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Proliferative versus Apoptotic Functions of Caspase-8 Hetero or Homo: The Caspase-8 Dimer Controls Cell Fate

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Abstract

Caspase-8, the initiator of extrinsically-triggered apoptosis, also has important functions in cellular activation and differentiation downstream of a variety of cell surface receptors. It has become increasingly clear that the heterodimer of caspase-8 with the long isoform of cellular FLIP (FLIP_L) fulfills these pro-survival functions of caspase-8. FLIP_L, a catalytically defective caspase-8 paralog, can interact with caspase-8 to activate its catalytic function. The caspase-8/FLIP_L heterodimer has a restricted substrate repertoire and does not induce apoptosis. In essence, caspase-8 heterodimerized with FLIP_L prevents the receptor interacting kinases RIPK1 and -3 from executing the form of cell death known as necroptosis. This review discusses the latest insights in caspase-8 homo- vs. heterodimerization and the implication this has for cellular death or survival.

Keywords

Apoptosis; Caspase; FLIP; Necroptosis; Receptor Interacting Protein Kinase

1 Introduction

1.1 Cell death and caspases

Caspases are cysteine proteases and the first member of this family was discovered some 20 years ago as the protease responsible for the maturation of interleukin-1 β [1,2]. Initially, this enzyme was called Interleukin Conversion Enzyme (ICE) but is now referred to as caspase-1. A related cysteine protease was discovered a few months later in the nematode worm *Caenorhabditis elegans* and called CED3 [3,4]. In *C. elegans*, CED3 was found to be responsible for the execution of developmental cell death and ever since, caspases have been almost synonymous with cell death [5]. However, it was soon recognized that in mammals at least two distinct branches of the caspase family have evolved with distinct functions; one branch involved in the initiation and execution of programmed cell death and one in the regulation of inflammation [6]. Caspases cleave their substrates exclusively after an aspartic acid residue (Asp/D). For a detailed overview of the activation mechanisms and substrate preferences of the caspases, we refer the reader to previous reviews [6,7]. Caspase-

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dependent programmed cell death is generally referred to as apoptosis, although caspase-1 is implicated in the induction of an inflammation-associated form of programmed cell death known as pyroptosis [8]. Two major pathways leading to apoptosis have been described, 'intrinsic' and 'extrinsic'. Intrinsic apoptosis is initiated by caspase-9 which, for its activation, depends on the release of cytochrome *c* and other pro-apoptotic factors from the mitochondria through a permeability transition pore formed by the pro-apoptotic members of the Bcl-2 family, primarily Bid and Bax. Pro-survival members of the Bcl-2 family counteract the formation of this pore and thus the release of apoptogenic proteins from the mitochondria, as reviewed in detail by Lindsay *et al.* [9]. Extrinsic apoptosis occurs downstream of death receptor signaling and is initiated by caspases-8 and -10, as will be discussed in greater detail below. Since Bid is a target of caspase-8/10 activity, the two pathways have been proposed to be inter-linked, although probably only in certain cells or tissues [10]. In the absence of caspase activation, another form of programmed cell death, now generally referred to as 'necroptosis', can be triggered [11,12]. Activation of this pathway is actively opposed by caspases, primarily by caspase-8. The objective of this review is to discuss the recent insights in the pro-survival role of caspase-8 that is linked to its catalytic function, primarily its role in T-cell activation and homeostasis by control of necroptotic death and the activation mechanism of caspase-8 by the inactive caspase homolog FLIP_L.

1.2 All the colors of the caspases

As mentioned above, human caspases seem to have evolved to be either involved in the regulation of apoptosis or inflammation. In Figure 1A, the pro-apoptotic members of the caspase family are marked in green, while the pro-inflammatory members are marked in red. Caspase-2 and -14, which have neither clear apoptotic nor clear inflammatory functions, are marked in black. The pro-apoptotic members of the caspase family are subdivided in the initiators of apoptosis (caspases-8, -9 and -10 in humans) and the executioners of apoptosis (caspase-3, -6 and -7). The difference between the two is that the initiators have a relatively large N-terminal dimerization domain, either a Death Effector Domain (DED; caspases-8 and -10) or a structurally related Caspase Recruitment Domain (CARD; caspase-9) and are expressed as monomers that require dimerization for activation. In contrast, the executioners only have short N-terminal pro-domains, exist as inactive dimeric zymogens in solution and require limited proteolytic cleavage between the large and small subunits for activation [6]. A general overview of the domain organization of the caspases is shown in Figure 1B. Caspase-2, which also has a CARD, is sometimes grouped among the initiators of apoptosis, however its function is hotly debated and it may either be involved in the DNA damage response, inducing either DNA repair or DNA-damage induced apoptosis, heat-shock induced apoptosis, the release of cytochrome *c* from the mitochondria [13-19] or none of the above. Another 'orphan' of the caspase family is caspase-14, which is exclusively expressed in keratinocytes and involved in their differentiation [20]. The most-studied member of the pro-inflammatory caspases is caspase-1, which is essential for the maturation of the pro-inflammatory cytokine interleukin-1 β (IL-1 β) as mentioned before [5]. The exact functions of the related caspases- 4 and -5 remain unknown, although they have been implicated in a number of processes [21-23]. Caspase-12, finally, is only expressed in a truncated, catalytically inactive form in most humans, with the exception of a small percentage of the

sub-Saharan African population that expresses the full-length enzyme. It is thought that full-length caspase-12 may have a protective effect against certain infections in neonates [24-26]. Alternatively, caspase-12 may act as a dominant negative in the regulation of caspase-1 activity [27]. Of late, it has become increasingly clear that the pro-apoptotic caspases, and in particular caspase-8, also have important functions in survival and development.

2 The developmental role of caspase-8

2.1 Caspase-8 knockout mice—It is the human condition to classify, subdivide and order. However, nature as such is not always as organized as we would like it to be. Therefore, when the first knock-out mice for apoptotic caspases were generated, it came as a bit of surprise that their phenotypes were not very apoptotic. The main defects of the caspase-3 and -9 knockout mice appeared to be in brain development, whereas apoptosis of hepatocytes or lymphocytes was not very much affected. For an extensive review of caspase knockout mice, we refer the interested reader to Los *et al.* [28]. The gene for caspase-10 was lost in the ancestor of the rodent lineage [29] – see Figure 2, but the caspase-8 knockout has the most dramatic phenotype of all caspase knockouts as these knockout mice die *in utero* at around day 11 of gestation with primary defects in cardiac development, growth retardation and hematopoietic progenitor deficiency [30]. Importantly, knockout of two other members of the complex in which caspase-8 is activated -FADD and FLIP_L- both revealed a very similar phenotype [31,32]. This indicates that formation of this complex, the Death Inducing Signaling Complex (DISC) and caspase-8 activation does not only trigger apoptosis but is also essential for embryonic development. Further studies on mice with tissue specific knockout of caspase-8 revealed an essential role for this caspase in the development of a variety of tissues, most notably the endothelial cells of the heart and vascular system, lymphocytes and monocytes. Only in the liver, it appeared, the apoptotic functions of caspase-8 were primarily affected [33]. Whereas catalytically active caspase-8 is required to rescue the lymphocyte development in caspase-8 deficient mice [34], the caspase-8 DEDs alone appear to be required to rescue the terminal differentiation defect of endothelial, epithelial and myeloid cells [35]. Furthermore, mice deficient in caspase-8 in basal epidermal keratinocytes suffer from chronic skin inflammation, as an apparent consequence of IRF3 hyper-activation [36]. This phenotype, however, is not rescued by catalytically inactive caspase-8. How expression of the caspase-8 DEDs alone can be sufficient to induce terminal differentiation in certain cells, is as yet unclear, but implies an important non-catalytic role for caspase-8 [37,38].

2.2 Caspase-8/10 deficiencies in humans—In humans, caspase-8 deficiency is compatible with development, as demonstrated by the case of a family with a detrimental mutation in the caspase-8 gene [39]. In homozygous individuals, lymphocyte apoptosis and activation is impaired, but overall development is normal. Besides caspase-8, humans, contrary to mice, also express the highly homologous caspase-10, which has similar substrate specificity to caspase-8 [29] and at least some overlapping functions, but can not completely substitute for loss of caspase-8 [40-42]. While mutations or deficiencies in caspase-10 are associated with autoimmune lymphoproliferative syndrome (ALPS) [43,44], expression of either caspase-8 or caspase-10 is apparently sufficient for normal human

development, but both are required to execute the apoptotic functions, at least in lymphocytes. It should also be noted that at least one mutation in caspase-10 that was originally associated with ALPS, V410I, is actually a common polymorphism in the healthy Danish population [45].

3 Activation of caspase-8/10

3.1 Dimerization vs. cleavage—In general, caspases can only be active as dimers, since neither the active site dyad nor the substrate pocket can be formed in the monomeric form [6]. In fact, it can not be stressed enough that dimerization is an absolute requirement for caspase activation. When caspase cleavage is detected, for example by Western blot, this does not signify that the caspase in question has been activated; merely that it has been cleaved. Active executioner caspases maintain the same core structure as zymogens, but undergo substantial re-arrangement of crucial surface loops after the intersubunit linker has been cleaved, leading to formation of the active site. Initiator caspases, on the other hand, have to undergo dimerization and subsequent activation by induced proximity in large multi-protein complexes [46-48]. Caspase-8 and -10 alike are activated at the DISC, downstream of death-receptor signaling [46]. Upon ligation, death-receptors such as the tumor-necrosis factor receptor (TNFR) or Fas, multimerize in the cell membrane which leads to a conformational change in the intracellular domain of the receptor [49,50]. This, in turn, leads to recruitment and multimerization of the adaptor protein FADD, which contains both a Death Domain (DD) that interacts with the receptor, and a DED that recruits caspase-8/10. At this point, we realize that many authors state in their papers that caspases-8 and -10 are activated by oligomerization. This, however, is incorrect. The DISC is a high order oligomeric ensemble, and provides a platform for *dimerization* of caspases-8 and 10. Thus, caspases-8 and -10 are recruited to the DISC as intact monomers [51] and recruitment of the caspases to the DISC subsequently leads to their dimerization and activation through induced proximity [29,48,52]. Although the caspase-8 dimer is active in the absence of proteolytic cleavage within the intersubunit linker, cleavage stabilizes the dimer and leads to a caspase species with increased activity [53-59]. Finally, caspase-8 also cleaves itself between the DEDs and the large subunit, resulting in release of the stable dimer from the DISC [60]. Cleavage in the absence of dimerization does not lead to activation, but cleavage after dimerization increases both the activity and the stability of the dimer [54,56]. However, it has been suggested in several cases that caspase-8 can be activated through cleavage by another protease. The serine protease granzyme B, which is known to activate both caspase-3 and -7 during cytotoxic T-cell induced death, was shown to cleave caspase-8 as well [61]. In another case, caspase-6 was shown to cleave caspase-8 between the large and small subunit which was associated with Bid-dependent cytochrome c release from isolated mitochondria in a cell free system [62]. In this latter study, it was not excluded that recombinant caspase-6 can also cleave Bid directly at high concentrations or that caspase-6-cleaved caspase-8 has a decreased threshold for self-dimerization, possibly even in 'mitosomes' on the mitochondrial membrane, as has been suggested in other studies [63-65]. Finally, it has been suggested that the lysosomal protease cathepsin D can cleave and activate caspase-8 during neutrophil apoptosis [66]. In all of these cases, dimerization is most likely a prerequisite for activation whereas cleavage stabilizes the dimers and increases their activity.

3.2 Activation by FLIP_L—A homologue of caspase-8, referred to as cellular Flice Inhibitory Protein (cFLIP) can also be recruited to the DISC. This homolog contains inactivating mutations in the catalytic site and is therefore catalytically dead. Heterodimerization with the long isoform of cFLIP (FLIP_L) leads equally to activation of caspase-8/10 [67-70]. The unprocessed heterodimer has a restricted substrate repertoire and is no longer capable of cleaving and activating the main pro-apoptotic targets downstream of caspase-8/10 activation, such as caspase-3 and Bid, as recently demonstrated in two independent studies utilizing *in vitro* generated caspase-8/FLIP_L heterodimers [57,71]. FLIP_L was simultaneously identified by several groups, and data presented in these publications were highly controversial regarding whether FLIP_L was pro- or anti-apoptotic. Some thought FLIP_L to be an inhibitor of caspase-8, hence the name, and others thought it to be an independent caspase-8-like cell death inducer [72-76]. This controversy was settled when it was recognized that FLIP_L only prevented cell death upon over-expression by occupying all the binding sites for caspase-8 at the DISC [77]. It now appears that caspase-8 has a wider array of functions besides inducing cell death and it appears to be the heterodimer, rather than the homodimer, which is involved in executing the non-death functions of caspase-8 [57,71,78]. Furthermore, the developmental phenotype observed in the caspase-8 knockout mouse is shared with both the FLIP_L knockout and the FADD knockout [31,32], and FLIP_L has been shown to be essential for cellular differentiation and activation in a number of studies [68,78-86]. Altogether, this indicates that it is FLIP_L-activated caspase-8 that fulfills the non-apoptotic functions of caspase-8 prior to autoprocessing, rather than the caspase-8 homodimer - see Figure 3 for a schematic of homo- versus heterodimerization of caspase-8. Interestingly, whereas the uncleaved caspase-8 heterodimer with FLIP_L can no longer cleave Bid and induce intrinsic apoptosis, the heterodimer of caspase-10 with FLIP_L still can [29,57]. This implies that at least in that respect, caspases-8 and -10 are functionally different, but in mice, who do not express caspase-10, these functions are apparently subsumed by caspase-8. It should be noted that whereas human caspase-8 is cleaved twice in the intersubunit linker, mouse caspase-8 only has one cleavage site. The caspase-8/FLIP_L heterodimer can process the first site with some difficulty, after which it can process the second site to become fully active. Since murine caspase-8 does not contain this first site, it is still an open question if and when murine caspase-8 dimerized with FLIP_L can be activated to form an apoptotic species or whether in mice a secondary event is required to fully activate caspase-8 in the heterodimer.

3.3 Structural insights into caspase-8 activation—Several crystal structures of the fully processed inhibitor-bound caspase-8 dimer have been published [87-91]. However, these structures allow little insight into the activation mechanism of caspase-8. The structure of monomeric un-processed caspase-8 as obtained by nuclear magnetic resonance (NMR) spectroscopy, was described only recently by Keller *et al.* [53]. Caspase-8 readily dimerizes and auto-processes during expression in *E. coli* and the unprocessed monomeric form is therefore hard to obtain in sufficient amounts to allow structural studies. The monomeric form of caspase-8 has a typical caspase fold, consisting of a six-stranded β sheet formed by five parallel and one anti-parallel β strand. This β sheet is flanked by a total of five α helices, two on one side of the structure and three on the other. Protruding from these structured regions are four loops and a short linker. The first of these loops, the one that

occupies the active site in monomeric caspases, contains a short α helix and upon dimerization and cleavage of caspase-8, this loop rotates along a perpendicular axis by $\sim 90^\circ$ to allow formation of the active site, as illustrated in Figure 4A. Even after the first loop has been re-arranged and the active site has formed in the dimer, the linker between the large and small subunits occupies the active site in the structure, hindering access to the active site cysteine (the red helix in Figure 4A). Keller *et al.* suggested that processing of the linker, possibly through autocatalysis, opened the active site and allowed full activation of caspase-8. However, even a mutant that could not be cleaved in either of the two cleavage sites in the linker generated some activity upon dimerization while a mutant incapable of dimer formation but with a cleavable linker remained completely inactive, stressing once more the importance of dimer formation [53,54]. The only way to generate robust activity from an unprocessed caspase, is to heterodimerize it with FLIP_L, as demonstrated by Pop *et al.* [57]. Interestingly, Boatright *et al.* [67], and later Yu *et al.* [70], showed that zymogen caspase-8 forms a much tighter interaction with FLIP_L than with another molecule of caspase-8. As many as 8 hydrogen bonds are formed in the dimer interface between FLIP_L and caspase-8, as compared to only 4 bonds in the caspase-8 homodimer [70]. The active site and the gross overall structural conformation of caspase-8 in this complex is very similar to active site of the homodimer, as illustrated in Figure 4, and therefore the restricted substrate specificity of the unprocessed heterodimer is likely caused by as yet undiscovered principles rather than a structurally different substrate cleft [57]. Since FLIP_L is catalytically inactive and can therefore not process its binding partner, the caspase-8/FLIP_L heterodimer is likely to remain unprocessed in cells until it is cleaved by an external protease, *e.g.* caspase-6. Within the caspase-8/FLIP_L heterodimer, caspase-8 prefers to process FLIP_L over itself and processing of free FLIP_L has been shown to markedly increase the recruitment of caspase-8 [70]. After cleavage, FLIP_L recruits other components to the complex with caspase-8, such as TRAF2, RIPK1 and NEMO, resulting in NF- κ B activation [79,80,82]. Therefore, the heterodimer of caspase-8/FLIP_L containing unprocessed caspase-8 signals towards cellular survival and activation, rather than towards apoptosis, and it is only after the caspase-8 subunit of the heterodimer has been processed that this caspase species becomes pro-apoptotic.

4. Programmed Necrosis

4.1 Necroptosis; death in the absence of caspase activation—By definition, forms of cell death that occur in the absence of caspases activation cannot be apoptosis, [92]. It was first observed in the murine fibroblastoma cell line L929 that if the cells were stimulated with TNF α in the presence of a broad spectrum caspases inhibitor the cells would die with a necrotic morphology [93]. This form of cell death was associated with high mitochondrial reactive oxygen species (ROS) production and loss of plasma membrane integrity [93,94], and was later recognized to occur in other cells as well upon stimulation with TNF α in the absence of caspase activation, as reviewed by Denecker *et al.* [95]. Further studies, performed by introducing the cowpox virus serpin and caspase-8 inhibitor CrmA [96] in the cells, revealed that in particular inhibition of caspase-8 triggered this form of cell death, now generally known as 'necroptosis' [93,97,98]. The serine/threonine kinase RIPK1 was discovered to be the initiator of necroptotic death in a screen for inhibitors of this form of cell death [11] and, more recently, three independent groups identified RIPK3

as a downstream effector of RIPK1 after extensive siRNA screening [99-101]. RIPK3 is thought to induce a switch in the cell's metabolism, leading to the increase of mitochondrial ROS production that culminates in cell death [101,102]. Altogether, this strongly implies that necroptosis is an alternative form of programmed cell death, rather than an accidental form of death as once thought. Caspase-8 has been shown to cleave, and presumably inactivate, both RIPK1 and RIPK3 and thus acts as a negative regulator of this pathway on at least two different levels [103-106]. It should be noted that RIPK1 was initially identified as an activator of the NF- κ B pathway of survival downstream of Fas/CD95 and TRAIL-receptor (DR4/5) signaling, but has later been suggested to be non-essential for activating NF- κ B downstream of TNFR1 signaling [105,107,108]. However, Jurkat cells deficient in RIPK1 fail to activate NF- κ B downstream of TNFR1, although it should be noted that these cells were selected for their inability to activate NF- κ B and it cannot be excluded that they lack one or more other components in the NF- κ B signaling cascade [109]. The main difference between the two death receptor signaling pathways appears to be that the TNFR1 receptor primarily signals through the adaptor TRADD and only secondarily through FADD [110], whereas DR4/5, similar to CD95/Fas primarily recruits the adaptor FADD [46]. The latter is disposable for activating RIPK1 as such, since FADD deficient T-cells or cells expressing dominant negative FADD are very susceptible to RIPK1-dependent necroptosis [111,112]. However, FADD is essential for the formation of both homo- and heterodimers of caspase-8. Thus, FLIP_L-dependent NF- κ B activation depends on FADD and, apparently, also on RIPK1. The main function of TRADD in the complex may be to attract the ubiquitin ligase TRAF2 which, through ubiquitination of RIPK1, may alter the outcome of DR signaling [113]. RIPK1 undergoes extensive ubiquitination on a single conserved lysine residue and whether it signals life or death appears to depend on whether the ubiquitin chain formed is K48, K63, K11-linked or linear [102,114-120]. A growing number of ubiquitin ligases and de-ubiquitinating enzymes regulate these functions of RIPK1, but discussing these is beyond the scope of this review. An excellent review on RIPK1 regulation by ubiquitination was recently published by Vandenabeele *et al.* [121]. However, novel regulators of the pathway are currently published on a regular basis.

4.2 Necroptosis versus Apoptosis—As mentioned before, both RIPK1 and RIPK3 have been identified as caspase-8 substrates [103,105,106]. Cleavage of RIPK1 prevents it from inducing necroptosis, while its C-terminal DD appears to be involved in DISC formation and can thus initiate apoptosis [95,122]. In both T-cell activation and macrophage development, caspase-dependent cleavage of RIPK1 has been shown to be an essential step [104,123-125]. In the absence of RIPK1 cleavage, activating T-cells undergo necroptotic cell death as an apparent consequence of massive macro-autophagy [124,125]. Normally, macro-autophagy, usually simply referred to as autophagy, is a starvation-induced response wherein a cell 'eats' its own organelles to conserve nutrients by enwrapping these organelles in a specialized compartment known as an autophagosome. It also represents a way for a cell to get rid of damaged organelles, such as mitochondria that produce excessive amounts of ROS [126]. Bell *et al.* have shown that caspase-8 forms a complex with RIPK1 and FADD on the autophagosomal membrane. Formation of this complex appeared to be initiated by the autophagosomal protein Atg5 modified by the ubiquitin-like protein Atg12 [127]. Presumably, the recruitment of RIPK1 leads to its activation in the complex, whereas the

role caspase-8 is to inactivate RIPK1 by cleavage. Only in the absence of caspase activation do the cells die as the apparent result of excessive, RIPK1-dependent, autophagy.

4.3 The heterodimer controls necroptosis—If caspase activation is required for development or activation, then how is apoptosis downstream of caspase-8 activity prevented? The answer seems to lie in the caspase-8/FLIP_L heterodimer. Some years ago, Dohrman and colleagues demonstrated that FLIP_L was essential for proper T-cell development [79]. More recently Leverrier *et al.* demonstrated that T-cell development in mice deficient in caspase-8 in the T-cell lineage could be rescued by a caspase-8 mutant that could no longer be processed in the inter-linker subunit, but retained catalytic activity [34]. By replacing the DEDs of caspase-8 and FLIP_L with inducible dimerization domains, Pop *et al.* have shown that the most favored way such a caspase-8 species can generate activity, is through heterodimerization with FLIP_L [57]. In addition, as mentioned above, this enzyme species has restricted substrate specificity and only poorly induces apoptosis. Several reports have recently been published that demonstrate that the embryonic defect of caspase-8 deficient mice can be rescued by back-crossing these mice on a RIPK3 or RIPK1 deficient background, thus demonstrating the deleterious effects of necroptosis during development [78,128-130]. In one of these reports, Oberst *et al.* demonstrate that FLIP_L expression is essential for preventing necroptosis [78]. This confirms what has been shown previously, that FLIP_L is essential for successful T-cell and monocyte differentiation and activation [32,79-85]. At this stage, it is not entirely clear whether the caspase-8/FLIP_L heterodimer controls the RIPKs by cleavage or whether cleavage of FLIP_L in this complex is essential for signaling towards NF- κ B, as has been suggested [79,80,82]. Caspase-8 also influences gene transcription directly by cleaving and inactivation histone deacetylase-7 (HDAC7), a repressor of genes controlled by the MEF2 family of transcription factors [131,132]. Inactivation of HDAC7 in T-cells results in transcriptional upregulation of the orphan nuclear receptor NuR77, which has been associated with their apoptotic demise [133]. However, the MEF2 transcription factors have multiple targets and their activity has been shown to be essential in monocyte differentiation [134], also a process wherein both caspase-8 and FLIP_L play a role [81,104]. HDAC7 is the most efficiently cleaved substrate for caspase-8 identified to date, at least in vitro, and is one of very few substrates known to be cleaved by the unprocessed caspase-8/FLIP_L heterodimer [57,132]. It may therefore represent an interesting target for further study into the pro-survival role of caspase-8.

4.3 Pro-survival functions of RIPK3?—Although the RIPK3/caspase-8 double knockout mice were born and matured relatively normal, older mice started suffering from a lymphoproliferative disorder, excessive proliferation of the T-cells caused by a lack of T-cell elimination through apoptosis [78,129,130]. This demonstrates the two-faced nature of caspase-8 which, like a game keeper, is on the one hand required to allow successful proliferation of the T-cell population in the first place and on the other hand to cull the herd of T-cells if proliferation gets excessive. Although the observation that the RIPK3 single knockout mice are relatively normal would suggest that RIPK3 is only required for the detrimental death of cells by necroptosis, these mice were also shown have an increased susceptibility to viral infections [99,135], though in a recent report Lu *et al.* observe no increased susceptibility of RIPK3^{-/-} mice to Murine Herpes Virus [136]. In addition, at least

one viral inhibitor of RIPK3 has been identified, indicating that, at least from the viral point of view, activation of RIPK3 is undesirable [135,137]. Finally, the intracellular anti-viral defense has been shown to depend on RIPK1-RIPK3 signaling [99,137,138]. It is as yet unclear whether RIPK3 is only required to execute necroptosis and that this alternate form of cell death itself is what prevents the spread of viral infection or whether the metabolic switch induced by RIPK3 activation is primarily a pro-survival, proliferative or pro-differentiation response, that only turns into necroptotic cell death if a secondary stimulus (*e.g.* caspase-8 activation) is not received. In a recent report, Biton and Ashkenazi demonstrate that RIPK1, in the absence of caspase activation, has a pro-survival role in the early response upon extensive DNA damage by activating NF- κ B through NEMO but induces the production of pro-inflammatory cytokines, such as IL8 and IL6, in a late-phase response through the JNK3 pathway that leads to activation of the transcription factor AP-1 [122]. In addition, they suggest a feed-forward signaling mechanism wherein RIPK1-dependent TNF α production induces cell death in an autocrine manner, as has been suggested previously for L929 cells [139]. In an even more recent paper, the same lab suggests that the same complex also induces TNF α production and subsequent tumor cell death after stimulating the cells with a TNF α related protein, TWEAK [140]. Thus, if RIPK1 is not inactivated by caspase-8 cell death can lead to sterile inflammation, similar to the phenotype observed in mice with caspase-8 deficient keratinocytes [36]. Whether or not RIPK3 has a role in this pathway was not discussed by the authors, since most of the experiments in the Biton study were performed with HeLa cells and these cells do not normally express RIPK3.

5 Post-translational modifications alter caspase-8 activity

5.1 Caspase-8 activity regulated by kinases—Caspase-8 is not only cleaved, but has also been shown to be phosphorylated and ubiquitinated. Several kinases have been shown to phosphorylate caspase-8 and suppress its activation. Whereas caspases-9, -3 and -2 appear to be regulated by serine or threonine phosphorylation, caspase-8 is mostly phosphorylated on a few conserved tyrosine residues. Thus, the serine/threonine kinases RIPK1 and RIPK3 can not control caspase-8 activity. For a detailed overview of the regulation of caspase activity by phosphorylation, we refer the reader to an excellent review by Kurokawa and Kornbluth [141]. It should also be noted here that phosphorylation of Bid, one of the main pro-apoptotic effectors downstream of caspase-8, on a conserved threonine residue in the caspase-8 cleavage motif by one of several kinases [142-146] prevents cleavage and by caspase-8. Considering that the caspase-8/FLIP_L heterodimer does not cleave Bid, but that the caspase-10/FLIP_L heterodimer does [29,57], phosphorylation of Bid may actually prevent premature induction of apoptosis by caspase-10.

5.2 Caspase-8 activity regulated by ubiquitination—So far we have seen a number of ways in which caspase-8 can remain anti-apoptotic, but how does caspase-8 turn pro-apoptotic? In an insightful study, Jin *et al.* suggested that caspase-8 undergoes extensive poly-ubiquitination in the DISC. C-terminal ubiquitination of caspase-8 was shown to be mediated by the ubiquitin ligase cullin-3, which was also found to be present in the DISC, downstream of DR4 signaling [147]. Poly-ubiquitination of caspase-8 increased its pro-death potential, apparently by stabilizing the dimers. The deubiquitinating enzyme A20

reversed the process. This is interesting, as A20 has also been shown to inhibit RIPK1 signaling towards NF- κ B by removing K63-linked polyubiquitin chains and replacing them with K48-linked chains [120,148,149]. Thus, A20 prevents caspase-8 from becoming pro-apoptotic while at the same time preventing RIPK1 from pro-survival signaling, apparently maintaining the status quo. This C-terminal poly-ubiquitination of caspase-8 observed by Jin *et al.* may also explain how caspase-8 turns into a pro-apoptotic protein in the first place. After all, as we have seen, unprocessed caspase-8 can only be activated through interaction with FLIP_L and, as such, it is incapable of auto-processing [57]. Therefore, a secondary stabilizer, such as a ubiquitin chain, may be required to 'zip up' and fully activate the caspase-8 heterodimer.

6 Conclusions

As we have seen, in the initial stages of its activation caspase-8 primarily has non-apoptotic, pro-survival functions. How then, does this good-natured caspase turn into a hardened killer? It all seems to depend on the company it keeps. The heterodimer of caspase-8 and FLIP_L is presumed to be formed preferentially upon death receptor ligation; since the K_d for dissociation of the heterodimer is significantly lower than for the homodimer [59,67]. However, the concentration of FLIP_L is rate-limiting; upon continued stimulation or when a very strong stimulus is received, homodimers will inevitably form. An alternative heterodimer of caspase-8 with MALT1 has also been suggested to form downstream of antigen receptor stimulation, which appears to have similar properties [150]. Progressive activation of the heterodimer also occurs, as illustrated in Figure 5. If caspase-activity remains absent, either because cells have been treated with a caspase inhibitor, because a viral infection prevents caspase activation or because some other condition prevents the formation of the FADD/caspase-8 DISC, active RIPK1 will eventually execute the cell by necroptosis. FLIP_L is good company in this respect, because it actively prevents RIPK1 from executing the cells by a pro-inflammatory type of cell death while simultaneously preventing full activation of caspase-8. In other words, FLIP_L keeps the peace and caspase-8 out of trouble. Cleavage of RIPK1 by the caspase-8 partner is essential for this function. Only cleaved FLIP_L activates NF- κ B, probably by recruiting TRAF2 and cIAP1 to the complex, which leads to K63-linked RIPK1 ubiquitination and interaction of RIPK1 with NEMO, instead of RIPK3 [82,122,151,152]. In this complex, caspase-8 can only be fully activated if it is cleaved by an external protease [57]. If the intrinsic pathway of apoptosis is activated and cytochrome *c* has been released from the mitochondria, caspase-6, which is activated downstream of caspase-3 and -9, will do so [62]. Alternatively, Cathepsin D appears to cleave caspase-8 upon lysosomal damage [66] while Granzyme B can do the job after a cell has been marked for death by a cytotoxic T-cell [61]. Thus, in a cell that has received extensive damage or has been infected the pro-life/pro-activation signal of the caspase-8/FLIP_L heterodimer can still be turned into an apoptotic signal, preventing the spread of an infection or, for example, tumor growth. Activation of the homodimer is similarly controlled from the outside, but this time by poly-ubiquitination of the C-terminus [147]. Altogether, activation of caspase-8 is not primarily a suicide mechanism. This explains the fast, and growing, number of events that rely on caspase-8 activity in the absence of cell death [153-156]. Caspase-8 primarily signals activation and proliferation,

only secondary events can turn this signal in a death-inducing mechanism. It is unlikely that RIPK1 and/or RIPK3 are the only relevant substrates of the caspase-8/FLIP_L heterodimer. HDAC7 has been mentioned before as an excellent substrate for this heterodimer, but other substrates are likely to turn up [57]. In pathological situations, such as tumor cell proliferation, activation of the heterodimer may be unwanted. Since the active site groove of the heterodimer is not essentially different from the groove in the homodimer, designing specific inhibitors for the heterodimer may be challenging. Active-site directed inhibitors will definitely target both homo- and heterodimers, resulting in necroptosis. Instead, allosteric inhibitors may have to be developed or, alternatively, allosteric activators of caspase-8 that turn the complex from pro-survival into pro-apoptotic, similar to the allosteric activator of caspase-3 recently described by Wolan *et al.*[157]. Altogether, the insight that caspase-8 can have different functions depending on its dimerization partners has proven to be a crucial insight and significantly improved our understanding of the complex processes of cell death and differentiation. Further research is pending into the exact targets of the heterodimer as compared to the homodimer and, not unimportantly, the role of caspase-10 in the heterodimer.

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Abbreviations

ALPS	Acute Lympho-Proliferative Syndrome
CARD	Caspase Recruitment Domain
CED	<i>Caenorhabditis elegans</i> Death protein
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DR	Death Receptor
FADD	Fas- Associated Death Domain protein
FLIP	FLICE Inhibitory Protein
HDAC	Histone Deacetylase
ICE	Interleukin Converting Enzyme
IL	Interleukin
IRF	Interferon Regulatory Factor
JNK	c-Jun N-terminal Kinase
NEMO	NF- κ B Essential Modulator

NF	Nuclear Factor
RIPK	Receptor Interacting Protein Kinase
ROS	Reactive Oxygen Species
TNF	Tumor Necrosis Factor
TNFR	TNF Receptor
TRADD	TNFR Associated Death Domain protein
TRAF	TNFR Associated Factor
TWEAK	TNF-Related Weak inducer of apoptosis

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1A.

1B.

Figure 1. The human caspases

- A: Phylogenetic tree of all the human caspases. Pro-apoptotic caspases are marked in green, pro-inflammatory caspases in red. Caspases with no clear apoptotic or inflammatory function are marked in black. Image generated with Genious Tree Builder using the full length protein sequence of the human caspases using Genious Pro v5.1.6, as developed by Biomatters Ltd.
- B: Overall organization of the initiator and executioner caspases. The yellow circle (●) on the 'large' subunit denotes the active site cysteine; blue circles (●) in the linkers denote caspase cleavage sites.

Figure 2. Schematic of the caspase-8 phylogeny

Two distinct caspase-8 family members arose in the chordate branch of the eukaryotes. Caspase-18 and the ancestor of -8 and -10 we call 'caspase-810' in this schematic, are still found in fishes. Later on in evolution, caspase-8 and -10 branched off from caspase-810. Birds and lizards express three apical caspases in the DR pathway; caspase-8, -10 and -18. Mammals subsequently lost caspase-18, while rodents lost caspase-10. See also Eckhart *et al.* [158].

Figure 3. Caspase-8 activation through homo- vs. heterodimerization

Caspase-8 (green) can either homodimerize with another molecule of caspase-8, leading to a homodimer wherein caspase-8 is fully processed and induces apoptosis (top) or heterodimerize with FLIP_L (blue) to form a heterodimer wherein FLIP_L is primarily processed to induce cell survival (bottom). In either case, dimerization is mediated by the adaptor protein FADD (violet).

Figure 4. Structural insights in caspase-8 activation

A: Structural overlay of the caspase-8 monomeric zymogen (green) and the substrate bound, fully-processed, caspase-8 dimer (orange; only one caspase-8 subunit is shown). During dimerization, a loop containing a small helix (in red) translocates from the active site (1), as indicated by the red arrow. Afterwards, the linker (blue) between the large and small subunits gets processed (2), opening up the active site completely for substrate binding. The inhibitor Z-EVD-CMK, in yellow, indicates the location of the active site cleft in the structure.

B: Structural overlay of the caspase-8 homo-dimer (earth colors) versus the caspase-8/FLIP_L heterodimer (blues). Over all structural changes upon formation of either the homodimer or the heterodimer are grossly similar.

C-E: Comparison of the substrate cleft in the monomer (C) versus the peptide-bound homodimer (D) and the peptide-bound heterodimer (E). The substrate cleft is closed in the monomeric zymogen, whereas the cleft is accessible for substrate binding in both dimers. The synthetic peptide Ac-IETD-CHO is shown in magenta bound in the substrate cleft of the heterodimer (E).

Based on PDB entries 1QDU, 2K7Z and 3H11 [53,70,88]. Images generated with PyMOL v1.4.

Figure 5. Progressive processing of caspase-8 determines the outcome of RIPK1-signaling

1) If caspase-8 is not activated, the complex of RIPK1 (top, orange), FADD (middle, violet) and caspase-8/FLIP_L (bottom, green/blue) induces necroptosis, as indicated in the left pane of the figure. 2) Moderate caspase-8 activation in the complex results in FLIP_L cleavage, possibly RIPK1 cleavage and signaling towards NF-κB. 3) Cleavage of caspase-8 inter subunit linker in the heterodimer, possibly by an external protease, results in a moderate apoptotic signal and RIPK1 cleavage. 4) Full activation and processing of caspase-8 in the homodimer results in efficient cleavage of RIPK1 and signaling towards apoptosis. Ubiquitination of both RIPK1 and caspase-8 controls the formation of the complex (not in the figure).