreased in caspase-3 deficient mice due to attenuation of osteogenic differentiation.⁷⁶ More recently, this protease was suggested to contribute to neural stem cell differentiation based on experiments with *ex vivo* cultured caspase-3 null neurospheres.⁷⁷ Similarly, the development of *Drosophila*

proneural clusters in the wing imaginal disc was recently shown to require tight control of caspase activation. This is achieved by the IKK-related kinase DmIKK ϵ , which phosphorylates and targets the caspase inhibitor DIAP for proteasomal degradation.⁷⁸ In contrast to these roles of caspases as positive regulators of cell differentiation, constitutive caspase-3 activity in immature dendritic cells was found to block dendritic cell maturation and cell-surface expression of peptide-loaded major histocompatibility complex (MHC) class II molecules.⁷⁹ Caspase-3-mediated cleavage of the β 1- and γ -subunits of the adaptor protein 1 (AP1) complex was discovered to be the underlying molecular event.⁷⁹ Treatment with pharmacological caspase inhibitors or LPS inhibited AP-1 cleavage and initiated dendritic cell maturation.⁷⁹ Finally, the *Drosophila* caspases DREDD, DRONC and drICE were all described to be essential for spermatid individualization and the proper differentiation of sperm cells.^{80,81} The impaired sperm differentiation resulting from caspase inhibition in *Drosophila* strikingly resembles one of the most commonly seen abnormalities of human spermatozoa, known as cytoplasmic droplet sperm.⁸² In this respect, mutations in several apoptotic mouse genes – although not identified in mammalian caspase genes – are known to produce defects in spermatogenesis and lead to male sterility.⁸³ Interestingly, spermatogenesis and fertility of male Apaf-1-deficient mice that reach adulthood depends on the genetic background of the animals: while Apaf-1 null mice in a mixed 129/ICR background are infertile, some male mutants kept in a C57BL/6 background succeed in impregnating females.⁸⁴ These data indicate that it would be wise to assess the effect of potential therapeutic caspase inhibitors on human fertility before entering the clinic.

Caspases in Cell Motility

Regulation of cell migration is another emerging non-apoptotic function of caspases. DIAP-mediated inhibition of the caspase-9 homologue DRONC has been found to be essential for border-cell migration in the *Drosophila* ovary.⁸⁵ Border-cells form clusters of supporting cells that assist in the proper development of oocytes in the *Drosophila* ovary. The cleavage of several adhesion- and cell motility-related proteins during mammalian apoptosis may also point to a role in the regulation of cell migration.⁸⁶ Recent findings are in support of this suggestion as cell migration is severely hampered in caspase-8-deficient cells and CrmA-overexpressing cells. The authors propose a model in which caspase-8 promotes Rac activation and subsequent cytoskeletal remodelling downstream of calpains.⁸⁷ Furthermore, the early-embryonic death of caspase-8^{-/-} mice is mainly attributed to their failure to assemble a functional circulatory system, possibly reflecting an underlying defect in endothelial cell migration.⁴⁴ These observations warrant further investigations of the molecular mechanisms of caspase-mediated regulation of cell migration.

Role of Caspase-Like Decoy Molecules

The discovery of human CARD-only proteins that resemble the CARD domain of caspase-1, such as COP/Pseudo-ICE,

INCA and ICEBERG, highlighted the existence of a possible mechanism for the modulation of the inflammasome complexes and the consecutive IL-1 β levels.^{88–91} COP, INCA and ICEBERG all bind the caspase-1 CARD and prevent the activation of caspase-1 and the subsequent generation of IL-1 β .^{88,90,91} COP, most related to the caspase-1 CARD, also binds RIP2 and activates NF- κ B.^{88,90} In contrast, the more distantly related CARD-proteins INCA and ICEBERG do not display these characteristics.^{88,90} Interestingly, orthologs of COP, INCA and ICEBERG cannot be retrieved in the genomes of the mouse and rat. On the other hand, putative orthologs of COP and INCA, respectively, sharing 97 and 94% protein sequence identity with their human counterparts, are encoded on chromosome 9 of the chimpanzee (Lamkanfi M., unpublished data). This observation suggests that COP and INCA must have emerged by gene duplication in an ancestral mammal somewhere between 90 and 5.4 million years ago during the divergence of rodents and chimpanzees from the human lineage. Paradoxically, no ortholog for ICEBERG could be identified in the chimpanzee genome (Lamkanfi M., unpublished data), although the low sequence homology to procaspase-1 suggests an earlier phylogenetic bifurcation as compared to the other two CARD-only proteins (Figure 3). However, as this locus on chromosome 9 of the chimpanzee still contains some gaps, it remains possible that a monkey ortholog for ICEBERG will be identified in the future.

Another recent evolutionary event (100–500 thousand years ago) is the incorporation of single nucleotide polymorphisms (SNPs) in the human *caspase-12* gene that lead to the production of either a CARD-only protein or a full-length caspase, although still lacking enzymatic activity.^{92,93} The presence of an intact QACRG motif in a rhesus monkey and chimpanzees (Genbank accession numbers DQ644576 and XM_522166, respectively) suggests that the SNP causing the loss of enzymatic activity in the human *caspase-12* gene must have arisen earlier during the bifurcation of apes and humans (around 5.4 million years ago). Some interesting parallels can be drawn between the catalytically inactive human caspase-12 and the caspase-8 inhibitor c-FLIP. Both proteins resemble full-length caspases with a large N terminal prodomain followed by a caspase domain. Indeed, caspase-12 is a close homologue of the inflammatory caspases-1, -4 and -5, while c-FLIP resembles the DED-containing caspases-8 and -10 (Figure 3). However, both human caspase-12 and c-FLIP lack the conserved QACXG box that is normally present in the catalytic site of genuine caspases. Furthermore, truncated prodomain-only-expressing splice variants have been described for both caspase-like decoy molecules.^{45,92} These common features suggest that catalytically inactive human caspase-12 may fulfil roles reminiscent of those of c-FLIP as described above. In this respect, it is intriguing that the full-length variant of caspase-12 is confined to specific populations of African descent and that the frequency of the full-length allele is apparently doubled in African American individuals who suffer from sepsis.⁹⁴ Furthermore, the mortality rate of patients with the read-through mutation suffering from severe sepsis is three times that of patients without the SNP.⁹⁴ In line with these results, caspase-12 deficient mice have recently been shown to be resistant to peritonitis and septic shock.⁹⁵ Therefore, human caspase-12

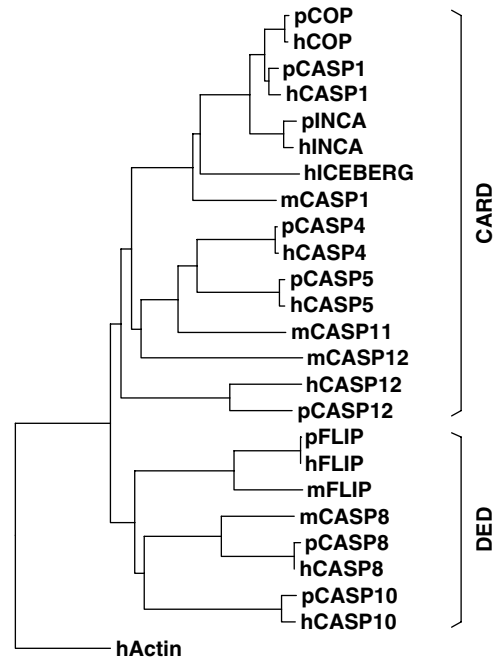


Figure 3 Phylogenetic analysis of caspase-like decoy molecules. The sequences of the short isoforms of the caspase-like decoy molecules were aligned with the prodomains of caspases-1, -4, -5, -8, -10, -11 and -12 using CLUSTAL X (gap weight = 20.00; gap length weight = 0.10). Trees were visualized in TreeCon. *Homo sapiens* (h), *Mus musculus* (m), (p) *Pan troglodytes*.

may function as a negative regulator of inflammatory signalling pathways, comparable to COP/Pseudo-ICE, INCA and ICEBERG.⁹³ The higher incidence of severe sepsis in patients with the caspase-12 read-through mutation as compared to individuals harbouring the CARD-only variant might be attributed to a less efficient negative regulation of the caspase-1-containing inflammasome complexes due to steric hindrance of the inactive caspase domain. In this model, the short human caspase-12 isoform may block caspase-1 or -5 activation in the inflammasomes more efficiently than the full-length isoform, reminiscent of the role of the prodomain-only isoform of c-FLIP in the DISC complex. Inhibition by the CARD-only isoform of human caspase-12 may thus render the inflammasome unable to process the pro-inflammatory cytokines pro-IL-1 β and -18, finally resulting in a less efficient innate immune response. Whether this or other functions are involved in caspase-12-mediated induction of sepsis needs further clarification.

Conclusions and Perspectives

The discovery that caspases also have non-apoptotic functions may change the widely accepted paradigm that caspase genes have been selected during metazoan evolution solely for their functions in programmed cell death. An interesting conceptual framework that reconciles the pleiotropic functions of caspases in an evolutionary perspective has been proposed recently.⁹⁶ Therefore, caspases may have been ideally positioned to play a central role in apoptosis through the integration of their pleiotropic functions in cell cycle and

innate immune system regulation, cell proliferation, cytokine release and cell differentiation. High levels of caspase activation achieved with or without mitochondrial amplification clearly lead to caspase-dependent apoptosis. In contrast, limited caspase activation may reveal mainly the non-apoptotic functions (proliferation, differentiation, intercellular communication through cytokine release and NF- κ B activation). These non-apoptotic functions partly involve prodomain-mediated and/or caspase activity-dependent activation of NF- κ B. In turn, NF- κ B could then augment the anti-apoptotic status of the cell, allowing the non-apoptotic functions of caspases to operate. A striking example of this model is provided by caspase-8 in humans and DREDD in *Drosophila*. By activating NF- κ B, these caspases can function both as crucial mediators of apoptosis and as activators of the innate immune system. The involvement of so-called 'apoptotic' caspases in cellular differentiation and immune and inflammatory responses demonstrates that therapeutic inhibition of caspase activity to prevent cell death may have broader implications than initially conceived. On the other hand, a better understanding of caspase functions could lead to the development of novel pharmaceutical strategies to prevent inflammation or to control autoimmune diseases.

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