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Caspase-10 is a negative regulator of caspase-8-mediated cell death with gene inductive properties

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Mannheim, 15.03.2016

Sebastian Horn

This thesis is dedicated to my family.

"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

- Albert Einstein

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III. Abstract

Death ligands such as CD95L and TRAIL initiate the extrinsic apoptotic signalling cascade by activation of the death-inducing signalling complex (DISC). Upon ligand binding to the receptor, the adaptor protein FADD is subsequently recruited to the receptor, thereby allowing for further recruitment of caspase-8 and its negative regulator cFLIP. In addition to the well-known caspase-8-mediated cell death induction, DISC-induced gene expression *via* the activation of NF-κB ruled out to be an important signalling pathway. Even if the major key players in DISC signalling have been described, the molecular mechanisms in DISC formation remain to be elucidated. In addition, the function of some DISC-associated proteins is unknown to date. To this day, the role of caspase-10, a homologue of caspase-8, in DISC signalling remains unknown and is controversially discussed.

In this study, we elucidated the function of caspase-10 in DISC-induced cell death signalling and unexpectedly observed anti-apoptotic features under endogenous protein conditions. In contrast to previous thoughts, our data reveal that caspase-10 negatively regulates caspase-8-mediated cell death signalling in the DISC by blocking the recruitment to the complex and thereby the activation of caspase-8. Furthermore, we demonstrate that caspase-10 functions independent of cFLIP for inhibition of caspase-8 activation in the DISC. In addition, we show that caspase-8 does not compete with other tandem DED proteins such as cFLIP or caspase-10 in binding *via* FADD to the receptor as current models suggest. By utilising CRISPR-Cas9 mediated homologous recombination, we generated caspase-8 knockout cell lines and were able to demonstrate that caspase-8 has to be placed upstream of both cFLIP and caspase-10 in the DISC. We found that even FADD association with the DISC was drastically reduced in the absence of caspase-8. Interestingly, reconstitution of wild type caspase-8 and its active site mutant rescued the phenotype, indicating that caspase-8 is indispensable for the formation and/or stability of the DISC independent from its enzymatic activity. Moreover, we identified caspase-10 to promote DISC-mediated NF-κB activation. Caspase-10 favours at least the degradation of IκBα upon DISC stimulation resulting in enhanced NF-κB activation and inflammatory gene expression.

Therefore, our data are consistent with a model in which caspase-10 rewires DISC signalling to NF-κB activation and cell survival. As a consequence, caspase-10 and cFLIP co-ordinately regulate caspase-8-mediated cell death signalling whereas both proteins contrast in their ability to induce gene expression upon death receptor activation.

IV. Zusammenfassung

Todesliganden wie CD95L und TRAIL initiieren die extrinsische Apoptose durch die Aktivierung des „Death-Inducing Signalling Complex“ (DISC). Nach Bindung des Liganden an den Rezeptor wird das Adaptermolekül FADD zum Rezeptor rekrutiert und ermöglicht so die Bindung der Caspase-8 und seines negativen Regulators cFLIP. Neben der wohl bekannten, durch Caspase-8 vermittelten Zelltodinitiierung, stellt die durch NF-κB-Aktivierung induzierte Genexpression einen wichtigen Signalweg unterhalb des DISCs dar. Obwohl die Hauptbestandteile des DISCs beschrieben wurden, so sind die zugrundeliegenden molekularen Mechanismen, die zur Formierung des Komplexes führen, bisher nicht vollständig aufgeklärt. Des Weiteren ist die Funktion mancher DISC-assoziierter Proteine bisher unklar. Bis heute wird kontrovers diskutiert welche Rolle die Caspase-10, ein Homolog der Caspase-8, im Signalweg des DISCs einnimmt.

In dieser Studie haben wir die Funktion der Caspase-10 im Signalweg des DISCs aufgeklärt und überraschenderweise unter endogenen Bedingungen antiapoptotische Eigenschaften beobachtet. Im Gegensatz zum heutigen Wissenstand zeigen unsere Daten, dass Caspase-10 den Caspase-8 vermittelten Zelltod negativ reguliert, indem sie die Rekrutierung mit einhergehender Aktivierung der Caspase-8 zum Komplex blockiert. Des Weiteren demonstrieren wir, dass Caspase-10 auch unabhängig von cFLIP die Aktivierung der Caspase-8 im DISC inhibiert. Zusätzlich zeigen wir, dass Caspase-8 nicht mit anderen Tandem DED Proteinen, wie cFLIP oder Caspase-10, um die Bindung an FADD konkurriert, wie bisherige Modelle vermuten lassen. Indem wir die CRISPR-Cas9 vermittelte homologe Rekombination nutzten, haben wir Caspase-8 knockout Zelllinien generiert und konnten zeigen, dass Caspase-8 oberhalb von cFLIP und Caspase-10 im DISC bindet. Wir fanden heraus, dass selbst die Assoziation von FADD mit dem DISC ohne Caspase-8 drastisch reduziert wurde. Interessanterweise hob die Rekonstitution von Wildtyp-Caspase-8 und einer Mutante, in der das aktive Zentrum mutiert ist, den Phänotyp auf. Dies deutet darauf hin, dass Caspase-8, unabhängig von seiner enzymatischen Aktivität, unabdingbar für die Formierung und/oder Stabilität des DISCs ist. Zusätzlich zeigen wir, dass Caspase-10 die DISC vermittelte NF-κB-Aktivierung fördert. Caspase-10 unterstützt dabei mindestens die Degradation von IκBα nach DISC-Stimulation, wodurch NF-κB verstärkt aktiviert und inflammatorische Zytokine exprimiert werden.

Unsere Daten gehen daher mit einem Model einher, in dem Caspase-10 den DISC-Signalweg in NF-κB-Aktivierung und zelluläres Überleben umwandelt. Dadurch koordinieren Caspase-10 und cFLIP den Caspase-8-vermittelten Zelltod, jedoch unterscheiden sich die Proteine in ihrer Fähigkeit die DISC vermittelte Genexpression zu induzieren.

V. Introduction

The first observation of naturally occurring cell death dates back to the middle of the 19th century. In 1842, Carl Vogt has first described cell death in the development of the midwife toad [1]. Over a century passed until this topic was rediscovered in 1972 when Kerr, Wyllie, and Currie first described the programmed form of cell death as 'apoptosis' [2]. Since then, the number of publications dealing with apoptosis or programmed cell death grew exponentially [3]. Ultimately, Sydney Brenner, H. Robert Horvitz, and John E. Sulston have been awarded with the Nobel Prize in Medicine in 2002 for the identification of genes regulating apoptosis in the nematode *C. elegans* [4].

V.1. Apoptosis

Apoptosis usually occurs in multicellular organisms, but has been described in unicellular organisms, too [5]–[8]. It is most likely that programmed cell death first developed in unicellular organisms as a defense strategy and was later adapted by multicellular organisms [9]. In higher organisms, apoptosis has been described to be important for the embryonic development, aging, and homeostasis of tissues, as well as a defense strategy against damaged cells caused by various diseases or poisonous agents [10]–[12]. Programmed cell death plays an important role in the development of the immune system, where it controls the maturation of lymphocyte progenitor cells into T and/or B cells [13]. Deregulated apoptosis is associated with immunodeficiency, several autoimmune diseases, AIDS, and degenerative disease of the central nerve system [14]. Furthermore, cell death resistance is one of the early described hallmarks of cancer and is a major obstacle in the treatment of patients (15). Tumour cells develop a variety of strategies to escape from cell death induction. They usually do so by either up-regulating anti-apoptotic regulators or by silencing pro-apoptotic factors [16]. As a consequence, the tumour increases its mass, ensures survival of the population, and renders resistance to therapy.

However, under normal terms the apoptosis-inducing machinery is powerful and tightly controlled. When cell death is initiated, pro-apoptotic factors are activated and switch the well-orchestrated balance between pro- and anti-apoptotic regulators. Whenever a certain point of no return is reached, each dying cell shows characteristic morphological features while it undergoes apoptosis. At the early stage, the whole cell shrinks and is rounded because of the disruption of the cytoskeleton [17]. Now the organelles are tightly packed and the cytoplasm appears dense. Next, the cell undergoes an event which is called pyknosis [2].

During pyknosis the nuclei shrink in size and chromatin is condensed into a structureless and solid mass. Afterwards, the nuclear envelope becomes discontinuous. The DNA inside is fragmented in a process known as karyorrhexis and the nucleus breaks into several discrete pieces [18]. Moreover, the plasma membrane shows irregular blisters (an event which is known as blebbing) and, at the end, cellular fragments are separated into vesicles called apoptotic bodies. These bodies consist of cytoplasm and dense packed organelles. The integrity of the organelles is maintained in the apoptotic bodies and the plasma membrane is still intact [19]. The remaining debris of a dead cell is most typically eliminated by macrophages through phagocytosis [20], thereby preventing the activation of dendritic cells and further immune response [21].

V.1.1. Molecular mechanism

Apoptosis can be initiated by two main pathways: the intrinsic or mitochondrial pathway and the extrinsic so-called death receptor pathway. Additionally, cytotoxic T-cells and natural killer cells can induce apoptosis *via* the perforin/granzyme A/B pathway [22]. The apoptotic signalling pathway is driven by a family of cysteine aspartate-specific proteases called caspases [23]. Caspases are commonly expressed as an inactive proenzyme and they can cleave other proteins at aspartic acid residues [24]. Once activated, caspases induce a deadly cascade in which they often activate other caspases or even undergo autoactivation [19]. Thus far, 14 caspases have been described, but for classification solely the caspases 1-10 have been considered. However, caspases are subdivided into initiator (caspase-2, -8, -9, -10), effector (caspase-3, -6, -7), and inflammatory caspases (caspase-1, -4, -5) [25]. The other caspases have been shown to be involved in the regulation of apoptosis and cytokine maturation under septic shocks (caspase-11) and the mediation of endoplasmic-specific apoptosis and amyloid-β cytotoxicity (caspase-12) [26], [27]. Caspase-13 is a bovine specific caspase and caspase-14 is highly expressed in embryonic but absent in adult tissues [28], [29]. However, the apoptosis inducing machinery always results in the activation of caspase-3 and subsequent DNA degradation [19], with one exception: solely granzyme A is able to induce a caspase-independent apoptotic pathway resulting in single-stranded DNA damage [30].

V.1.1.1. The intrinsic signalling pathway

Diverse intracellular signals are able to initiate the intrinsic apoptotic signalling pathway. These include physico-chemical stresses such as chemotherapeutic agents (i.e. doxorubicin), DNA damage-inducing agents, and free radicals. Furthermore, the removal of nutrients, oxygen or growth factors, alterations in temperature and osmolarity, as well as pro-inflammatory cytokines can trigger the intrinsic signalling pathway [31]–[34]. These stress signals activate pro-apoptotic B-cell lymphoma-2 (Bcl-2) homology 3 (BH3)-only proteins [35] which in turn activate the Bcl-2-associated X protein (Bax) and Bcl-2 antagonist or killer (Bak) [36], [37], as shown in figure 1. Upon activation, Bax and Bak subsequently undergo conformational changes, resulting in the translocation of Bax to mitochondria. Furthermore, both proteins homo-oligomerise, thereby enabling the formation of pores in the outer membrane of mitochondria [38], [39]. This process is called mitochondrial outer membrane permeabilization (MOMP) and finally leads to the release of proteins from the intermembrane space (IMS) into the cytosol. The activity of Bax and Bak is largely controlled by other anti-apoptotic Bcl-2 family members to prevent spontaneous cell death induction [40]. After disruption of the mitochondrial membrane potential, cytochrome *c* is released into the cytosol [41]–[43]. Cytochrome *c* binds the apoptotic protease-activating factor 1 (APAF1), which thereby oligomerises and forms the so-called apoptosome. This complex recruits and activates the initiator caspase-9 which cleaves and further activates the executioner (also known as effector) caspases-3 and -7 [44]. Once activated, caspase-3 and -7 initiate the last phase of the apoptotic cascade. Endonucleases such as the caspase-activated DNase (CAD) are activated and cytokeratins, as well as nuclear and plasma membrane cytoskeletal proteins are cleaved, finally leading to the previously described morphological features of apoptosis [45], [46].

It is important to control the activity of the apoptosome as well as caspase-3 and -7 therewith cell death is not spontaneously induced. The main regulator in this process is the anti-apoptotic protein called X-linked inhibitor of apoptosis protein (XIAP). It regulates the activity of caspase-3, -7, and -9 by binding and thereby inhibiting these proteins [47]. The inhibitory function of XIAP is neutralized by the second mitochondria-derived activator of caspases (Smac; also known as DIABLO) and the serine protease HtrA2 (also known as OMI). Alike cytochrome *c*, both proteins are released from the IMS upon permeabilization of mitochondria and promote the intrinsic signalling cascade [48], [49].

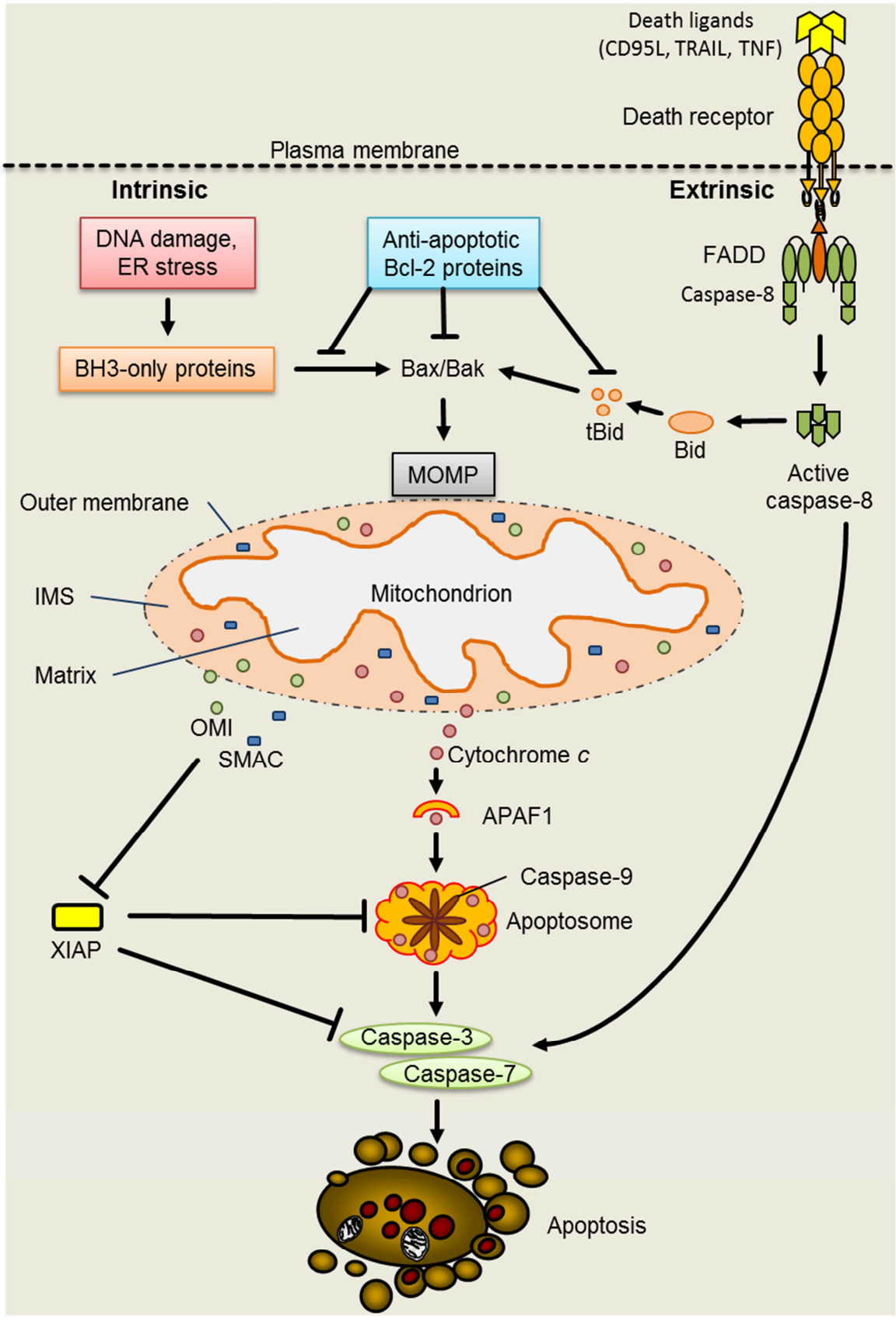

Figure 1 Molecular mechanisms of the intrinsic and extrinsic signalling pathways in apoptosis. The intrinsic pathway is induced *via* diverse intracellular stress signals that lead to MOMP, release of cytochrome *c*, formation of the apoptosome and effector caspase activation. Death ligands induce the extrinsic pathway by caspase-8 activation, which can either directly activate caspase-3 and-7 or induce MOMP by Bid cleavage.

V.1.1.2. The extrinsic signalling pathway

Formation of a membrane bound so-called death-inducing signalling complex (DISC) initiates the extrinsic apoptotic signalling cascade [50]. Therefore, death ligands such as the cluster of differentiation 95 ligand (CD95L; also known as FasL and Apo-1L), tumor necrosis factor (TNF), and TNF-related apoptosis inducing ligand (TRAIL) bind to their respective receptors. The receptors become activated leading to subsequent recruitment of Fas-associated protein with death domain (FADD). Homotypic interaction mediates the recruitment of the adaptor molecule FADD *via* its death domain to the cytosolic domain of the receptor [51]. Next, the initiator caspase-8 binds *via* its two death effector domains (DED) to the DED of FADD [52]. The large and small catalytic subunits of caspase-8 are cleaved and assemble the highly active caspase-8 homodimer. Upon activation, caspase-8 can directly activate the executioner caspase-3 and -7 [53] to induce further apoptotic events which were described above (Figure 1). Interestingly, some cell types, are incapable to propagate the signalling cascade from caspase-8 directly to caspase-3/-7 [54]. These cells are called type II cells and need in contrast to type I cells the circuit over mitochondria to trigger cell death signalling. The crosstalk between the extrinsic and intrinsic pathways is orchestrated by the pro-apoptotic Bcl-2 family member BH3 interacting-domain death agonist (Bid) [55]. Bid is activated by cleavage through active caspase-8 (Figure 1). Truncated Bid (tBid) then in turn induces conformational changes in Bax, leading to MOMP, the release of cytochrome *c* from mitochondria, the formation of the apoptosome, and the activation of caspase-3 and -7 [56].

The following chapters will focus on the extrinsic apoptotic signalling cascade, the initiation of cell death signalling by DISC formation and diverse signalling beyond the DISC.

V.2. TNF superfamily

Death ligands, such as TNF, TRAIL, and CD95L belong to the TNF superfamily. This superfamily consists of 19 different ligands and 29 receptors [57]. As the ratio of ligand to receptor indicates, some ligands solely interact with their respective receptor such as CD95L, but others are able to bind up to five different receptors, for instance TRAIL [58]. The ligands are mostly expressed as type II membrane proteins and organised as homotrimers, whereas the receptors are type I membrane proteins. Soluble forms of the ligands exists as well, which result from proteolytic cleavage of the membrane bound form or from alternative splicing [59]. The ligand trimer interacts with three receptor molecules as cross-linking and crystallographic experiments revealed [60]. Notably, ligands preferentially interact with preformed receptor trimers which assemble an N-terminal pre-ligand-binding assembly domain (PLAD) [61], [62].

Without any exception, all members of the TNF superfamily have pro-inflammatory activity which is mainly driven by the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). On top, some members have proliferative effects on hematopoietic cells, play a role in differentiation, and some function in the initiation of apoptosis [63]–[65]. Characteristic for the members of the TNF receptor superfamily is the presence of up to six copies of an extracellular cysteine-rich domain (CRD). The receptors can be further classified into two subclasses depending on the existence of an intracellular death domain (DD). This is a cytoplasmic, 45 amino acid long protein-protein interaction domain, which is essential for the induction of apoptosis [57], [66]. Interestingly, some receptors do not contain a DD, such as CD40 or TNF-receptor 2 (TNF-R2), but they are nonetheless able to induce apoptosis by transcriptionally up-regulating other death ligands [67].

V.2.1. CD95 and TRAIL receptors

As mentioned before, TRAIL interacts with five different receptors. These are the TRAIL receptors (TRAIL-R) 1-4 and osteoprotegerin (OPG). OPG does not preferentially bind TRAIL and it seems that OPG blocks rather than promotes the apoptotic potential of TRAIL [68]. All four TRAIL receptors contain 2 CRDs domains, but only TRAIL-R1 and -2 are able to transfer apoptotic signalling. The lack of a DD in TRAIL-R3 and the presence of a truncated form in TRAIL-R4 avoids apoptotic induction [69]. Whereas TRAIL-R1 can be activated by soluble as well as membrane-bound TRAIL, TRAIL-R2 can solely be efficiently activated by membrane-bound ligand [70]. Similar to TRAIL-R2, CD95 is only activated by its membrane-bound ligand. Even if soluble CD95L, which is cleaved by metalloproteases, exists under normal physiological conditions, it is not effective in inducing apoptosis [71]. However, it is possible to crosslink six CD95L monomers to form a hexameric protein which contains two CD95L trimers. The resulting ligand is highly efficient in inducing apoptosis even in its soluble form [72].

In contrast to TRAIL receptors, the CD95 receptor contains three CRDs in its extracellular tail [59], but both receptor types share analogical signalling machineries. TRAIL and CD95 receptors activate the apoptotic signalling in a similar manner. In the first step, the receptors assemble the PLAD domain to allow efficient ligand binding. Then the adaptor molecule FADD is recruited *via* its C-terminal DD to the DD of the receptors by homophilic interaction [73]. Now, caspase-8 can be recruited to the DISC by binding with its N-terminal DED to the likewise N-terminal DED of FADD. This protein-protein interaction is also driven by homophilic interaction [59]. Interestingly, DEDs and DDs share structural similarities; both domains consist of six conserved α-helices [74]. The DISC is composed of at least three death receptor molecules but it is most likely that the ligands and receptors form highly stable

supra-molecular clusters by their extracellular domains [75]. In addition, it has been shown that death receptors are able to form oligomeric structures with FADD *via* DD interaction [76]. These facts indicate that several caspase-8 molecules can be recruited to the DISC, are brought into close proximity, and can therefore be activated by dimerisation. Recent studies showed that the interaction of FADD and caspase-8 is even more complex as previously suggested. By utilising mass spectrometry, two independent studies showed that a single FADD molecule is able to recruit a multitude of caspase-8 molecules [77], [78]. Different caspase-8 molecules can assemble in chains by interactions between their DEDs after being recruited to the DISC. Thus, the recruitment of a single FADD molecule to the receptor is sufficient to induce autoprocessing of caspase-8 and further cell death signalling.

Mature caspase-8 protein consists of two N-terminal DEDs and a large (p18) plus a small (p10) catalytic subunit (Figure 2). Two different isoforms are expressed ('a' and 'b') that differ solely in a flexible linker region between the DEDs and the catalytic domain. If caspase-8 proform dimerises in the DISC, the mature protein is processed by autoproteolytic cleavage into the p18 and p10 subunits [79], [80]. As the initial step, the p10 subunit is cleaved from the proform. The resulting intermediate p43/41 fragment is still bound the DISC *via* FADD. It contains the two DEDs, as well as the p18 subunits and differs in size depending on the isoform of caspase-8 (Figure 3). Importantly, the p10 subunit is still associated with the p43/41 fragment by non-covalent interaction. In the next step, the p18 subunit is released from the intermediate fragment. Once these processing steps occurred at least in two caspase-8 molecules, two p18 and two p10 subunits assemble to form the highly active caspase-8 homodimer which is ultimately released from the DISC.

V.2.1.1. DISC regulation by cFLIP

DISC-mediated caspasse-8 activation is negatively regulated by the cellular FLICE (FADD-like IL-1β-converting enzyme)-like inhibitory protein (cFLIP). It is a caspase-like protein without enzymatic activity [81]. Several isoforms of cFLIP have been described but cFLIP-short ($cFLIP_s$) and cFLIP-long ($cFLIP_L$) are the best studied and most frequently detected in cellular systems. Both isoforms contain two N-terminal DEDs, but solely $cFLIP_L$ contains a caspase-like domain without protease activity. The short isoform consist solely of the two DEDs (Figure 2). Both isoforms are recruited to the DISC upon ligand stimulation and, similar to caspase-8, associates with FADD *via* the DEDs. In the DISC, cFLIP blocks caspase-8-mediated cell death signalling (Figure 3) [82]–[84]. Furthermore, knockout of cFLIP causes embryonic lethality in mice and even the transient knockdown in cell culture systems is reported to induce spontaneous cell death [85]–[87], indicating the indispensable role of cFLIP in regulating cell death processes.

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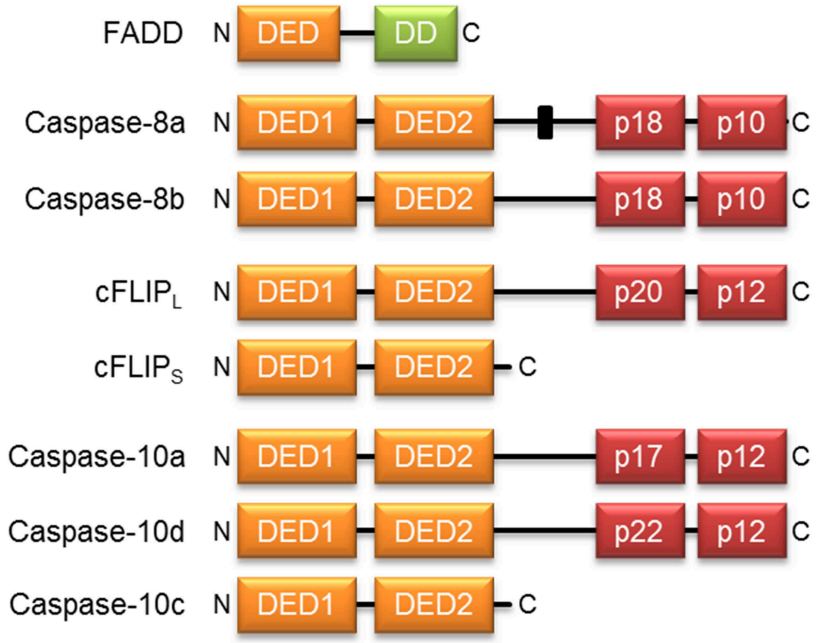


Figure 2 Structural organisation of the DED containing protein family members FADD, caspase-8, cFLIP, caspase-10, and their isoforms
DED: death effector domain; DD: death domain

Compared to caspase-8, cFLIP is less present in the DISC. Computational modelling in line with mass spectrometry data show that the ratio of caspase-8 to cFLIP molecules in the DISC is around 1:10 [88]. It appears that cFLIP regulates caspase-8 activation in the DISC through insertion into the caspase-8 DED chains, without terminating chain elongation [88]. While cFLIP$_s$ fully blocks processing and thereby activation of caspase-8 in the DISC, the role of cFLIP$_L$ in inhibiting caspase-8 is more complex and not fully elucidated to date. When overexpressed, cFLIP$_L$ is functionally blocking cell death [89]. Heterodimerisation between cFLIP$_L$ and caspase-8 with reduced proteolytic activity has been described. These heterodimers can process caspase-8 into the p43/41 and p10 fragment, however as they are not released from the DISC, they are not sufficient to induce apoptosis [90]. Furthermore, cFLIP$_L$ has independently been reported in several studies to have caspase-8 activating abilities [90]–[93]. In contrast, selective knockdown of solely cFLIP$_L$ promotes the formation of the DISC, as well as activation, processing, and release of caspase-8 [86]. Thus, endogenous cFLIP$_L$ inhibits caspase-8 activation in the DISC and further effector caspase-mediated cell death. However, presented data are highly controversial and it is still under debate whether cFLIP$_L$ has pro- or anti-apoptotic abilities. Nevertheless, it seems that caspase-8 has a broader function spectrum than solely inducing apoptosis. It appears that the caspase-8/cFLIP heterodimer in contrast to the caspase-8 homodimer has the ability to trigger non-apoptotic signalling [94], [95].

A multitude of other signalling proteins have been reported that are associated with the DISC or bind directly to the CD95 or TRAIL receptor. However, the function of these proteins in death receptor signalling is presently unclear and remains to be elucidated [96].

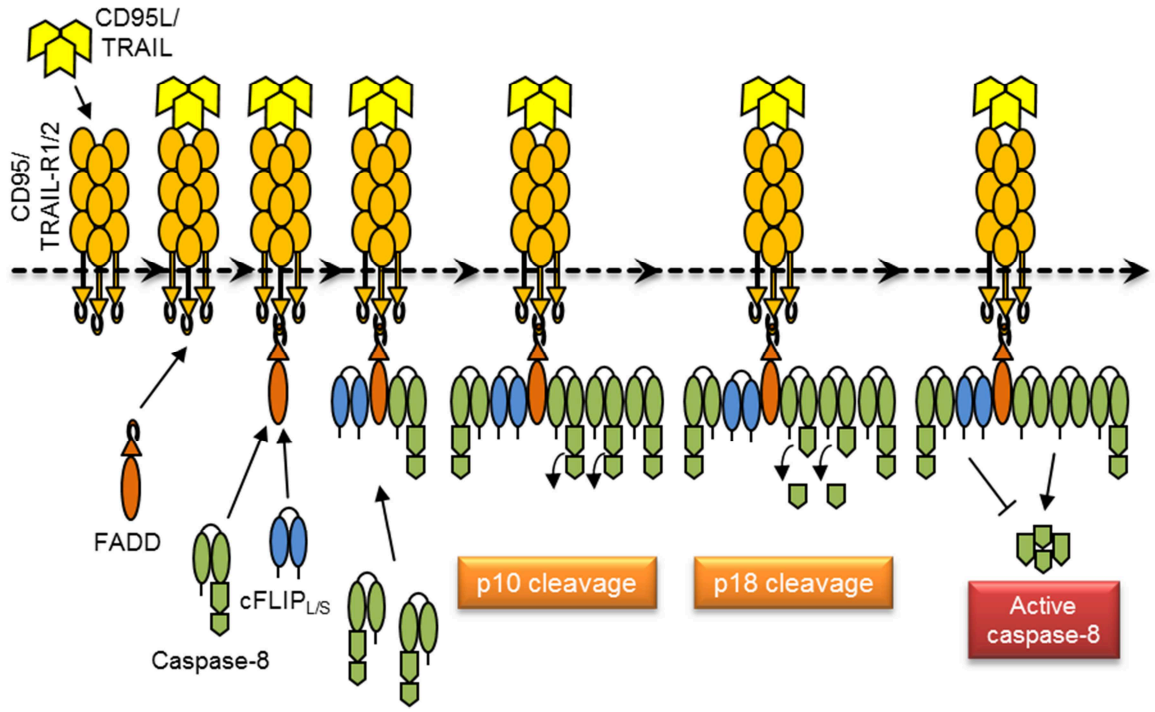


Figure 3 Caspase-8 activation and its regulation by cFLIP in the DISC. After ligand binding, the adaptor molecule FADD is recruited to the death receptor. Next, caspase-8 and its regulator cFLIP assemble the complex *via* FADD. Several caspase-8 molecules assemble the DISC by forming DED chains. Through their close proximity, p10 subunits of caspase-8 are autoprocessed and stay associated with the complex. Finally, the p18 subunits of caspase-8 are cleaved from the prodomain and assemble with the small subunits of the active caspase-8 homodimer. cFLIP$_{L/S}$ inhibit either the processing of caspase-8 (cFLIP$_S$) or build an inactive caspase-8-cFLIP$_L$-heterodimer.

V.2.1.2. Non-apoptotic cell death signalling of the DISC

The induction of apoptosis is the best characterised function of the CD95- and TRAIL-DISC. However, death ligand-induced formation of the DISC can trigger other signalling cascades leading to non-apoptotic outcomes (Figure 4). Recently, another form of programmed cell death, namely necroptosis, was discovered. This cell death mechanism lacks the morphological hallmarks of apoptosis [97]. Instead, it is a programmed form of necrosis and is driven by deregulation of the death domain-containing serine-threonine kinase RIPK1 (receptor-interacting protein kinases 1) and RIPK3 [98], [99]. This deregulation leads to the activation and oligomerisation of the pseudokinase mixed lineage kinase domain-like (MLKL) within the plasma membrane and thereby to the loss of membrane integrity [100].

Necroptosis is driven by a multiprotein complex named the ripoptosome. Complex formation can be induced by a variety of stimuli including genetoxic stress, Toll-like receptor (TLR) ligation, the depletion of cellular inhibitor of apoptosis proteins (cIAPs) which control the ubiquitination of RIPK1, and of course by death receptor stimulation [101]. Importantly, necroptotic cell death solely occurs if caspase activity is actively blocked by the use of synthetic or viral inhibitors [97].

V.2.1.3. Pro-inflammatory signalling of the DISC

As previously described, all members of the TNF superfamily harbour pro-inflammatory activity. Upon ligand stimulation, the CD95- and TRAIL-DISCs are able to induce the activation of NF-κB resulting in cytokine gene induction [102]–[106]. The molecular mechanisms rewiring apoptosis induction into cytokine production remain largely unknown, although it appears to act similar to the well-described TNF signalling pathway. Even if TNF is able to induce apoptosis in some cell lines, its deadly activity has a minor function *in vivo* compared to its gene inducing capacities [107]. Upon ligand binding to the TNF-R1, the TNF receptor type 1-associated DD protein (TRADD) is recruited to the complex by interacting with the DD of the receptor [108]. The adaptor protein TRADD represents an assembly platform to allow further recruitment of TNF receptor-associated factor 2 (TRAF2), RIPK1 and cIAP1/2 [109]. RIPK1 undergoes polyubiquitination in the complex, a reaction which is catalysed by TRAF2, cIAPs and the ubiquitin conjugating enzyme E2 D1 (UbcH5) [110]. TNFα-induced protein 3 (TNFAIP3) or better known as A20, is a zinc finger protein with deubiquitinase as well as E3 ubiquitin ligase domains. A20 regulates RIPK1 ubiquitination *via* interfering with cIAP1 and TRAF2 activity by antagonising the interaction with UbcH5 [111]. RIPK1 ubiquitination leads to the recruitment of the inhibitor of NF-κB kinase (IKK) complex to the receptor. The IKK complex consists of three components named IKK1, IKK2, and NF-κB-essential modulator (NEMO). Whereas IKK1 and -2 are catalytically active, NEMO is essential for the association of the IKK complex to the ubiquitin chains [112], [113]. In addition to the IKK complex, the TNF-R1 complex recruits another complex to the membrane *via* the ubiquitin chains. This complex consists of the transforming growth factor β-activated kinase 1 (TAK1), the TAK1 binding protein 1 (TAB1), and TAB2. TAK1 then mediates the activation of the IKK complex [114]. As this activation is usually not sufficient to fully propagate downstream signalling, the linear ubiquitin chain assembly complex (LUBAC), consisting of heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1L), HOIL-1L interacting protein (HOIP) and shank-associated RH domain-interacting protein (SHARPIN), is recruited to the ubiquitin chains in the receptor complex [115]. HOIP and HOIL catalyses ubiquitination of NEMO, leading to enhanced association and full activation of the IKK complex [116]. The activated IKK complex phosphorylates inhibitor of NF-κB (IκB) proteins, thereby marking them for proteasomal degradation [117]. IκB proteins inhibit NF-κB dimers by masking their

nuclear localisation sequence, thereby keeping them in an inactive state. The best studied member of this inhibitory family is IκBα. Upon activation of the TNF signalling pathway, IκBα is rapidly degraded leading to the release and nuclear translocation of NF-κB [118]. NF-κB induces the transcription of a variety of genes, which are mainly involved in immune response, inflammation, cell survival, differentiation, and proliferation [119].

The above described TNF-R1 complex which leads upon ligand engagement to the activation of NF-κB and subsequent gene induction is called complex I. It is important to dissect it from the TNF-R1 complex II which triggers the apoptotic signalling pathway and consists of RIPK1, TRADD, FADD, caspase-8, and cFLIP. In contrast to other death receptors, FADD is unable to directly bind the intracellular DD of TNF-R1 [120]. RIPK1 and TRADD have to be released from complex I to induce cell death. Deubiquitination of RIPK1, the inhibition of protein translation, and inhibition of cIAPs are possible factors to induce the release of RIPK1 and TRADD [121], [122]. Detached from the receptor, FADD is able to assemble the complex to further recruit caspase-8 and its regulator cFLIP [123]. Cell death initiation occurs similar to CD95L- and TRAIL-induced apoptosis. Caspase-8 is activated and transfers the signal either directly or *via* cleavage of Bid to effector caspases.

TNF-R1 signalling has been studied intensively in the past. Due to its complexity, many other proteins have been shown to be involved in the activation of NF-κB. Especially the importance of ubiquitin-editing to activate or regulate the different kinase complexes is currently under research. In addition to the above described canonical or classical NF-κB activation pathway, TNF is able to induce NF-κB by the non-canonical or alternative pathway [124]. The canonical NF-κB activation is a rapid process which occurs independent from protein synthesis. In contrast, the non-canonical NF-κB activation is time consuming and depends on protein synthesis [125]. Whereas stimulation of the TNF-R1 leads to canonical NF-κB activation, stimulation of the TNF-R2 activates the non-canonical pathway [124]. The central protein which propagates the signal from the TNF-R2 to NF-κB activation is the NF-κB-inducing kinase (NIK). TRAF2 and TRAF3 are associated with NIK and cIAPs under normal conditions and induce the cIAP-mediated polyubiquitination of NIK. This leads to the proteasomal degradation of NIK and prevents NF-κB activation [126]. Stimulation of the TNF-R2 leads to the degradation of cIAPs, TRAF2, and TRAF3, thereby preventing NIK from degradation [127]. NIK is now able to phosphorylate IKKα dimers which further activate NF-κB [128].

Many other facts increase the complexity of research on TNF signalling and NF-κB activation. For example, many stimuli have been described to induce either the canonical or the non-canonical NF-κB pathway, besides TNF [129]. On top, five NF-κB isoforms have been described to have differential gene-inducing possibilities [130]. Furthermore, TNF

signalling is known to activate gene transcription independent of the NF-κB pathway. Activation of mitogen-activated protein kinases (MAPK) signalling pathways, including p38, extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) have been reported upon TNF stimulation [107], [131]. These diverse signalling possibilities downstream the receptor indicate how complex this machinery is. However, it is not relevant for this thesis to dissect these pathways in more detail.

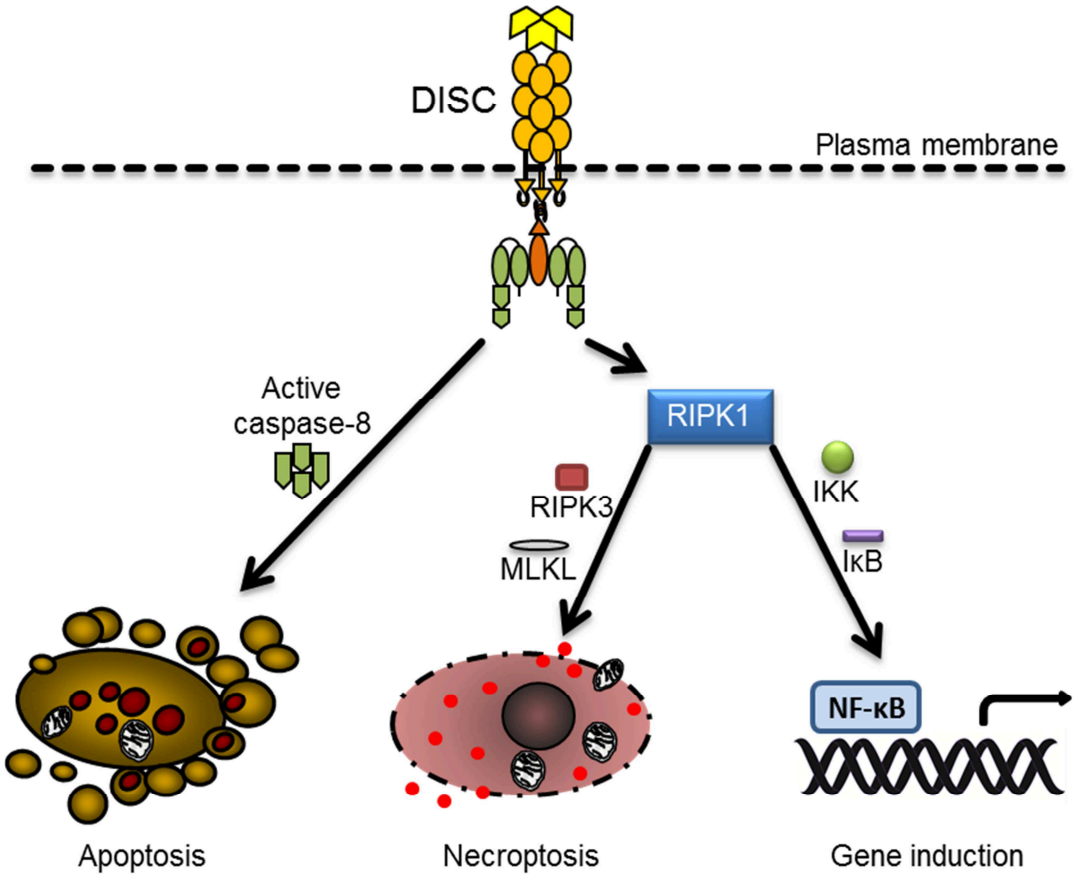


Figure 4 Diverse DISC-induced signalling pathways. Formation of the DISC mainly results in apoptosis through the activation of caspase-8. Once caspase activity is blocked, necroptotic cell death can be induced by the activation of RIPK1, RIPK3, and MLKL. Additionally, RIPK1 is able to induce the degradation of IκBα upon DISC stimulation resulting in NF-κB activation and subsequent gene induction.

As described above, the CD95- and TRAIL-DISCs are able to induce the activation of NF-κB as well. Even if the molecular mechanisms are not fully elucidated to date, it appears that RIPK1 has a central function in propagating the signal from the DISC to the IKK complex resulting in the degradation of IκBα and translocation of NF-κB to the nucleus [132]. It is thus far unknown how and especially where RIPK1 is activated upon DISC formation. However,

Cullen and colleagues showed that knockdown of RIPK1 fully abrogates NF-κB activation upon DISC stimulation. Interestingly, the association of RIPK1 to the DISC is in general very weak, but the blockade of caspase activity could drastically enhance the recruitment and binding of RIPK1 to the complex [132]. Upon death ligand stimulation, RIPK1 accumulates in a soluble complex containing caspase-8, FADD, and solely the long isoform of cFLIP [133]. Compared to DISC-bound protein levels, the relative amount of RIPK1 bound to this soluble complex is much higher and can again be enriched by caspase inhibitors [132]. Of note, cFLIP is not only inhibiting caspase-8 mediated cell death signalling, it is also reported to be a negative regulator of DISC-induced NF-κB activation [134]. It appears that DISC signalling functions in an opposite fashion compared to the TNF-R1 signalling. The TNF-complex mainly drives NF-κB activation and cytokine production through the receptor bound complex I and only induces apoptosis under some circumstances in the soluble complex II. In contrast to the TNF complex, DISC signalling favours cell death induction from the receptor (complex I) and activates NF-κB in a RIPK1-dependent manner *via* formation of a soluble complex (complex II).

V.3. Caspase-10

Another DED-containing protein which is involved in DISC signalling is caspase-10. This enzyme is a close homologue of caspase-8 with structural similarities and thought to be an initiator caspase as well. Like caspase-8, it contains two N-terminal DEDs plus a large and a small catalytic active subunit (Figure 2) [135]. Caspase-10 is a highly conserved caspase throughout evolution, but interestingly absent in rodents [136], [137]. Thus far, seven human caspase-10 isoforms (caspase-10a-g) have been described [138]. However, only the isoforms ‘a’, ‘d’, and ‘c’ can be detected under endogenous protein conditions in cellular systems. The other four isoforms have solely been described by polymerase chain reaction (PCR) or overexpression studies. Like caspase-8 and cFLIP, the caspase-10 gene is located on chromosome 2q33-q34 and alternative splicing results in the different isoforms [139]. Interestingly, despite the presence of two DEDs in all isoforms, only caspase-10a and –d are catalytically active. Whereas caspase-10c is structurally similar to $cFLIP_S$ and solely consists of the two DEDs and a small overhang, the isoforms ‘a’ and ‘d’ contain a large and small subunit (Figure 2). Both isoforms harbour an equal small p12 fragment, but differ in size of the large subunit. The isoform ‘a’ contains a p17 subunit, whereas caspase-10d is larger and contains a p22 subunit, instead.

Mutations in the caspase-10 gene are associated with an autoimmune lymphoproliferative syndrome (ALPS) type II [140]. This disease is characterised by abnormal lymphocyte and

dendritic cell homeostasis and defective immune regulation [141], [142]. The same clinical hallmarks can be observed in ALPS type I patients, but this disease is characterised by mutations in mainly CD95 or, less likely, its respective ligand [143], [144]. Mutations in caspase-10 which cause ALPS type II lead to diminished caspase activity and death receptor-induced apoptosis. Mutations in the caspase-10 gene seem to affect the enzymatic subunits and not the DED structure. Therefore, mutated caspase-10 is most likely recruited to the DISC and blocks caspase-8 processing by its decreased enzymatic activity [140].

A few reports have described the function of caspase-10 outside the DISC. Caspase-10 has been reported to be associated with the ripoptosome [98]; however, its function in this complex remains to be elucidated. In addition, caspase-10 has been described to be involved in an alternative intrinsic apoptotic pathway. After permeabilization of mitochondria, adenylate kinase 2 translocates into the cytosol and forms a complex with FADD and caspase-10 that triggers caspase-3 activation. Furthermore, caspase-10 has recently been described to be an inhibitor of autophagic cell death and removal of caspase-10 causes autophagy induction [145].

In general, the main function of caspase-10 seems to be related with DISC signalling. Caspase-10 has been shown to be recruited to the native TRAIL- as well as CD95-DISC [146]. Processing-dependent activation of caspase-10 in the DISC occurs analogue to caspase-8 activation. First, the small p12 subunit is cleaved *via* proximity-induced autoprocessing and remains afterwards associated with the complex. Next, the large p17/22 subunit is cleaved and finally two subunits of p12 and p17/22 are able to assemble the active caspase-10 homodimer [147]. Even if the substrate repertoire of caspase-8 and -10 is similar, the substrate specificities of both caspases vary. For example, caspase-10 is more efficient in cleaving RIPK1 compared to caspase-8. Interestingly, caspase-10 cleaves Bid at a distinct position in contrast to caspase-8, but it is still unknown which consequences differential Bid cleavage has for MOMP initiation upon death ligand stimulation [139]. Heterodimerisation of caspase-10 with $cFLIP_L$ has been reported, but its function is as controversial as caspase-8/cFLIP heterodimer formation. Interestingly, the caspase-10/cFLIP heterodimer is in contrast to the caspase-8/cFLIP heterodimer able to cleave Bid [148], [149]. Furthermore, heterodimers between caspase-8 and -10 have been observed by the use of chemical dimerisers [150]. The resulting heterodimers are most likely catalytically inactive and thus far it has not been ruled out if caspase-8 and -10 are able to build heterodimers under native conditions.

Studies analysing the functional substitution potential of caspase-10 for caspase-8 obtained conflicting results. Sprick and colleagues reported that caspase-10 is unable to functionally substitute for caspase-8 in cell death signalling [146], whereas others observed a functional

redundancy [147]. In general, the function of caspase-10 in DISC signalling is highly controversial. Most of the data base solely on overexpression experiments which putatively heavily derail the stoichiometry of the complex and lead to artificial results. However, the absence of caspase-10 in rodents and the homology to caspase-8 led to a kind of omission of research on this caspase.

VI. Material and Methods

All materials not listed below are commercially available analytical reagents or laboratory-grade materials.

VI.1. Materials

Table 1 Reagents and kits

Reagents and kits	Company	Catalog #
4-(2-Aminoethyl)-benzenesulfonyl Fluoride (AEBSF) Hydrochloride	AppliChem	A1421
Agarose Low Melt	Roth	6351.5
Ampicillin sodium salt	Roth	K029.1
Aprotinin	Roth	A162.3
BD OptEIA™ Human IL-8 Set	BD Biosciences	555244
BD OptEIA™ Reagent Set B	BD Biosciences	550534
Benzamidine	Sigma-Aldrich	12072-10G
Bis-Tris	Applichem	A1025,0500
Bovine Serum Albumin (BSA)	Santa Cruz	sc 2323A
Bromophenol blue	Sigma-Aldrich	B8026
Calcium chloride dihydrate	Sigma-Aldrich	C3306
cOmplete Protease Inhibitor Cocktail Tablets	Sigma-Aldrich	11836145001
Crystal violet	Applichem	A0691.0250
di-Sodium hydrogen phosphate dihydrate	Roth	4984.1
Deoxynucleoside triphosphate (dNTPs)	Thermo Scientific	R0193
Dithiothreitol (DTT)	Applichem	A2948.0025
E.Z.N.A. FastFilter Plasmid Maxi Kit	OMEGA	D6924-04
E.Z.N.A. Gel Extraction Kit	OMEGA	D2500-02
Ethanol denatured	Roth	K928.4
Ethanol ROTIPURAN	Roth	9065.3
FACS Shutdown solution	BD Biosciences	334224

FACSFlow Sheath Fluid	BD Biosciences	342003
FastDigest BamHI	Thermo Scientific	FD0054
FastDigest EcoRI	Thermo Scientific	FD0274
FastDigest HpaI	Thermo Scientific	FD1034
FastDigest NheI	Thermo Scientific	FD0973
FastDigest SwaI	Thermo Scientific	FD1244
GeneChip® Human Gene 2.0 ST Array	Affymetrix	902112
GeneJET Plasmid Miniprep Kit	Thermo Scientific	K0503
GeneRuler 1 kb DNA Ladder	Thermo Scientific	SM0312
GeneRuler 100 bp DNA Ladder	Thermo Scientific	SM0242
Glycerin ROTIPURAN	Roth	3783.2
iBlot 2 Transfer Stacks	Life technologies	IB24001
KAPA SYBR FAST Universal	Peqlab	07-KK4600-03
LB-Agar (Lennox)	Roth	X965.2
LB-Medium (Lennox)	Roth	X964.2
Leupeptin	Sigma-Aldrich	L2884
Luminata Forte Western HRP substrate	Merck Millipore	WBLUF0500
Methanol AnalaR NORMAPUR	VWR	20847.360
Microlance 3 27 G ¾''	BD	302200
MOPS for buffer solutions	AppliChem	A1076,1000
Nancy-520	Sigma-Aldrich	01494
NuPAGE Novex 4-12 % Bis-Tris Protein Gels	Invitrogen	NP0329BOX
Pacific Blue Annexin V	Biolegend	640918
PageRuler Prestained Protein Ladder	Life technologies	26617
Phusion High-Fidelity DNA Polymerase	New England BioLabs	M0530L
Pierce ECL Western Blotting Substrate	Thermo Scientific	32106
Potassium chloride	Roth	6781.3
Potassium dihydrogen phosphate	Roth	3904.1

Propidium iodide (PI)	Sigma-Aldrich	81845-100MG
Protein Assay Reagent A	Bio-Rad	5000113
Protein Assay Reagent B	Bio-Rad	5000114
Protein Assay Reagent S	Bio-Rad	5000115
Protein G Agarose	Roche	05015952001
Restore Western Blot Stripping Buffer	Thermo Scientific	21063
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen	10777-019
RNeasy Mini Kit	Qiagen	74106
SDS Pellets	Roth	CN30.3
Skim Milk Powder	Sigma-Aldrich	70166-500G
Sodium chloride (NaCl)	Roth	3957.2
Sodium citrate dihydrate	Sigma-Aldrich	W302600
Sodium hydroxide (NaOH) solution	Roth	KK71.1
Sodium orthovanadate	Sigma-Aldrich	S6508
SuperScript II Reverse Transcriptase	Invitrogen	18064-071
T4 DNA Ligase	Thermo Scientific	EL0016
Tris ultrapure	AppliChem	A1086,1000
Triton X-100	AppliChem	A1388,0500
Tween 20	Roth	9127.3
UltraPure DNase/RNase-Free Distilled Water	Invitrogen	10977-049
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich	G9422-10G
β-Mercaptoethanol	Merck Millipore	805740

Table 2 Cell culture reagents

Cell culture reagents	Company	Catalog #
(Z)-4-Hydroxytamoxifen	Sigma-Aldrich	H7904
AllStars Negative Control siRNA	Qiagen	1027281
Corning™ cell scraper	Sigma-Aldrich	CLS3010
Dimethyl sulfoxide (DMSO)	AppliChem	A3672,0100
DMEM, high glucose, GlutaMAX	Thermo Scientific	61965-059
DoxyHEXAL solution	Hexal	Pharmacy-only
DPBS, calcium, magnesium	Thermo Scientific	14040-174
Ethylenediaminetetraacetic acid (EDTA) (Versen)	Merck Millipore	L 2113
Fetal Bovine Serum (FBS)	Thermo Scientific	10270-106
Hepes solution	Sigma-Aldrich	H0887
Hygromycin B	A&E Scientific	P21-014
Lipofectamine 2000 Transfection Reagent	Invitrogen	11668-019
Necrostatin-1 (Nec)	Sigma-Aldrich	N9037-25MG
Opti-MEM I Reduced Serum Medium	Thermo Scientific	31985-047
Polyprene (Hexadimethrine bromide)	Sigma-Aldrich	H9268-5G
Puromycin Dihydrochloride	Thermo Scientific	A11138-03
RPMI 1640 Medium	Thermo Scientific	21875-091
Sodium Pyruvate	Thermo Scientific	11360-088
Sterile Cellulose Nitrate Membranes	GE Healthcare	10401170
TRIPZ Human CASP10 shRNA	GE Healthcare	RHS4696-200761700
TRIPZ Inducible Lentiviral Non-silencing shRNA Control	GE Healthcare	RHS4743
Trypsin 2,5%	Invitrogen	15090-046
Zeocin Selection Reagent	Thermo Scientific	R250-01
z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk)	Bachem	N15100025

Table 3 Stimulatory cytokines

Stimulatory cytokines	Source
CD95L-Fc	M. Feoktistova [133]
His-Flag-TRAIL (HF-TRAIL)	P. Diessenbacher [151]

Table 4 Antibodies

Target	Species/Feature	Application	Company	Catalog #
Primary Antibodies				
A20 (TNFAIP3)	Mouse IgG1	WB	Imgenex	IMG-161
APO-1 (CD95)	Mouse IgG3	IP	Provided by P.H. Krammer	
Caspase-10	Mouse IgG1	WB	MBL	M059-3
Caspase-8	Rabbit	WB	Millipore	04-574
Caspase-8	Mouse IgG2b	WB	Provided by P.H. Krammer	
Caspase-8	Goat	IP	Santa Cruz	sc-6136
CD95	Rabbit	WB	Santa Cruz	sc-715
CD95	Mouse IgG1	FACS	Provided by P.H. Krammer	
cFLIP	Mouse IgG1	WB	Enzo Life Science	ALX-804-961-0100
c-Jun	Rabbit	WB	Santa Cruz	sc-44
FADD	Mouse IgG1	WB	BD Biosciences	F36620
IκBα	Rabbit	WB	Santa Cruz	sc-371
JNK	Rabbit	WB	Cell Signaling	9252
p38	Rabbit	WB	Santa Cruz	sc-535
Phospho-IκBα	Rabbit	WB	Cell Signaling	9246
Phospho-JNK	Rabbit	WB	Cell Signaling	9251
Phospho-p38	Rabbit	WB	Cell Signaling	9215

Phospho-RIPK1 (S166)	Rabbit	WB	Provided by P. Gough	
RIPK1	Mouse IgG2a	WB	BD Biosciences	R41220
TRAIL-R1	Mouse IgG1	FACS	Biomol	AG-20B-0022-C100
TRAIL-R2	Mouse IgG1	FACS	Biomol	AG-20B-0023-C100
β-Tubulin	Mouse IgG1	WB	Sigma-Aldrich	T4026
Secondary Antibodies				
mouse IgG1	Goat/HRP	WB	SouthernBiotech	1070-05
mouse IgG2a	Goat/HRP	WB	SouthernBiotech	1080-05
mouse IgG2b	Goat/HRP	WB	SouthernBiotech	1090-05
rabbit	Goat/HRP	WB	SouthernBiotech	4030-05
rat	Goat/HRP	WB	SouthernBiotech	3030-05
goat IgG	Rabbit/HRP	WB	SouthernBiotech	6160-05
mouse IgG	Goat/Biotinylated	FACS	SouthernBiotech	1030-08
Tertiary Reagents				
Streptavidin	PE-Cy5	FACS	BD Bioscience	554062

VI.2. Buffer solutions:

PBS (pH 7.4)

2.7 mM KCl

1.5 mM KH_2PO_4

137 mM NaCl

8 mM Na_2HPO_4

Lysis buffer

30 mM TRIS-HCL (pH 7.5)

120 mM NaCl

10 % (v/v) Glycerol

1 % (v/v) Triton X-100

2 tablets cOmplete Protease Inhibitor/100 ml

5x Laemmli

250 mM Tris-HCl (pH 6.8)
10 % (w/v) SDS
50 % (v/v) Glycerol
0.1 % (w/v) Bromophenol blue

2x HBS buffer (pH 7.05)

280 mM NaCl
50 mM Hepes
1.5 mM Na_2HPO_4

Annexin buffer

10 mM Hepes
140 mM NaCl
2.5 mM $CaCl_2$

Hypotonic fluorochrome solution

0,1 % (w/v) Sodium citrate
0,1 % (v/v) Triton X-100
50 µg/ml Propidium iodide (PI)

Crystal violet dye

0.5 % (w/v) crystal violet
20 % (v/v) methanol

FACS buffer

1 % (w/v) BSA in PBS

Phospho-lysis buffer

20 mM Tris (pH 7,4)
137 mM NaCl
10 % (v/v) Glycerol
1 % (v/v) Triton X-100
2 mM EDTA
50 mM β-Glycerophosphate disodium salt hydrate
1 mM Na orthovanadate
1 mM AEBSF Hydrochloride
5 µg/ml Aprotinin
5 µg/ml Leupeptin
5 mM Benzamidine

VI.3. Software and devices

Table 5 Analysis software

Analysis software	Source
BD FACSDIVA Software	BD Biosciences
FCS Express V3	De Novo Software
GraphPad Prism 5	GraphPad Pris
Image J	National Institute of Health (NIH)
MxPro QPCR Software	Agilent
Primer3	Whitehead Institute for Biomedical Research
Wallac 1420 WorkOut Data Analysis software	PerkinElmer

Table 6 Devices

Devices	Company
Curix 60	AGFA
FACSCanto II	BD Biosciences
Gel iX20 Imager	INTAS
Mx3005P QPCR System	Agilent
NanoDrop 2000 Spectrophotometer	Thermo Scientific
T100 Thermal Cycler	BioRad
VICTOR^3 1420 Multilable Reader	PerkinElmer

VI.4. Methods

VI.4.1. Cell culture

All human cell lines used in this study (Table 7) were cultured in a humidified atmosphere at 37 °C and 5 % CO_2. Except Jurkat cells, the cell lines were grown in Dulbecco´s Modified Eagle Medium (DMEM) containing 4,500 mg/l glucose and 4 mM L-glutamine, supplemented with 1 % Hepes, 1 % sodium pyruvate, and 10 % heat inactivated fetal bovine serum (FBS), from here on referred as DMEM medium. Routinely tests were performed to exclude mycoplasma contaminations of the cell lines. Cells were grown in respective medium and further subcultured when they reached a confluence of 90-100 %. Therefore, cells were washed in PBS and detached from the cell culture dish using 2.5 % trypsin solution. By the addition of DMEM medium, trypsinisation was stopped and cells were resuspended until a homogeneous suspension was reached. The cell suspension was centrifuged for 5 min at 400 x g and room temperature (RT) to pellet the cells. The supernatant was removed and the cell pellet was resuspended in fresh medium. The dilution of the cells depended on the used cell line and the period of cultivation, but was in general between 1:1 and 1:15. Importantly, HaCaT cells were never diluted less than 1:5.

VI.4.1.1. Cultivation of Jurkat cells

Jurkat cells were grown in RPMI 1640 medium supplemented with 10 % FBS. As these cells grow in suspension, cells were splitted when they reached a confluence of 1 million cells/ml. Therefore, cells were collected and centrifuged for 5 min at 400 x g. Pelleted cells were resuspended in fresh medium and diluted in a ration between 1:1 and 1:10 depending on the period of cultivation.

Table 7 Cell lines

Cell line	Origin
HaCaT	Keratinocytes
HEK-293	Embryonic kidney cells
HeLa	Cervical carcinoma
Jurkat	T lymphocytes
MC	Melanoma
SK-Mel	Melanoma

VI.4.2. Polymerase chain reaction (PCR)

To amplify the respective DNA fragment, vectors already containing the gene of interest were used as a template. Plasmids containing caspase-10 constructs in pEF6/V5-His-TOPO were kindly provided by Martin Sprick, and Marion McFarlane kindly provided us with caspase-8

constructs in pcDNA3.1 [94]. Specific forward and reverse primer pairs were designed (Table 8) for each PCR which were complementary to the template DNA and had an equal melting temperature (T_m). Thereby, each primer contained the respective restriction site for later ligation with the plasmid.

A master mix with a final volume of 50 µl was used for the PCR. It contained 0.01 µg template DNA, 0.2 mM dNTPs, 0.5 µM of each primer, 1 U Phusion High-Fidelity DNA Polymerase, and 10 µl 5x reaction buffer (New England Biolabs).

The PCR program was carried out as follows:

- 98 °C for 3 min

30 cycles of:

- 98 °C for 10 s
- T_m+3 °C for 30 s
- 72 °C for 45 s

Final extension:

- 72 °C for 10 min

The samples were kept at 4 °C for storage to exclude nuclease activity.

Table 8 PCR primers

Primer	forward	reverse	T_m [°C]	Restriction site
Caspase-10 in pCFG5-IEGZ	ACTGGAATTCATGAAATCTCAAGGTCAACA	CCGCATTTAAATCTATATTGAAAGTGCATCCA	58	EcoRI SwaI
Caspase-10 in pF 5 × UAS W SV40 Puro	GCGAGGATCCATGAAATCTCAAGGTCAACA	GCGTGCTAGCCTATATTGAAAGTGCATCCA	62	BamHI NheI
Caspase-8 in pF 5 × UAS W SV40 Puro	GTCAGCTAGCATGGACTTCAGCAGAAATCT	GCCGGTTAACTCAATCAGAAGGGAAGACAA	62	NheI HpaI

To visualize and separate the amplified DNA fragments, samples were run on agarose gels containing 0.5x Nancy-520 (Sigma-Aldrich) and exposed to UV light. Successfully amplified samples were cut out from the gel and purified using the E.Z.N.A. Gel Extraction Kit (Omega) according to manufacturer´s instructions and eluted in DNase free water.

VI.4.3. Restriction digest and ligation

The PCR DNA fragments and the corresponding empty vectors (pCFG5-IEGZ kindly provided by B Baumann; pF 5x UAS MCS W SV40 Prom. kindly provided by J. Silke) were digested by restriction enzymes. Therefore, a master mix of 20 µl was used. It contained 10-30 µg of the PCR DNA fragment or 1.5 µg plasmid, 1 µl of each FastDigest restriction enzyme (Table 8), and 2 µl 10x reaction buffer (Thermo Scientific). The samples were incubated for 15 min at 37 °C and cleaned up *via* agarose gel electrophoresis as described above.

To combine the digested DNA fragments and plasmids, a master mix of 10 µl was used. Therefore, 1 µl plasmid, 5-7 µl DNA fragment, 1 µl T4 DNA ligase, and 1 µl ligation buffer (Thermo Scientific) were incubated over night at 15 °C.

VI.4.4. Transformation and plasmid isolation

To introduce the ligated plasmids into chemically competent TOP10F *E. coli* (Invitrogen) , the whole ligation mixture was incubated together with 100 µl bacteria on ice for 30 min. Afterwards, cells were heat shocked for 45 s at 42 °C and rested again for 2 min on ice. Next, 500 µl lysogeny broth (LB) medium was added and the samples were further incubated gently shaking for 1 h at 37 °C. Transformed bacteria were plated on pre-warmed LB agar plates containing 100 µg/ml ampicillin and incubated over night at 37 °C. Single colonies were picked and transferred into new reaction tubes containing approximately 5 ml LB medium and 100 µg/ml ampicillin. The tubes were again incubated over night at 37 °C. Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to manufacturer´s instructions. The constructs were verified by restriction digest and sequencing. Successfully generated plasmids were again transformed into bacteria as described above and plated on LB agar plates. Single colonies were picked and incubated over night at 37 °C in a sterile Erlenmeyer flask containing 200 ml LB medium supplemented with 100 µg/ml ampicillin. Plasmids were isolated using the E.Z.N.A Fastfilter Plasmid Maxi Kit (Omega) according to manufacturer´s instructions. Plasmids were eluted in 1.5 ml DNase free water and analysed with a NanoDrop 2000 spectrophotometer. Solely samples complying with the quality recommendations (260 nm/ 280 nm ratio: 2.0 +/- 0.2; 260 nm/ 230nm ratio: 2.0 +/- 0.2) were further used.

VI.4.5. Transfection and transduction

To generate viral particles, cells were seeded at various concentrations to receive a confluence of 50-60 % at the day of transfection or transduction.

VI.4.5.1. Generation of retroviral particles

To generate retroviral particles, the retroviral helper cell line Phoenix-AMPHO which originates from HEK-293 cells was cultured in 10 cm dishes. Cells were transfected with the pCFG5-IEGZ vector [152], which constitutively overexpresses the gene of interest, by calcium phosphate transfection. Therefore, 20 µg DNA was diluted in in a total volume of 500 µl containing 250 mM $CaCl_2$. 500 µl 2x HBS buffer were prepared in a separate tube. By carefully blowing air inside the HBS buffer to increase the surface and the oxygen uptake, the DNA $CaCl_2$ solution was dropwise added into the mixture. The combined solution was incubated for 25 min at RT. In the meanwhile, medium was removed from the cells and 5 ml fresh medium containing 25 µM chloroquine was added. The transfection solution was dropwise added to the cells and they were incubated at 37 °C (5 % CO_2). On the next day, medium containing retroviral particles was collected, filtered (0.45 µm), shock frozen in liquid nitrogen, and stored at -80 °C. Fresh medium was added to the cells for a second collection of viral supernatants on the next day. All steps described were carried out according to the safety class two requirements.

VI.4.5.2. Generation of lentiviral particles

HEK-293 cells were cultured in 10 cm dishes to generate lentiviral particles *via* the 2nd generation packaging system. Cells were cotransfected with 3 µg pMD2.G, 7.5 µg pSPax2, and 3 µg pcDNA3.1/p35 of lentiviral packaging vectors together with 10 µg of the transfer vector. To inducible overexpress the gene of interest the transfer vector pF 5 × UAS W SV40 Puro [153] which expresses the gene of interest in a Gal4-dependent fashion was used. For inducible shRNA expression, the pTRIPZ lentiviral shRNAmir system was applied. Calcium phosphate transfection and collection of viral supernatants was carried out as described above.

VI.4.5.3. siRNA-mediated knockdown

For transient knockdown experiments the following siRNA duplexes were used: FlexiTube siRNA for caspase-8 (Hs_CASP8_11), caspase-10 (Hs_CASP10_8, Hs_CASP10_9, Hs_CASP10_10, and Hs_CASP10_11), cFLIP (Hs_CFLAR_9) and the respective control siRNA (AllStars Negative Control siRNA 1027281). All siRNA preparations were from QIAGEN. For transient transfection 2x10^5 cells per well were seeded in a 6-well plate and incubated over night. Prior to transfection, cells were incubated with Opti-MEM medium for 20 min followed by transfection according to the manufacturer´s recommendations using Lipofectamine 2000 and the respective siRNA species. In case of single cFLIP knockdown, the molarity of cFLIP siRNA was reduced to 500 pM (HeLa) and 1 nM (HaCaT).

VI.4.5.4. Generation of stable cell lines

To generate stable cell lines, viral particles were added to the cells containing 5 µg/ml polybrene and cells were spin-infected for 1.5 h at 30 °C. The day after, cells were at least washed 3 times and successfully transduced cells were selected using antibiotic resistance. Therefore, cells were cultured in medium containing the lowest concentration of antibiotics which was sufficient to kill 100 % of untransduced control cells (Table 9). All steps were carried out according to the safety class two requirements until the cells were washed at least 6 times in virus-free medium after transduction.

Stable cell lines, inducible overexpressing the gene of interest by addition of 4-Hydroxytamoxifen, were transduced with a virus containing the pF GEV16 Super PGKHygro vector [153] prior to transduction with pF 5 × UAS W SV40 Puro.

Table 9 Antibiotics used for selection

Vector	Antibiotic	Concentration	Time [d]
pCFG5-IEGZ	Zeocin	3 µl/ml	10-20
pF 5 × UAS W SV40 Puro	Puromycin	1 µg/ml	3-7
pF GEV16 Super PGKHygro	Hygromycin B	300 µg/ml	5-10
pTRIPZ	Puromycin	1 µg/ml	3-7

VI.4.6. Generation of knockout cell lines

Caspase-8 deficient HeLa cells were kindly generated by Tencho Tenev (Pascal Meier lab, ICR, London) using the CRISPR-Cas9 system. Therefore, HeLa cells were seeded in a 24-well plate and transiently cotransfected with the pMA-T vector (carrier of the cassette-U6-gRNA(casp8)-TTTTT; Life technologies) and hCas9-pcDNA3.3-TOPO (Addgene) by the use of Lipofectamine LTX Reagent with PLUS™ Reagent (Thermo Fischer Scientific) due to manufacturer´s recommendations. The following gRNA sequences were used:

Casp8-1: GCCTGGACTACATTCCGCAA
Casp8-2: GCTCTTCCGAATTAATAGAC

Two days after transfection, cells were sorted with a BD FACSAria I (BD Bioscience) and plated as single clones in 96-well plates. Arising clones were cultured for 2-3 weeks and analysed for successful caspase-8 knockout by Western blotting.

VI.4.7. Immunoblotting

Cells were collected by trypsinisation to extract proteins. Pelleted cells were washed in PBS and lysed in lysis buffer for 1 h on ice. The samples were centrifuged for 30 min at 20,000 x g

and 4 °C to remove cellular debris. Protein concentration was measured using the DC™ Protein Assay (BioRad) according to manufacturer´s instructions.

For Western blotting, protein lysates were prepared in Laemmli buffer under reducing conditions (5 % DTT (1M)) at a concentration of 0.5-2 µg/µl and boiled for 10 min at 96 °C. Samples were loaded on NuPAGE 4-12 % BisTris gels (Invitrogen) and proteins were separated according to manufacturer´s protocol using MOPS buffer. To control the molecular weight and the separation of the proteins PageRuler Prestained Protein Ladder (Life Technologies) was loaded on each gel.

Proteins were transferred on polyvinylidene difluoride (PVDF) membranes using the iBlot 2 Transfer Stacks (Life Technologies) according to manufacturer´s instructions. The membranes were blocked with 5 % non-fat dried milk in PBS + 0.1 % Tween 20 (from here on referred as PBST) for 1 h at RT. Primary antibodies were diluted in 5 % milk in PBST or 5 % bovine serum albumin (BSA) in PBST with the respective concentrations (Table 10). Membranes were incubated gently shaking over night at 4 °C with the primary antibodies. Membranes were brought to RT and washed four times in PBST. The respective secondary antibodies were diluted 1:10,000 in 5 % milk in PBST and incubated with the membranes for 1 h at RT. Membranes were washed three times in PBST and once in PBS and signals were visualised depending on the strength of the signal using Pierce ECL Western Blotting substrate (Thermo Scientific) or Luminata Forte Western blot HRP (Millipore) and the Curix 60 (AGFA) developing machine.

VI.4.7.1. Detection of phospho-proteins

To analyse the phosphorylation state of the protein of interest, cells were harvested as described above. The washed cell pellet was lysed by the use of a special lysis buffer containing phosphatase inhibitors (phospho-lysis buffer). Therefore, phospho-lysis buffer was added to the cells and they were quickly homogenised by pushing the cells three times through a 0.4 mm Microlance needle (BD). The samples were then centrifuged and further processed exactly as described above, with the exception that the PVDF membranes were blocked in PBST containing 5 % BSA.

VI.4.8. Immunoprecipitation

For precipitation of the CD95-DISC $1.5x10^7$ cells were used for each condition. Cells were treated with the indicated concentrations of CD95L-Fc for the indicated times. Afterwards, cells were washed three times in ice-cold PBS and scraped off using a cell scraper. Cells were lysed in 1 ml lysis buffer and incubated gently shaking for 45 min at 4 °C. Lysates were centrifuged at 20,000 x g for 5 min and 4 °C. A minor fraction was used to determine the respective protein concentration as described above. Therefore, a total protein amount of

1-2.5 mg protein was used and each lysate was set to the same concentration. To precipitate the CD95 receptor and its associated proteins, 1.5 µg Apo-1 IgG3 antibodies (kindly provided by P.H. Krammer) were added to each lysate. 40 µl protein G beads (Roche) were used to precipitate the complexes. Samples were incubated gently shaking for 16-24 h at 4 °C. Beads were washed 4 times with ice cold lysis buffer before the protein complexes were eluted from dried beads by addition of reducing Laemmli buffer. Sample preparation and detection of respective proteins were carried out as described above.

A second precipitation of caspase-8 was subsequently prepared after depleting CD95 from the lysate. Therefore, supernatants were carefully removed from the protein G beads after CD95 precipitation and incubated with 1 µg caspase-8 antibodies (Santa Cruz) for 16-24 h at 4 °C followed by precipitation as described above.

Table 10 Antibodies for Western blotting

Antibody	Dilution	Diluent
A20	1:1000	Milk
Caspase-10	1:2000	Milk
Caspase-8 (C-term.)	1 µg/ml	Milk
Caspase-8 (N-term.)	1:1000	Milk
CD95	1:1000	Milk
cFLIP	1:100	Milk
c-Jun	1:1000	Milk
FADD	1:1000	Milk
IκBα	1:1000	Milk
JNK	1:1000	Milk
p38	1:1000	Milk
Phospho-IκBα	1:1000	BSA
Phospho-JNK	1:1000	BSA
Phospho-p38	1:1000	BSA
Phospho-RIPK1	1:1000	BSA
RIPK1	1:2000	Milk
β-Tubulin	1:5000	Milk

VI.4.9. Quantification of specific proteins

To quantify specific proteins, respective protein bands were densitometrically analysed with *ImageJ* software. Therefore, non-saturated exposures of blots were used. Protein band

intensity was calculated in respect to either β-Tubulin as the loading control or to precipitated CD95 as control of the immunoprecipitation.

VI.4.10. RNA isolation and reverse transcription

Total RNA isolation from human cell lines was performed using the RNeasy Mini Kit (Quiagen) according to manufacturer´s instructions. In brief, cells were washed in PBS and lysed in RLT buffer containing 1 % β-mercaptoethanol. RNA was extracted using spin column purification. The samples were washed twice and RNA was eluted using RNase free water.

RNA quality and concentration was measured using a NanoDrop 2000 spectrophotometer as described above.

1.5 µg RNA of each sample was used for the reverse transcription reaction. Therefore, RNA was diluted in RNase free water to a final volume of 9.8 µl. 1 µl 10 mM deoxynucleoside triphosphate (dNTP), 1 µl 100 µM random nonamers and 0.2 µl 100 µM oligo dT primer were added to the sample. The probes were incubated in a thermocycler for 5 min at 65 °C. Afterwards, 4 µl 5x First Buffer, 2 µl 0.1 M DTT, 1 µl RNase out (Life Technologies), and 0.7 µl Superscript II (Life Technologies) was added into the reaction tube. The samples were further incubated in a thermocycler for 2 min at 42 °C, 12 min at 25 °C, 50 min at 42 °C, and finally 15 min at 70 °C. Before the use, cDNA was diluted to a final concentration of 10 ng/µl.

VI.4.11. Quantitative real time polymerase chain reaction (qPCR)

Primers (Table 11) used for this study were designed using Primer3 software. qPCR experiments were performed in triplicates, each having a final volume of 20 µl, using the KAPA SYBR FAST Universal master mix (Peqlab) and a Mx3005P QPCR System (Agilent). 100 ng cDNA per sample was used for each condition. Equal cycling conditions were used to amplify the genes of interest.

The qPCR program was set as follows:

- 15 min at 95 °C

followed by 42 cycles of:

- 95 °C for 15 s
- 55 °C for 30 s
- 72 °C for 30 s

Melting curve analysis was used to confirm the specific amplification of a single product of the expected size for each gene. Furthermore, all primers used for qPCR studies have been

tested before by cDNA dilution curves to show an efficiency within 80-110 %. In addition, all samples were tested to have a low variance in the housekeeping gene (*Gapdh*) expression (< 2 cycles). Serial dilutions of cDNA (1, 1/5, and 1/25) were amplified for the construction of a standard curve and used for the estimation of the pPCR efficiency using MxPro software. The relative quantification was calculated after dividing the standard curve value of the respective genes by that of the housekeeping gene (*Gapdh*) for each individual sample using Excel and GraphPad Prism 5.

Table 11 qPCR primer

Primer	Forward	Reverse
IL-8	CACCCCAAATTTATCAAAGA	ACTGGCATCTTCACTGATTC
TNF	TCAGATCATCTTCTCGAACC	TGGTTATCTCTCAGCTCCAC
GAPDH	CCTGGTATGACAACGAATTT	AGTGAGGGTCTCTCTCTTCC
Caspase-10	AGAAGGCATTGACTCAGAGA	CTCCAGGCATGTCAGATTAT

VI.4.12. Cell death quantification by different assays

To induce cell death, constructs for expression of CD95L-Fc [154] (kindly provided by P. Schneider, Switzerland) and His-FLAG-TRAIL (HF-TRAIL) [151] were used. 1 U of CD95L-Fc was determined as 1 U/ml supernatant that was sufficient to kill 50 % (LD50) of parental HeLa cells within 16-20 h. Ligand-mediated cell death was fully blocked by addition of soluble CD95-Fc or TRAIL-R2-Fc protein.

VI.4.12.1. Crystal violet staining

Crystal violet staining of attached living cells was performed after stimulation with the indicated concentrations and time points of death ligands. Therefore, 1-1.5x10^4 cells were seeded in triplicates per condition in 96-well plates. Cells were treated with the respective inhibitors and death ligands as indicated. Medium was removed and cells were carefully washed with PBS. After removing the PBS, plates were put upside down on a tissue to remove remaining liquids. 50 µl 0.5 % crystal violet dye was added into each well and the plates were incubated on a shaker for 20 min at RT. Plates were carefully washed with water until unbound crystal violet was removed, put upside down on a tissue, and dried at least for 2 h at RT to remove remaining liquids. 200 µl methanol was added per well to dissolve the crystal violet and plates were incubated for 20 min on a shaker at RT. Optical density of the samples was analysed with a VICTOR^3 1420 Multilable Reader and optical density of control conditions (cells treated with diluents) was normalised to 100 % to allow comparison of independent experiments. Calculations were performed using Excel and graphs were visualised in GraphPad Prism 5.

VI.4.12.2. Nicoletti staining

HeLa cells were seeded in 6-well plates and treated with CD95L-Fc as indicated. Medium, containing dead cells, was collected and cells were washed in PBS. The PBS was collected as well and cells were harvested by trypsinisation. The medium, PBS, and the cell solution were combined and the samples were centrifuged for 5 min at 400 x g. The cell pellet was washed twice in PBS and carefully resuspended in 0.5 ml hypotonic fluorochrome solution containing 50 µg/ml PI. The samples were stored at 4 °C in the dark for 48 h. Afterwards, samples were again carefully resuspended and finally analysed by flow cytometry using a FACSCanto II (BD Biosciences) and the BD FACSDIVA software. Visualisation of the results was done using FCS Express V3 software and summary of independent experiments was carried out using GraphPad Prism 5.

VI.4.12.3. Annexin V staining

To analyse the externalisation of phosphatidylserine, $1x10^5$ HeLa cells were seeded per well in a 6-well plate and stimulated with CD95L-Fc as indicated. Cells were collected by trypsinisation, washed twice in PBS, and once in Annexin buffer. The cell pellet was resuspended in 800 µl Annexin buffer. 5 µl Pacific Blue Annexin V (Biolegend) was added to 100 µl of the cell suspension. Samples were vortexed and incubated for 15 min at RT in the dark. 400 µl Annexin buffer was added to the samples before they were analysed *via* flow cytometry and the FACSCanto II as described above.

VI.4.13. Enzyme-linked immunosorbent assay (ELISA)

To measure the concentration of IL-8 in the supernatant of CD95L-Fc stimulated HeLa cells, the BD OptEIA™ Human IL-8 Set (BD Biosciences) was used. Therefore, $6x10^4$ cells were seeded per well in 24-well plates. Cells were treated with starvation medium (0.5 % FBS) containing CD95L-Fc concentrations as indicated for 24 h. Supernatants were collected, centrifuged for 5 min at 400 x g to get rid of dead cells and diluted 1:10 for further use. The ELISA was performed according to manufacturer´s instructions. In brief, 96-well plates were coated with capture antibody, blocked with assay diluent, standard and samples were added in duplicates, detection antibody was added, and enzyme reaction was applied. The plates were washed multiple times between the individual step using a multichannel pipette. The plates were finally analysed by absorbance measurement using the VICTOR^3 1420 Multilable Reader and the Wallac 1420 WorkOut Data Analysis software.

VI.4.14. Receptor expression staining

HeLa cells were cultured in 6-well plates and harvested by trypsinisation. Cells were pelleted and resuspended in fresh medium. $2x10^5$ cells were dissolved in 100 µl FACS buffer and incubated with 10 µg/ml of the respective primary antibodies (anti-CD95, anti-TRAIL-R1, and anti-TRAIL-R2) for 1 h at 4 °C. Next, cells were washed in FACS buffer and resuspended in

100 µl FACS buffer containing 1 µl of the secondary antibodies (anti-mouse IgGI-Biotin). Samples were incubated for 30 min at 4 °C and again washed in FACS buffer. Then, 100 µl FACS buffer containing 1 µl of the tertiary staining reagent (PE-Cy5 Streptavidin) was added to the cells and the samples were incubated for 30 min at 4 °C. Cells were again washed with FACS buffer, dissolved in 500 µl FACS buffer, and measurement was performed *via* flow cytometry and the FACSCanto II as described above.

VI.4.15. Microarray analysis

HeLa cells were seeded in 6-well plates and respective shRNA expression was induced by the addition of 0.5 µg/ml doxycycline for 72 h. In three independent experiments, cells were pre-starved for 4 h in starvation media followed by zVAD-fmk treatment (10 µM) for 1 h. Afterwards, cells were stimulated with 0.1 U/ml CD95L-Fc for 3 h. Total RNA from stimulated and control cells was isolated as described above. The following steps were kindly performed by the group of Carsten Sticht (ZMF, Mannheim). In brief, RNA was tested by capillary electrophoresis on an Agilent 2100 bioanalyzer (Agilent) and high quality was confirmed. Gene expression profiling was performed using arrays of human Hugene-2_0-st-type (Affymetrix) according to manufacturer´s instructions. Bioinformatic evaluations were done as previously described [155]. Significant regulated genes (adjusted p values (FDR) < 0.05) were considered by a log2 fold change > 1 compared to unstimulated control cells. The complete data set is available in the GEO database (http://www.ncbi.nlm.nih.gov/geo; GSE number: GSE75365)

VI.4.16. Statistical analysis

Statistical analysis was carried using GraphPad Prism 5. Statistical significance (p values) was analysed using paired Student´s *t*-test and assumed for p values < 0.05.

VII. Aims of the thesis

The discovery of the extrinsic apoptosis pathway enabled new possibilities in the treatment of various diseases, including cancer. First clinical trials using death ligands to target malignant cells showed inauspicious results. It ruled out that primary cells are usually more sensitive to death stimulations than their malignant counterpart. Indeed, most tumour cells develop resistance mechanisms to undergo cell death induction by either extrinsic or intrinsic stimuli. Nevertheless, there is also evidence that death ligands promote tumour growth even if these cells are fully resistant to cell death. Furthermore, many autoimmune diseases have been linked to deregulations in the extrinsic signalling pathway. It is of high relevance to understand the molecular mechanism undergoing cell death induction by death ligands to generate potential therapies for patients.

In the last years, many studies showed that the DISC is much more complex than previously thought. However, the processes regulating the diverse DISC signalling pathways are poorly understood. Especially caspase-10 remains to be a mystery in the DISC. Its function has solely been analysed by overexpression studies and it is still controversial if it can substitute for caspase-8. Its absence in rodents exacerbates research on it but may be highly important for the differences between species.

Therefore, this thesis aims to address the following outstanding questions:

- **What is the general function of caspase-10 in DISC-induced cell death signalling and gene expression?**

- **How are the three tandem DED proteins caspase-8, caspase-10, and cFLIP interconnected in propagating the signal downstream the death receptor?**

By utilising overexpression, knockdown, and knockout approaches in several human cell lines, this thesis examines the surprising function of caspase-10 in rewiring DISC signalling to NF-κB activation and cell survival. It will furthermore highlight the interplay between caspase-8 and its two regulators cFLIP and caspase-10 und will point out the importance of caspase-8 as a central element in DISC formation and stability.

VIII. Results

VIII.1. Caspase-10 promotes CD95L-induced and spontaneous cell death upon overexpression

To initially study the impact of caspase-10 on DISC-induced cell death signalling, stable HeLa cell lines were generated which constitutively overexpress caspase-10. Therefore, the isoforms ‘a’, ‘d’, and ‘c’ as well as the respective active site mutants (ASM; from ‘a’ and ‘d’) were cloned into a retroviral vector system. Thereby, caspase-10a ASM carried a cysteine to serine mutation at amino acid position 358 (C358S) and caspase-10d ASM a C401S mutation. Each construct was successfully overexpressed upon transduction (Figure 5A), but the overexpression of the long caspase-10 isoforms led to spontaneous cell death induction after 48-72 hours (Figure 5A, indicated by crosses). Interestingly, even the ASMs killed the cells and solely the short isoform ‘c’ did not alter cell survival when overexpressed.

To avoid spontaneous cell death induction by the long isoforms of caspase-10, new stable cell lines were generated *via* lentiviral transduction. These cell lines allowed an inducible overexpression of the gene of interest by the addition of 4-hydroxytamoxifen (4-HT). As seen before, overexpression of the long isoforms induced spontaneous cell death independent from their enzymatic activity (data not shown). To circumvent this surprising toxicity, the induction of caspase-10 expression was adapted. Within four hours of 4-HT stimulation, caspase-10a and its respective ASM were highly overexpressed in HeLa cells (Figure 5B). The inducible system showed a strong leakiness and led to a drastic caspase-10 expression even if the cells were not treated with 4-HT (Figure 5B). Based on the strong overexpression and the leakiness of the system, endogenous caspase-10 expression could only be detected weakly (Figure 5B). However, overexpression of caspase-10a resulted in a sensitisation to CD95L stimulation which was fully blocked by the expression of the ASM (Figure 5C). Thereby, caspase-10 promoted DISC-induced cell death only when protein expression was further up-regulated by the addition of 4-HT. Cell death induction was so strong that the pan-caspase inhibitor zVAD-fmk failed to completely block it. Although caspase-10 was strongly overexpressed solely by the leakiness of the vector system, cell death response was unchanged under these conditions (compare Figure 5B and C). Figure 5D illustrates that the overexpression was appropriately adapted to exclude any spontaneous cell death induced by caspase-10.

These data confirm the previously observed function of caspase-10 as an initiator caspase in DISC-mediated cell death signalling. However, we strongly wondered that caspase-10 has to be highly overexpressed to promote cell death and that the overexpression caused by the leakiness had no effect on cell death response.

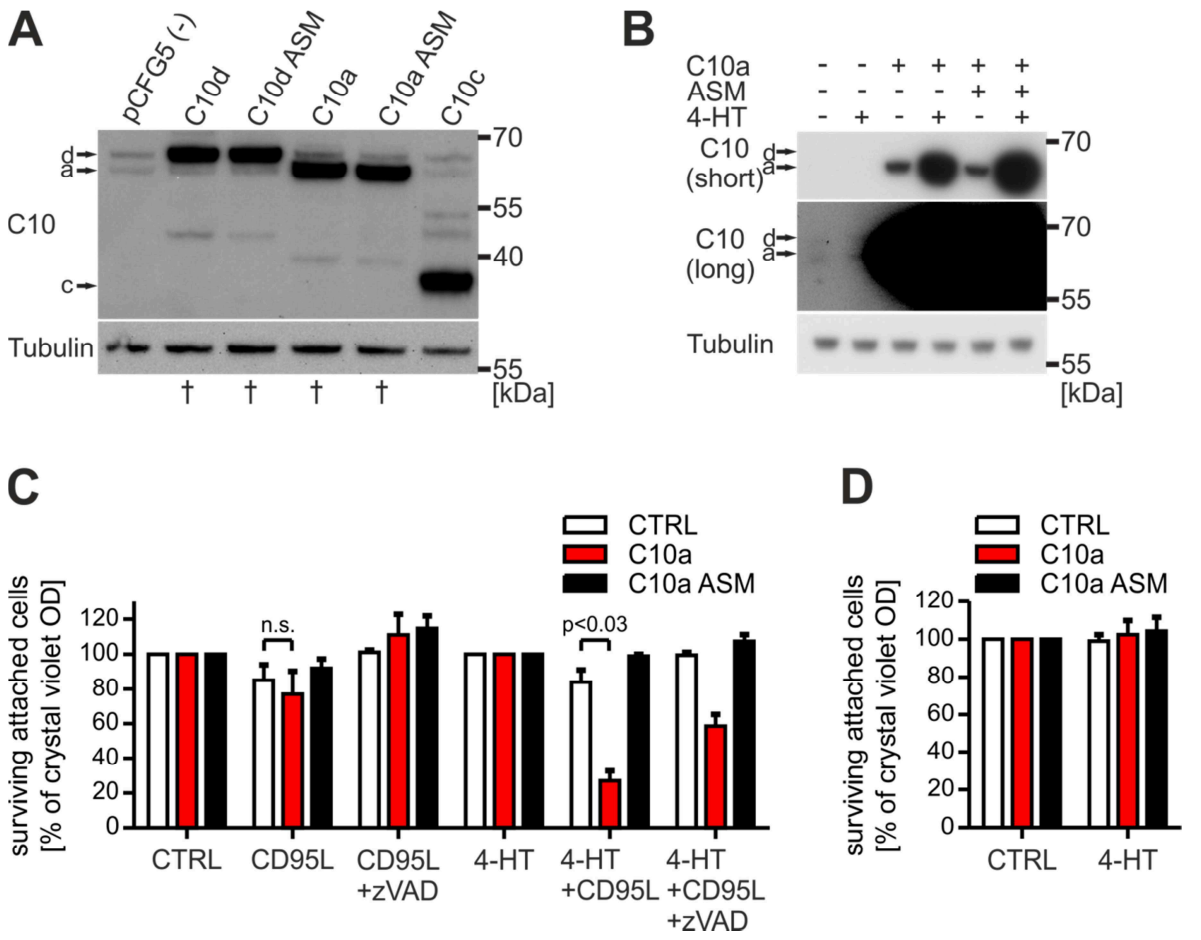


Figure 5 Caspase-10 overexpression promotes CD95L-induced apoptosis
A HeLa cells were transduced with retroviruses containing different caspase-10 (C10) isoforms and their respective active site mutants (ASM) in pCFG5. 24 h after transduction, cells were tested by Western blotting for the constitutive overexpression of the respective caspase-10 variants. Crosses mark the cell lines which spontaneously died after transduction. **B** HeLa cells inducible overexpressing either caspase-10a (C10a), its respective active site mutant (C10a ASM), or the empty vector were incubated for 4 h with 100 nM 4-hydroxytamoxifen (4-HT). The overexpression and the leakiness of the inducible vector system were analysed by Western Blotting. **C** Triplicates of 4-HT treated cells from B were additionally incubated with 10 µM zVAD-fmk (zVAD) for 1 h and stimulated with 2 U/ml CD95L-Fc for 80 min. Cell viability was analysed by crystal violet staining. **D** Cell viability of 4-HT treated cells from C were analysed in respect to untreated cells. Shown are mean values ± SEM of three independent experiments. Significance levels (p values) were measured by Student´s *t*-test (n.s.: not significant).

VIII.2. Caspase-10 inhibits death receptor-induced cell death

As the overexpression of caspase-10 might drastically derail the stoichiometry of the DISC and potentially leads to artificial results, the impact of caspase-10 on DISC signalling was next analysed by utilising knockdown approaches. Therefore, caspase-10, caspase-8, or the combination of both were knocked down by siRNA in HeLa cells and analysed for their

impact on CD95L stimulation (Figure 6A). The single and combined knockdown of caspase-8 fully prevented cells from cell death, but loss of caspase-10 surprisingly resulted in an enhanced cell death response. The observed effect was reproducibly investigated in independent experiments and was fully apoptotic as zVAD-fmk fully prevented from cell death, whereas the blockade of RIPK1 by Necrostatin-1 (Nec) had no impact on the phenotype (Figure 6A).

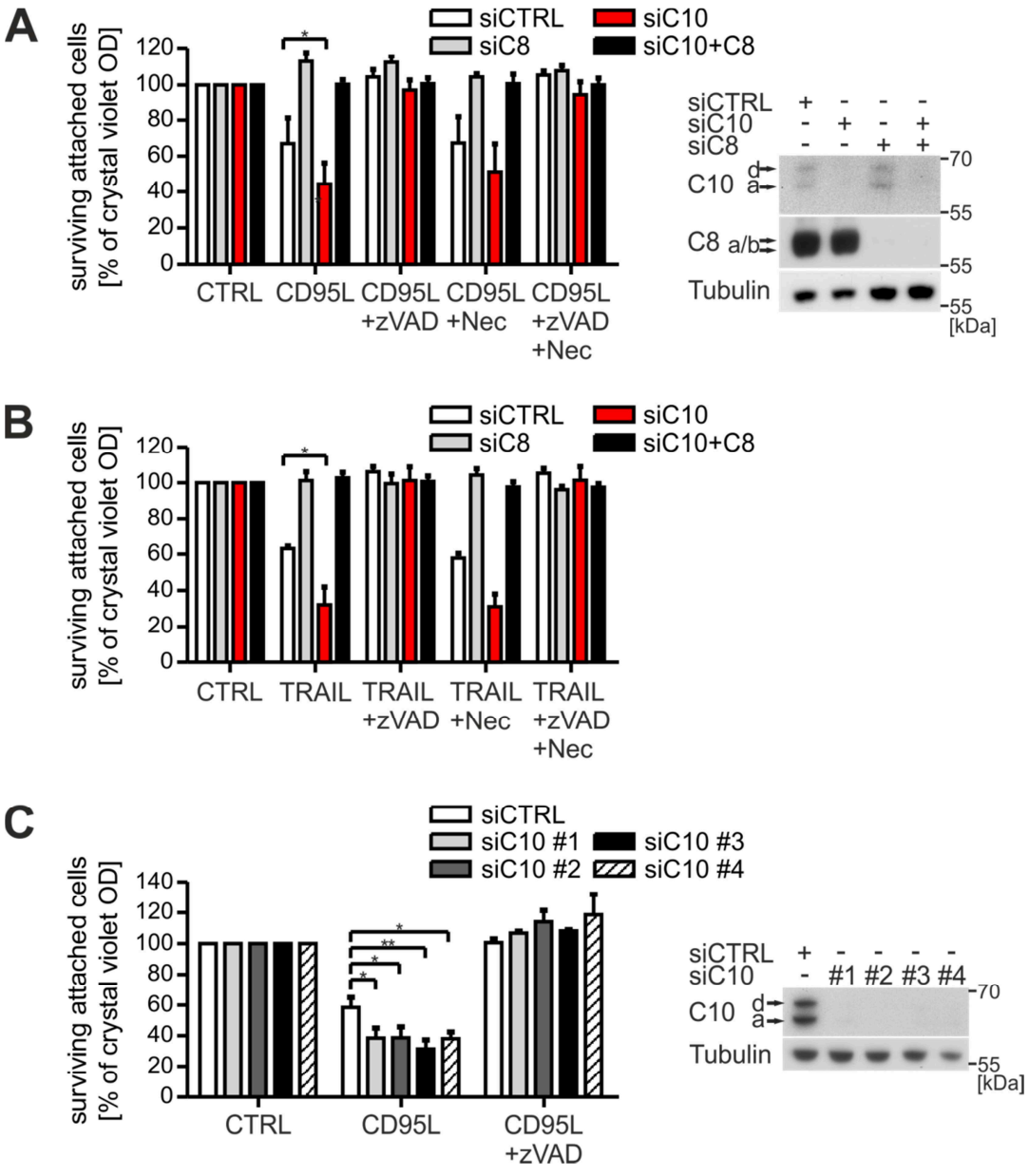


Figure 6 Caspase-10 protects from death ligand-induced cell death
A HeLa cells were treated with caspase-10 (siC10), caspase-8 (siC8), the combination of both, or control siRNA (siCTRL) for 72 h. Triplicates were pre-incubated with 10 µM zVAD-fmk (zVAD) or 50 µM Necrostatin-1 (Nec) or the combination of both for 1 h followed by stimulation of 1 U/ml CD95L-Fc for 16-20 h. Knockdown efficiency was controlled by Western blotting. **B** HeLa cells were treated as described above and stimulated with 1 µg/ml HF-TRAIL for 16-20 h. Cell viability was analysed by crystal violet staining. **C** HeLa cells were treated with four different siRNAs against caspase-10 (siC10 #1-4) or control siRNA (siCTRL) for 72 h. Triplicates were pre-incubated with 10 µM zVAD for 1 h followed by stimulation of 1 U/ml CD95L-Fc for 16-20 h. Cell viability was analysed by crystal violet staining. Knockdown efficiency was controlled by Western blotting. Shown are mean values ± SEM of three independent experiments. Significance levels (p values) were measured by Student´s *t*-test. Asterisks indicate the p values in comparison to the respective reference (* $p<0.05$; ** $p<0.01$).

To rule out if the sensitisation to death ligands by loss of caspase-10 is a general phenomenon, we next performed the same experiments as described above but induced cell death by stimulating the TRAIL-receptors (Figure 6B). As observed before, caspase-10 inhibited death receptor-induced cell death. The mode of cell death was again caspase-8-mediated as the single knockdown of caspase-8 and the combined knockdown of caspase-8 and -10 fully prevented from death induction (Figure 6B). To exclude any off-target effect of the used siRNA, we next knocked down caspase-10 using four different siRNAs. Whenever caspase-10 was lost by RNA interference, cells showed an enhanced response to CD95L (Figure 6C). Thus, the observed sensitisation to death ligands following knockdown of caspase-10 is exclusively driven by the loss of caspase-10.

Furthermore, stable HeLa cell lines were generated which inducible expressed a shRNA against caspase-10 by the addition of doxycycline. These cells additionally confirmed the inhibitory function of caspase-10 in DISC-induced cell death signalling in a time-dependent CD95L stimulation (Figure 7A). As a next consequence, we analysed the function of caspase-10 in extrinsic apoptosis in other cell death assays to exclude any assay-specific effects. Therefore, the externalisation of phosphatidylserine upon apoptosis induction was investigated by annexin V staining after the knockdown of caspase-10 by shRNA and CD95L stimulation (Figure 7B). In addition, specific cell death was measured by analysing DNA fragmentation by PI staining following loss of caspase-10 and death ligand stimulation (Figure 7C). Independent from the applied apoptosis assay, knockdown of caspase-10 sensitised to death receptor-induced cell death under all conditions.

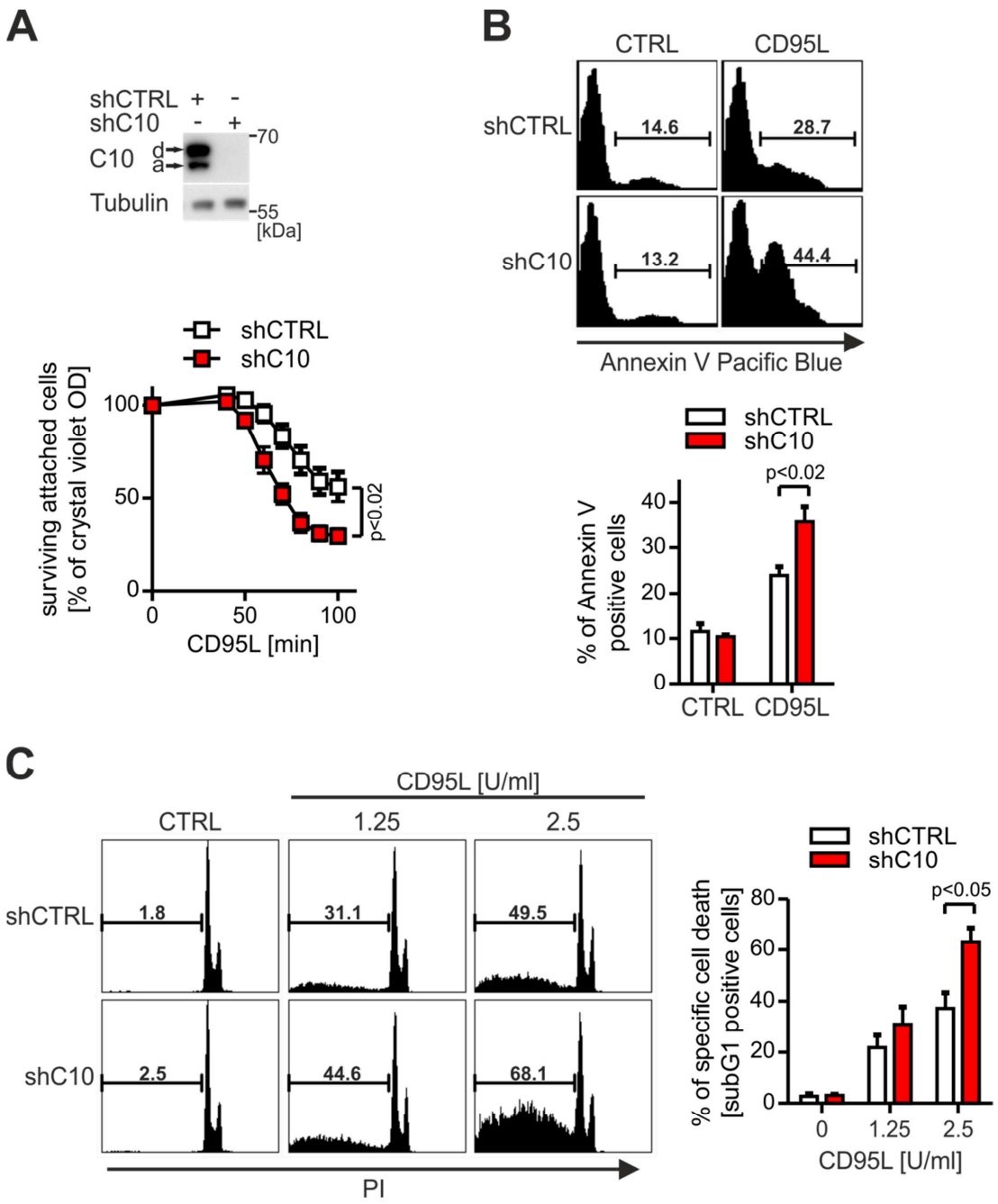


Figure 7 shRNA-mediated knockdown of caspase-10 confirms its anti-apoptotic role in DISC-signalling

HeLa cells inducible expressing a shRNA against caspase-10 (shC10) or control shRNA (shCTRL) were treated with 0.5 µg/ml doxycycline for 72 h. **A** Cells were stimulated in triplicates for the indicated times with 2 U/ml CD95L-Fc. Cell viability was analysed by crystal violet staining. Knockdown efficiency was controlled by Western blotting. **B** Cells were stimulated with 1 U/ml CD95L-Fc for 3 h. Annexin V-Pacific Blue positive cells were measured using flow cytometry. **C** Cells were stimulated with the indicated CD95L-Fc concentrations for 7 h. DNA degradation was quantified by flow cytometry using PI staining for sub G1 populations. Shown are mean values ± SEM of at least three independent experiments. Significance levels (p values) were measured by Student´s *t*-test.

The increased sensitisation to the death ligands was not caused by a caspase-10 knockdown-induced alteration in the expression pattern of the death receptors. Following knockdown of caspase-10, receptor surface expression pattern of the CD95, TRAIL-R1, as well as TRAIL-R2 death receptors were unaltered in HeLa cells (Figure 8).

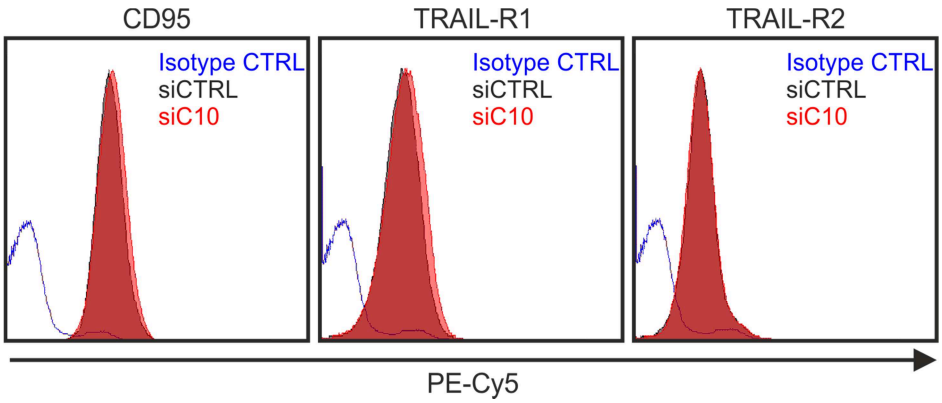


Figure 8 Death receptor expression is unchanged after knockdown of caspase-10
HeLa cells were treated with caspase-10 (siC10) or control siRNA (siCTRL) for 72 h and analysed for CD95, TRAIL-R1, and TRAIL-R2 surface expression by flow cytometry. Isotype control staining was used to verify the specificity of the antibodies.

Taken together, our data demonstrate that caspase-10 is an inhibitor of death receptor-mediated cell death signalling in HeLa cells. Cell death is thereby exclusively mediated by caspase-8 and independent from other cell death modes.

VIII.2.1. High expression of caspase-10 abolishes its anti-apoptotic features

Thus far, the impact of caspase-10 on DISC signalling has only been studied in HeLa cells. To spread our analysis on other cellular systems, we screened other cell lines (diverse melanoma (SK-Mel, IGR, WK, and MC), B-cell and T-cell lymphoma (BJAB and Jurkat), and spontaneously transformed keratinocytes (HaCaT)) for their impact of caspase-10 on death receptor stimulation. As previously observed in HeLa cells, SK-Mel melanoma cells were significantly sensitised after the knockdown of caspase-10 against CD95L stimulation (Figure 9A). In sum, caspase-10 protected from CD95L-induced cell death in 3 out of 8 cell lines that we screened (data not shown for the other cell line). Interestingly, the cell lines which were not sensitised to CD95L by the knockdown of caspase-10 were also not protected against the death ligand. These cells were unaffected by the depletion of caspase-10 in respect to cell death response (exemplarily shown for MC (Figure 9B) and HaCaT (Figure 9C) cells). Remarkably, all cell lines which were unaffected by the knockdown of caspase-10 showed a much higher expression pattern of caspase-10 compared to the cell lines that were sensitised upon death ligand stimulation (exemplarily shown for HaCaT/MC

vs. HeLa/SK-Mel in Figure 9D). Noticeable, protein levels remaining after successful knockdown of caspase-10 in HaCaT or MC cells were comparable to endogenous protein levels in HeLa or SK-Mel cells (Figure 9D).

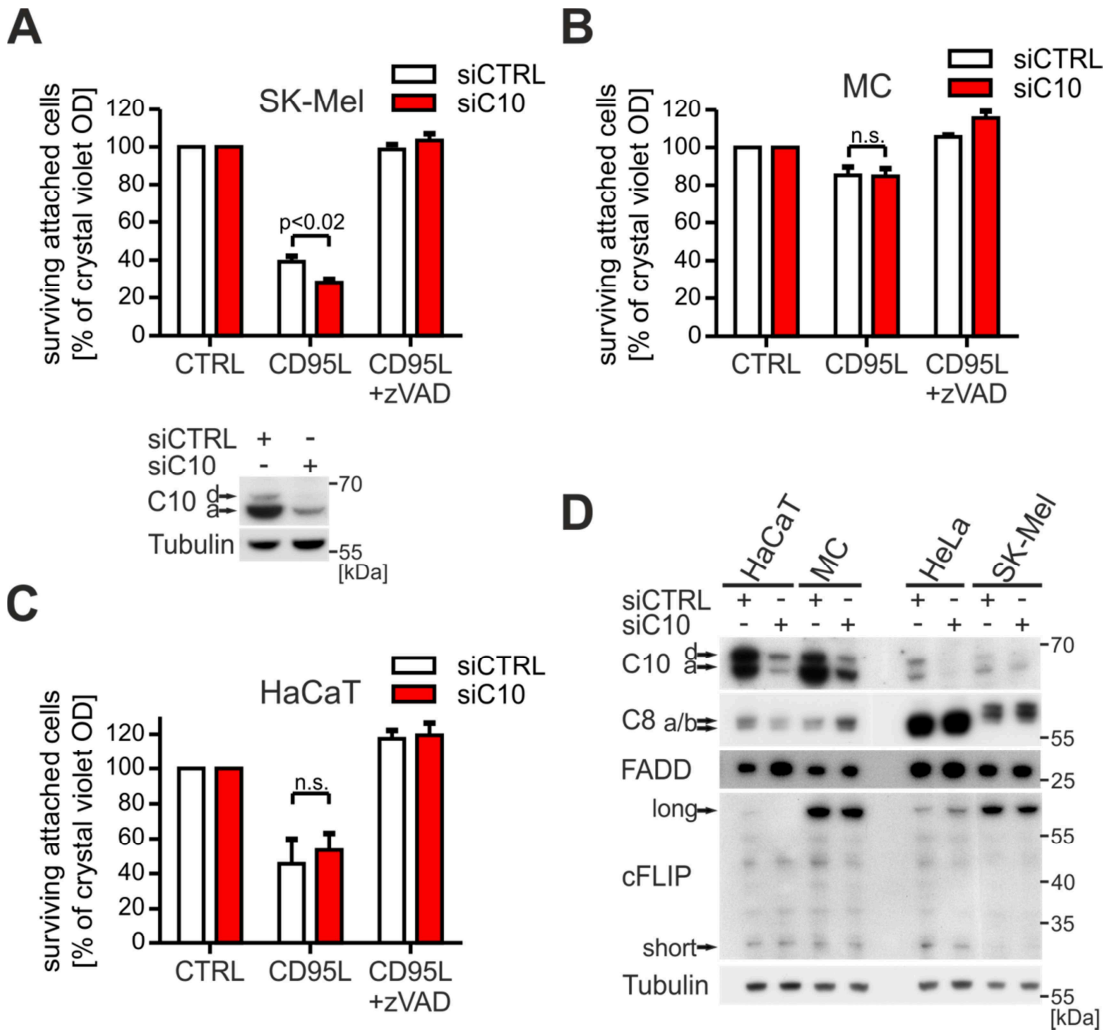


Figure 9 Caspase-10 expression levels predict its anti-apoptotic function
A SK-Mel cells were treated with caspase-10 siRNA (siC10) or control siRNA (siCTRL) for 72 h. Triplicates were pre-incubated with 10 µM zVAD-fmk (zVAD) for 1 h followed by stimulation of 0.5 U/ml CD95L-Fc for 16-20 h. Caspase-10 knockdown efficiency was analysed by Western blotting. Cell viability was analysed by crystal violet staining. **B** Triplicates of siRNA treated MC cells as outlined in A were pre-incubated for 1 h with 10 µM zVAD and stimulated for 16-20 h with 0.5 U/ml CD95L-Fc. Cell viability was analysed by crystal violet staining. **C** HaCaT cells were treated with siRNA as described in A. Triplicates were pre-incubated with 10 µM zVAD for 1 h followed by stimulation of 0.1 U/ml CD95L-Fc for 16-20 h. Cell viability was analysed by crystal violet staining. **D** Different cell lines were treated with siC10 or siCTRL for 72 h. Knockdown efficiency and proteins involved in DISC signalling were analysed by Western blotting. Cell lines with high expression of caspase-10 (HaCaT and MC) were compared to low expressing cell lines (HeLa and SK-Mel). Shown are mean values ± SEM of three independent experiments. Significance levels (p values) were measured by Student´s *t*-test (n.s.: not significant).

This stoichiometry of caspase-10 between the different cell lines was also reflected in the DISC. The CD95-DISC was precipitated in HaCaT cells following knockdown of caspase-10 and compared to the DISC of parental HeLa cells (Figure 10). When caspase-10 was depleted by shRNA in HaCaT cells by the addition of doxycycline, the levels of DISC-associated caspase-10 were comparable to endogenous levels in HeLa cells. This finding indicates that caspase-10 is functional relevant in DISC signalling and led us to the initial hypothesis that the remaining caspase-10 levels after the knockdown in HaCaT or MC cells might be sufficient to protect from the observed phenotype in HeLa and SK-Mel cells.

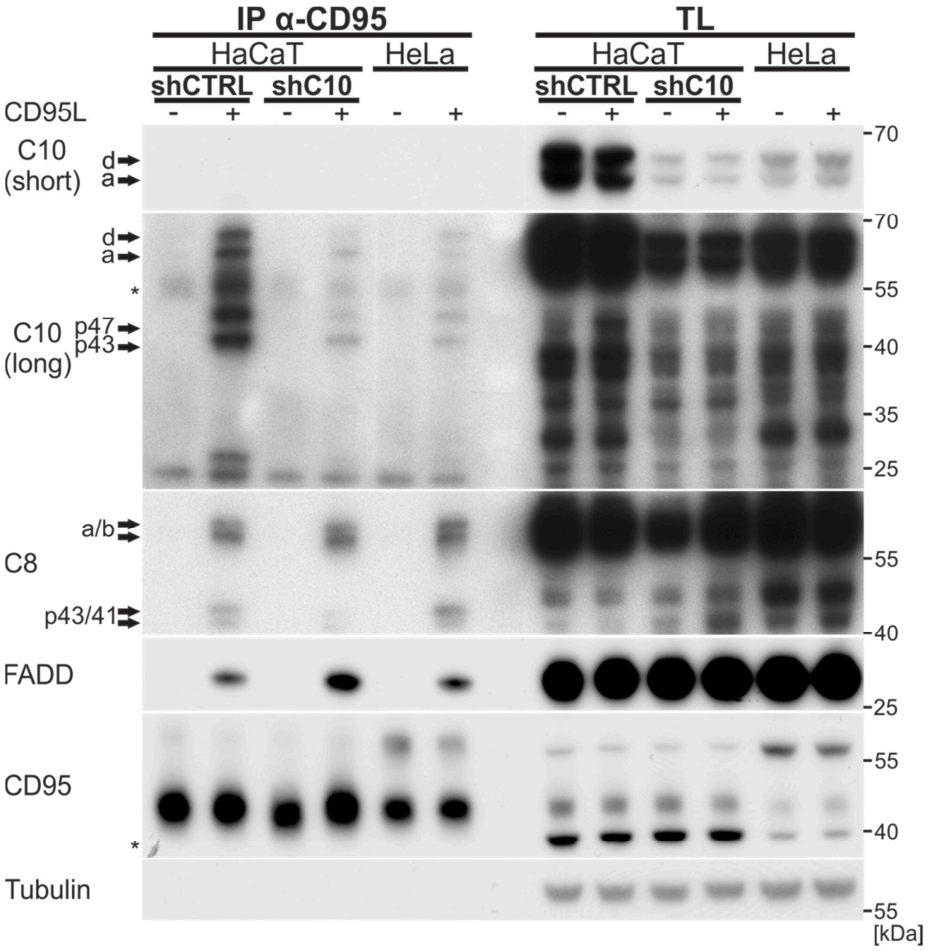


Figure 10 Caspase-10 is functional relevant for death receptor signalling
Caspase-10 (shC10) or control (shCTRL) shRNA expression was induced in HaCaT cells by the addition of 0.5 µg/ml doxycycline for 72 h. Cells were stimulated with 1 U/ml CD95L-Fc for 40 min and compared to equally treated HeLa cells. CD95 was immunoprecipitated from total cell lysates (TL) and coprecipitated proteins were analysed by Western blotting. The asterisks mark unspecific bands.

VIII.2.2. HaCaT cells counter-regulate loss of caspase-10 by up-regulation of cFLIP

However, the above described experiment represented solely a snapshot into the stoichiometry of the DISC. A closer look into the formation of the DISC in HaCaT cells revealed a surprising, reciprocal counter regulation of cFLIP after inducible knockdown of caspase-10 (compare cFLIP expression in total lysate (TL) in Figure 11A). These changes in cFLIP expression were also reflected in the DISC. When we precipitated the CD95-DISC in a time-dependent CD95L stimulation, DISC-associated cFLIP(p43) fragments were reproducibly enriched at the late time points after the knockdown of caspase-10 (Figure 11A). In contrast, FADD as well as caspase-8 association with the receptor was unchanged. A time kinetic to induce the expression of the shRNA against caspase-10 showed that cFLIP is up-regulated in response to loss of caspase-10 in HaCaT cells (Figure 11B). These data indicate that caspase-10 and cFLIP have a critical and synergistic protective function in DISC-induced cell death signalling. To address this assumption, the knockdown of caspase-10 and cFLIP was combined in HaCaT cells and analysed in respect to CD95L stimulation (Figure 11C). The siRNA-mediated knockdown of caspase-10 had again no influence on cell death (Figure 11C, bright red columns). As HaCaT cells express very low levels of cFLIP (compare Figure 9D), depletion of cFLIP slightly sensitised to CD95L stimulation (Figure 11C, black columns). Remarkably, combined knockdown of both cFLIP and caspase-10 could further sensitise to death receptor stimulation than the single knockdown (Figure 11C, compare dark red to black columns at a concentration of 0.5 U/ml CD95L).

Taken together, in contrast to previous thoughts, caspase-10 is a negative regulator of death receptor-induced cell death. Independent from the applied assay or used cell line, caspase-10 never showed any pro-apoptotic features in death receptor-mediated cell death induction. As exemplified by HaCaT cells, caspase-10 and cFLIP are able to cooperate their function to protect from caspase-8-mediated cell death.

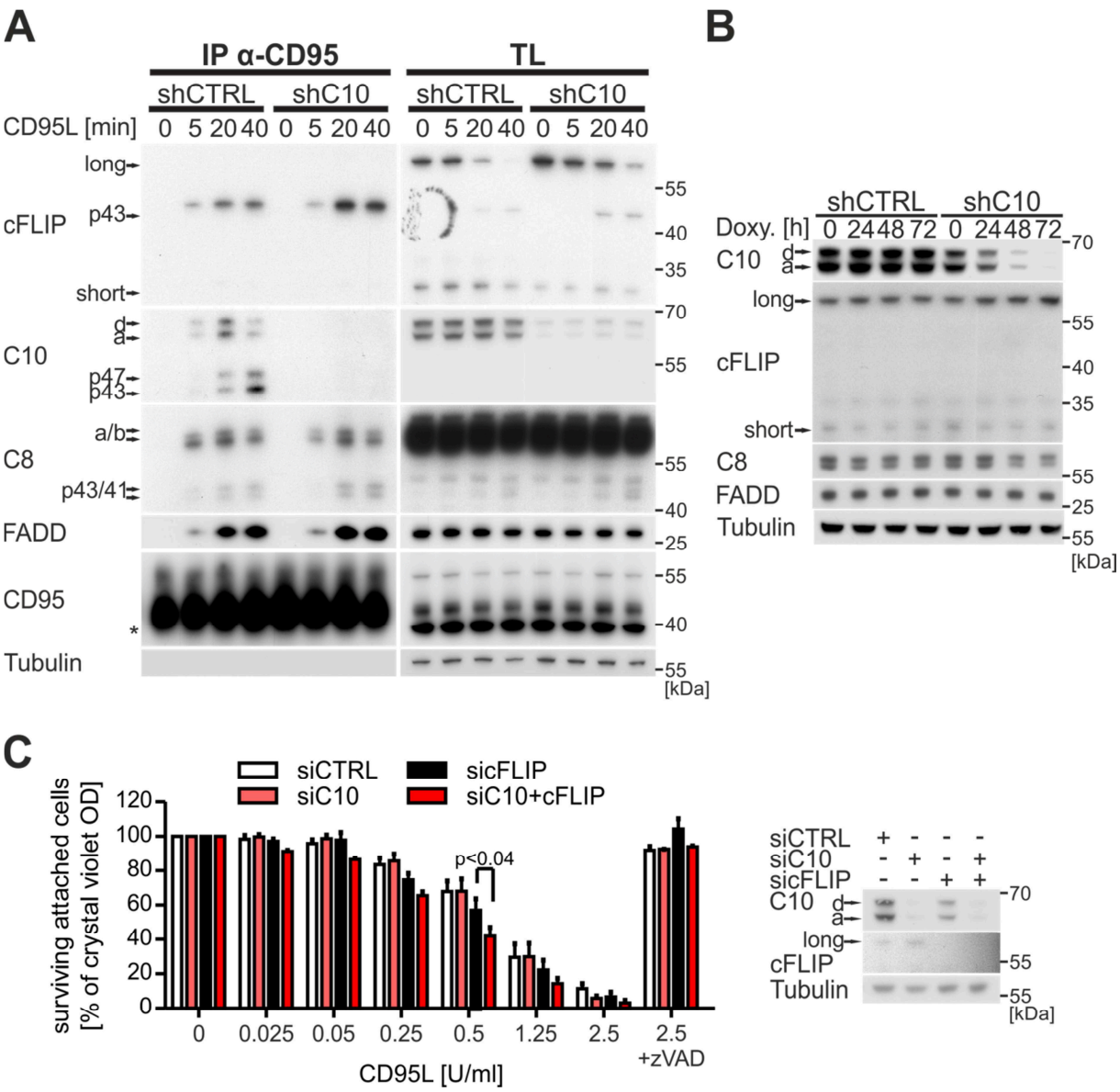


Figure 11 cFLIP compensates for caspase-10 in HaCaT cells
A Caspase-10 (shC10) or control shRNA (shCTRL) expression was induced in HaCaT cells by the addition of 0.5 µg/ml doxycycline for 72 h. Cells were stimulated with 1 U/ml CD95L-Fc for the indicated time points. CD95 was immunoprecipitated from total cell lysates (TL) and coprecipitated proteins were analysed by Western blotting. The asterisk marks an unspecific band. **B** Respective shRNA expression was induced in HaCaT cells by the addition of 0.5 µg/ml doxycycline (Doxy.) for the indicated time points. DISC-related proteins were analysed by Western blotting. **C** HaCaT cells were treated with caspase-10 (siC10), cFLIP (sicFLIP), the combination of both, or control (siCTRL) siRNA for 48 h. Cells were pre-incubated in triplicates with 10 µM zVAD-fmk (zVAD) for 1 h followed by stimulation with the indicated concentrations of CD95L-Fc for 4 h. Cell viability was analysed by crystal violet staining. Knockdown efficiency was controlled by Western blotting. Shown are mean values ± SEM of three independent experiments. Significance level (p value) was calculated by Student´s *t*-test.

VIII.3. Caspase-10 blocks caspase-8 recruitment to and activation in the DISC

The above presented functional data show that caspase-10 does not favour pro-apoptotic DISC signalling. To analyse weather caspase-10 modulates the assembly of the DISC and therefore cell death outcome, the CD95-DISC was precipitated in a time-dependent kinetic in the presence and absence of caspase-10 in HeLa cells (Figure 12A). Whenever caspase-10 was knocked down by shRNA, full-length and p43/41 caspase-8 fragments were enriched in the DISC. In contrast, association of FADD and cFLIP to the complex was unaffected by the depletion of caspase-10. The enhanced sensitisation to death ligands following knockdown of caspase-10 was also confirmed by increased processing of caspase-8. When caspase-10 was depleted, a higher processing rate of caspase-8 to the p43/41 fragments was detected (Figure 12A; total lysate). To demonstrate the significance of the enhanced caspase-8 recruitment to the DISC in the absence of caspase-10, the relative ratio of co-precipitated full-length and p43/41 caspase-8 in the DISC was quantified in respect to precipitated CD95 from three independent experiments (Figure 12B, left panel). The quantification showed a significant enrichment of caspase-8 in the DISC at early time points which was caught up in control cells over time. Furthermore, FADD and cFLIP(p43) association to the DISC proved to be unaffected by knockdown of caspase-10 (Figure 12B, middle and right panel). Of note, a slight up-regulation of cFLIP after the knockdown of caspase-10 in HeLa cells was detectable in some of the experiments (compare total lysates in Figure 12A). However, this up-regulation did not increase the association of cFLIP to the DISC or alter the increased recruitment of caspase-8. These findings even further strengthen the inhibitory function of caspase-10 in death receptor signalling.

VIII.4. Caspase-10 and cFLIP independently block the association of caspase-8 to the DISC

The data collected in HaCaT cells (Figure 11) revealed a close correlation of caspase-10 and cFLIP in cooperating the inhibition of caspase-8-mediated cell death signalling. However, it has to be mentioned that the expression of cFLIP in HaCaT cells is very low (Figure 9). To address the interplay between cFLIP and caspase-10 in more detail, the analyses were therefore extended to cell lines with higher cFLIP expression levels. Thus, caspase-10 and cFLIP were knocked down by siRNA in HeLa cells and analysed for their impact on CD95L-induced cell death (Figure 13A). Knockdown of caspase-10 again confirmed its anti-apoptotic characteristics (Figure 13A, bright red columns), but the effect was much weaker compared

to the depletion of cFLIP (Figure 13A, black columns). Importantly and alike the data in HaCaT cells, the combined knockdown of cFLIP and caspase-10 (Figure 13A, dark red columns) resulted in a further sensitisation to CD95L as the cFLIP knockdown alone.

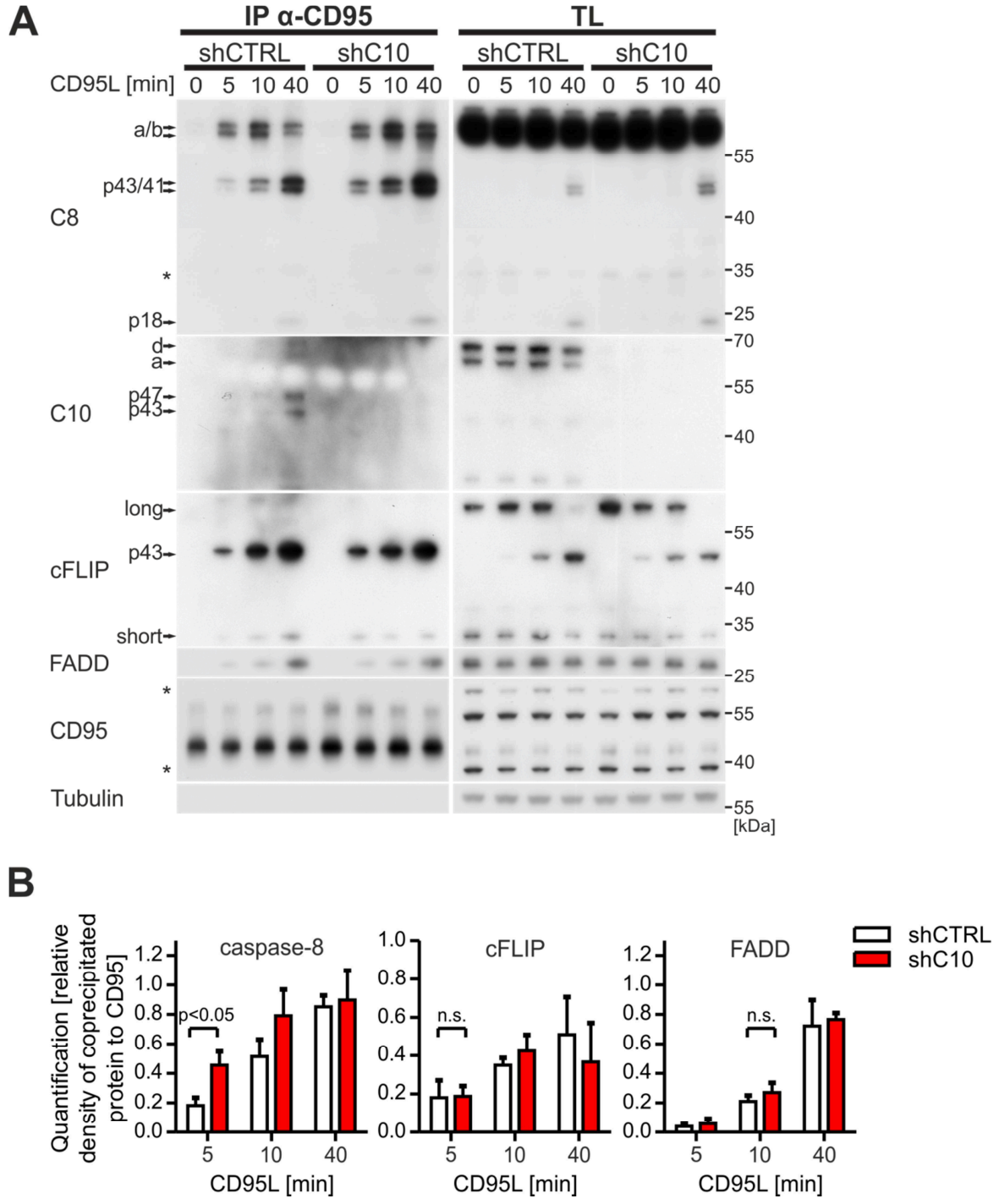


Figure 12 Caspase-10 represses caspase-8 recruitment to the DISC
A Control (shCTRL) or caspase-10 shRNA (shC10) expression was induced for 72 h by 0.5 µg/ml doxycycline in HeLa cells. Cells were then incubated for the indicated time points with 2 U/ml CD95L-Fc. CD95 was immunoprecipitated from total cell lysates (TL) and DISC associated proteins were analysed for DISC recruitment by Western blotting. The asterisks mark unspecific bands. **B** Relative densities of coprecipitated caspase-8 (full length a/b and p43/41), cFLIP p43, and FADD were quantified and calculated in respect to precipitated CD95 in the CD95L time kinetic of the above described experiment. Shown are mean values ± SEM of three independent experiments. Significance level (p value) was calculated by Student´s *t*-test (n.s.: not significant).

To explore the impact of cFLIP and caspase-10 in modulating the assembly of the DISC, the short half-life of cFLIP protein was utilised. cFLIP is known to be rapidly degraded when protein translation is blocked [82]. Therefore, HeLa cells were treated for up to five hours with the ribosomal inhibitor cycloheximide (CHX) to block protein translation and analysed for the stability of DISC-associated proteins (Figure 13B). Whereas caspase-10, caspase-8, and FADD levels were unaffected, cFLIP was substantially degraded within two hours of CHX treatment. This mimicked cFLIP knockdown confirmed the siRNA data shown in figure 13A. When caspase-10 knockdown was combined with CHX treatment (Figure 13C, dark red columns), cell death response to CD95L stimulation was significantly increased compared to CHX treatment alone (Figure 13C, black columns). Of note, CHX treatment has obviously further effects on cell death signalling than solely the degradation of cFLIP. When cFLIP knockdown was combined with CHX, sensitisation to CD95L was further increased as compared to either the single knockdown or CHX treatment alone (Figure 13D).

Being aware of its artificial characteristics, we reasoned that the impact of CHX on the composition of the DISC is mainly limited to its capacity to degrade cFLIP. As a next step, the formation of the CD95-DISC was analysed after the knockdown of caspase-10 by shRNA in combination with CHX treatment (Figure 14). When cFLIP was degraded by CHX, caspase-8 as well as caspase-10 recruitment to the DISC was enhanced (Figure 14, lanes 1-8). Knockdown of caspase-10 once more confirmed the increased recruitment of caspase-8 to the complex (Figure 14, lanes 9-12). Importantly, the combination of CHX treatment and caspase-10 knockdown resulted in a more rapid enrichment as well as cleavage of caspase-8 in the DISC (Figure 14, lanes 13-16).

Taken together, caspase-10 has an anti-apoptotic function in death receptor-induced cell death signalling. Our data demonstrate that caspase-10 blocks the recruitment and thereby activation of caspase-8 in the DISC. Furthermore, caspase-10 and cFLIP act independent in inhibiting the extrinsic apoptotic pathway. Both proteins interfere with the recruitment of caspase-8 to the DISC and thereby block its activation for further downstream signalling.

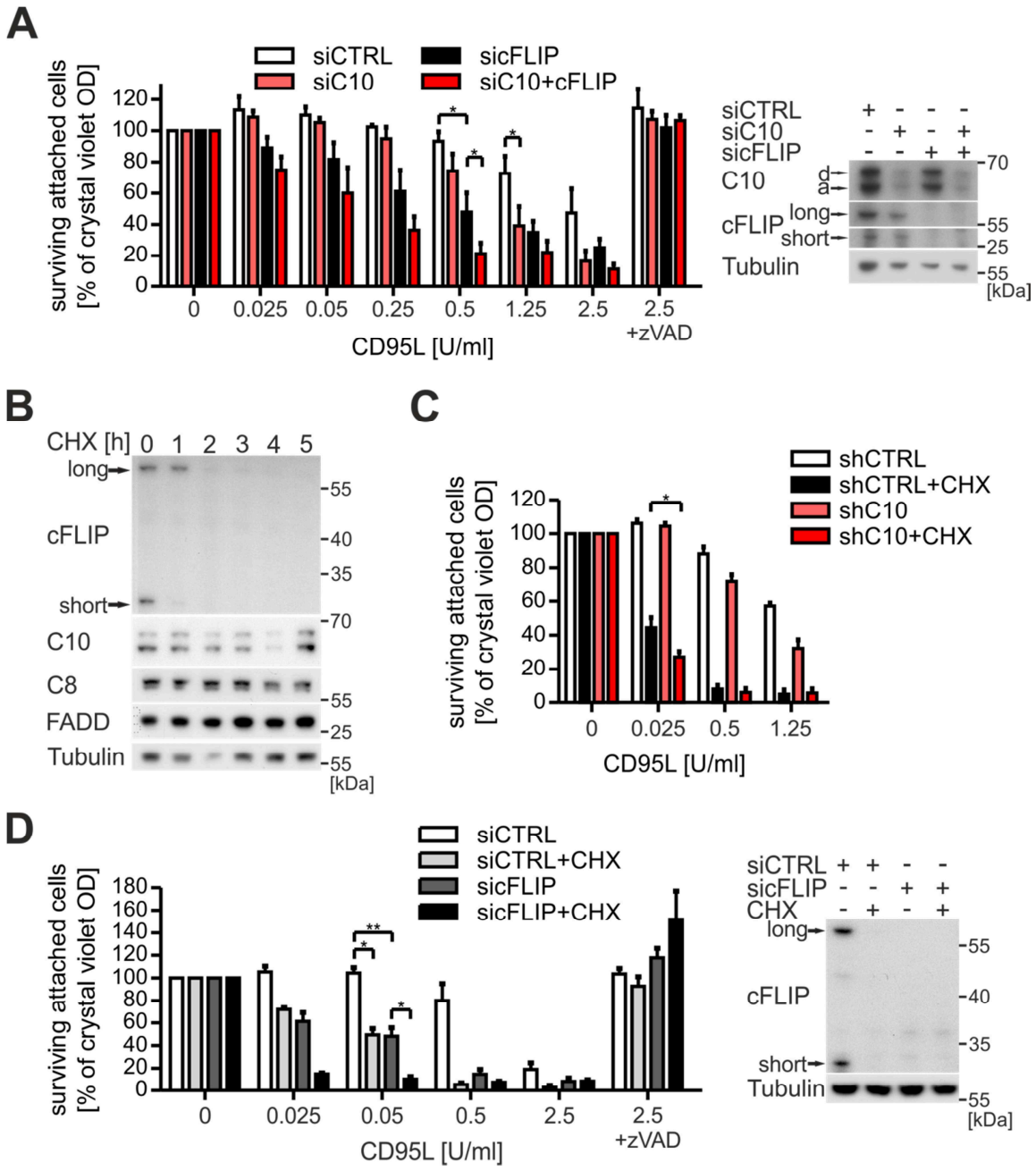


Figure 13 cFLIP and caspase-10 independently inhibit DISC-signalling
A HeLa cells were either treated with control (siCTRL), caspase-10 (siC10), cFLIP (sicFLIP), or the combination of both siRNAs for 48 h. Cells were pre-stimulated in triplicates with 10 µM zVAD-fmk (zVAD) for 1 h followed by stimulation with the indicated concentrations of CD95L-Fc for 4 h. Knockdown efficiency was controlled by Western blotting. **B** HeLa cells (shCTRL as outlined in C) were treated with 5 µg/ml cycloheximide (CHX) for the indicated time points. Cell lysates were analysed for DISC proteins by Western blotting. **C** Caspase-10 (shC10) or control (shCTRL) shRNA expression was induced for 72 h in HeLa cells by the addition of 0.5 µg/ml doxycycline. Cells were pre-stimulated in triplicates with 5 µg/ml CHX for 2 h followed by stimulation with the indicated concentrations of CD95L-Fc for 4 h. **D** shCTRL-expressing HeLa cells were treated with either siCTRL or sicFLIP for 24 h. Triplicates of cells were pre-stimulated for 2 h with 5 µg/ml CHX followed by stimulation with the indicated concentrations of CD95L-Fc for 4 h. Knockdown efficiency was controlled by Western blotting. Cell viability was measured using crystal violet staining. Shown are mean values ± SEM of three independent experiments. Significance level (p value) was measured by Student´s *t*-test. Asterisks indicate the p values in comparison to the respective reference (* $p<0.05$; ** $p<0.01$).

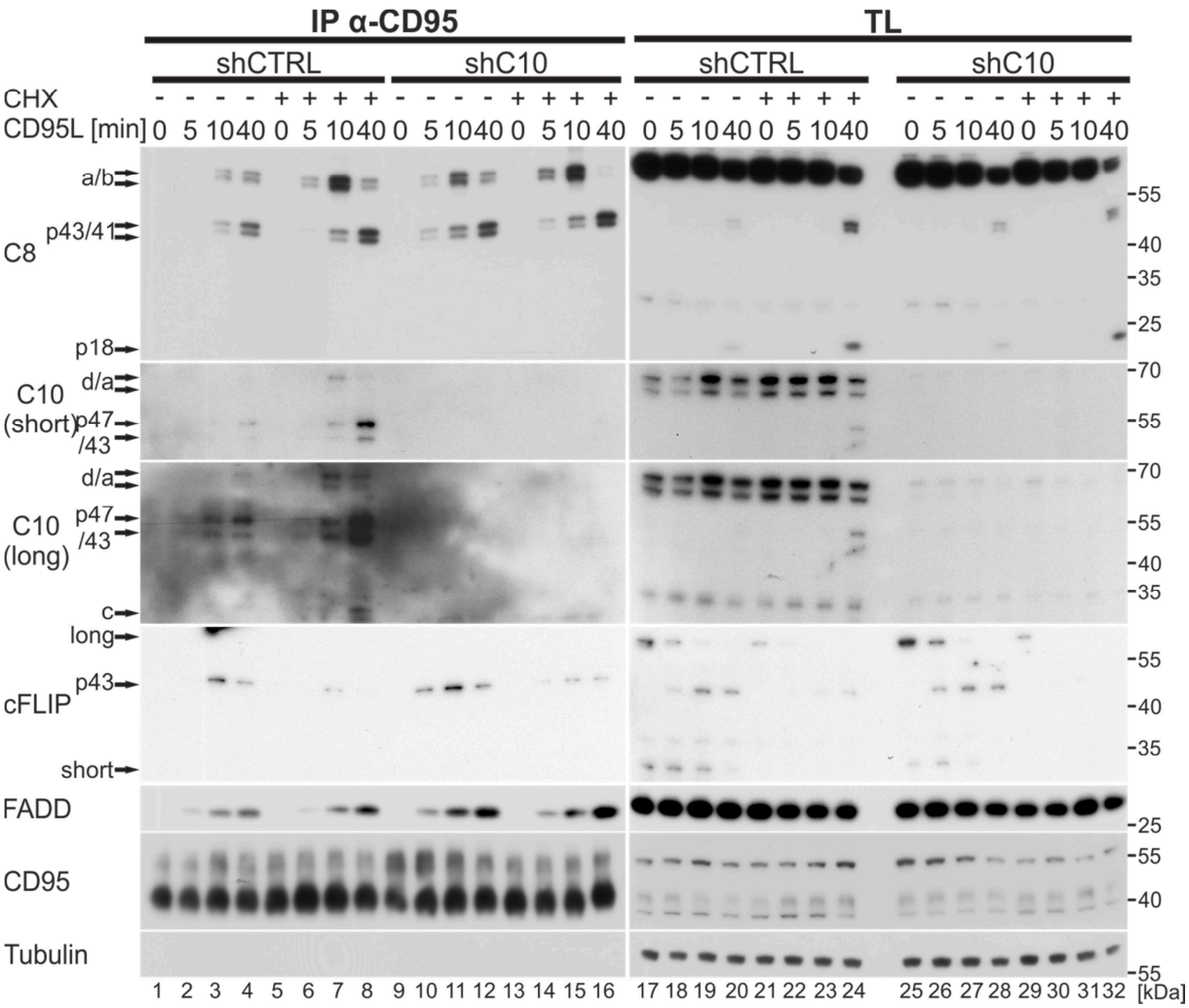


Figure 14 Caspase-10 and cFLIP compete with caspase-8 for DISC recruitment
Control (shCTRL) or caspase-10 (shC10) shRNA expression was induced in HeLa cells by the addition of 0.5 µg/ml doxycycline. Cells were pre-stimulated for 2 h with 5 µg/ml cycloheximide (CHX) followed by stimulation with 2 U/ml CD95L-Fc for the indicated time points. CD95 was immunoprecipitated from total cell lysates (TL) and coprecipitated proteins were analysed by Western blotting.

VIII.5. Caspase-8 is indispensable for the assembly and stability of the CD95-DISC

Caspase-10 and cFLIP independently cooperate in inhibiting caspase-8 activation. However, it still remains elusive if caspase-10 is generally able to substitute for caspase-8. To address this question and to investigate how caspase-10 and cFLIP behave in the DISC in the absence of caspase-8, caspase-8 knockout HeLa cells were generated using CRISPR-Cas9-mediated recombination. Therefore, two independent cell clones (C8 CRISPR) were generated using two different gRNA sequences. Both cell lines fully lacked caspase-8 expression as exemplified by Western blotting and were resistant against CD95L stimulation

(Figure 15A). The inducible shRNA system for knockdown of caspase-10 was also introduced in one of the clones to analyse if any specific cell death can be related to caspase-10. Irrespective of the expression of caspase-10, caspase-8 knockout cells did not respond to CD95L stimulation (Figure 15B). Even if extremely high concentrations of the death ligand were applied, the cells fully survived in the absence of caspase-8 (Figure 15B, compare black and dark red columns). Of note, C8 CRISPR cells showed repressed cFLIP levels compared to parental HeLa cells (Figure 15B).

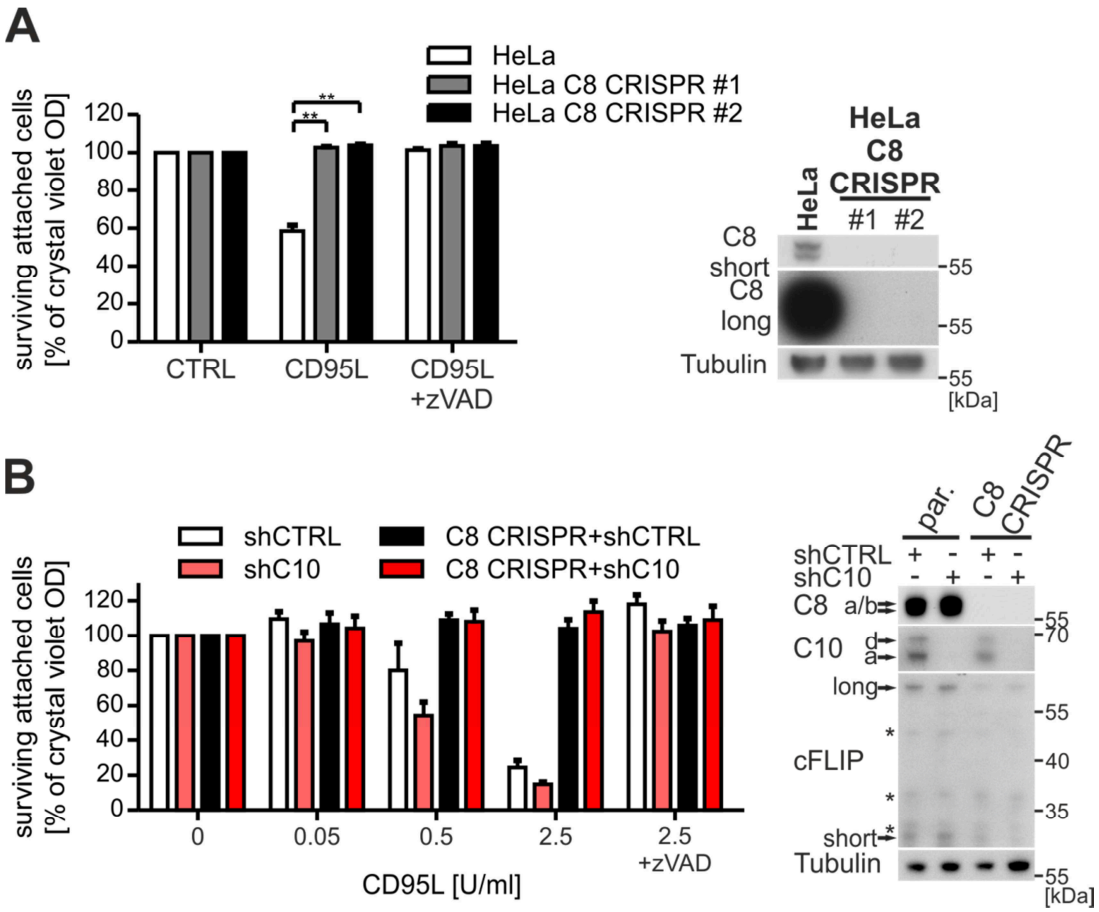


Figure 15 CD95L-induced cell death depends on caspase-8
A Caspase-8 deficient HeLa cell lines were generated using the CRISPR-Cas9 system with two different gRNAs targeting caspase-8. Parental HeLa cells as well as caspase-8 deficient cells (HeLa C8 CRISPR) were pre-stimulated with 10 µM zVAD-fmk (zVAD) for 1 h followed by stimulation with 1 U/ml CD95L-Fc for 16-20 h. Knockout efficiency was controlled by Western blotting. **B** Parental and caspase-8 deficient HeLa cells were treated for 72 h with 0.5 µg/ml doxycycline to induce the expression of either control (shCTRL) or caspase-10 (shC10) shRNA. Cells were pre-stimulated for 1 h with 10 µM zVAD followed by stimulation with the indicated concentrations of CD95L-Fc for 4 h. Knockdown efficiency of caspase-10, loss of cFLIP, as well as the depletion of caspase-8 were controlled by Western blotting. Cell viability was analysed in triplicates by crystal violet staining. Shown are mean values ± SEM of three independent experiments. Significance level (p value) was measured by Student´s *t*-test. Asterisks indicate the p values in comparison to the respective reference (** $p<0.01$).

Next, the impact of caspase-10 and cFLIP in DISC formation was studied in the absence of caspase-8. Therefore, assembly of the CD95-DISC was compared between parental and C8 CRISPR HeLa cells in a time-dependent CD95L stimulation. Surprisingly, this experiment revealed that the DISC did not form in the absence of caspase-8 (Figure 16). Whereas CD95 was effectively precipitated, caspase-10 as well as cFLIP were not associated with the CD95-DISC in caspase-8 knockout cells. Strikingly, even FADD did not assemble with the receptor. Prolonged exposures of the FADD immunoblots were needed to detect a weak interaction between FADD and the DISC in the absence of caspase-8 (data not shown). This striking observation indicates that caspase-8 is first indispensable for the formation of the DISC and second necessary for its stability.

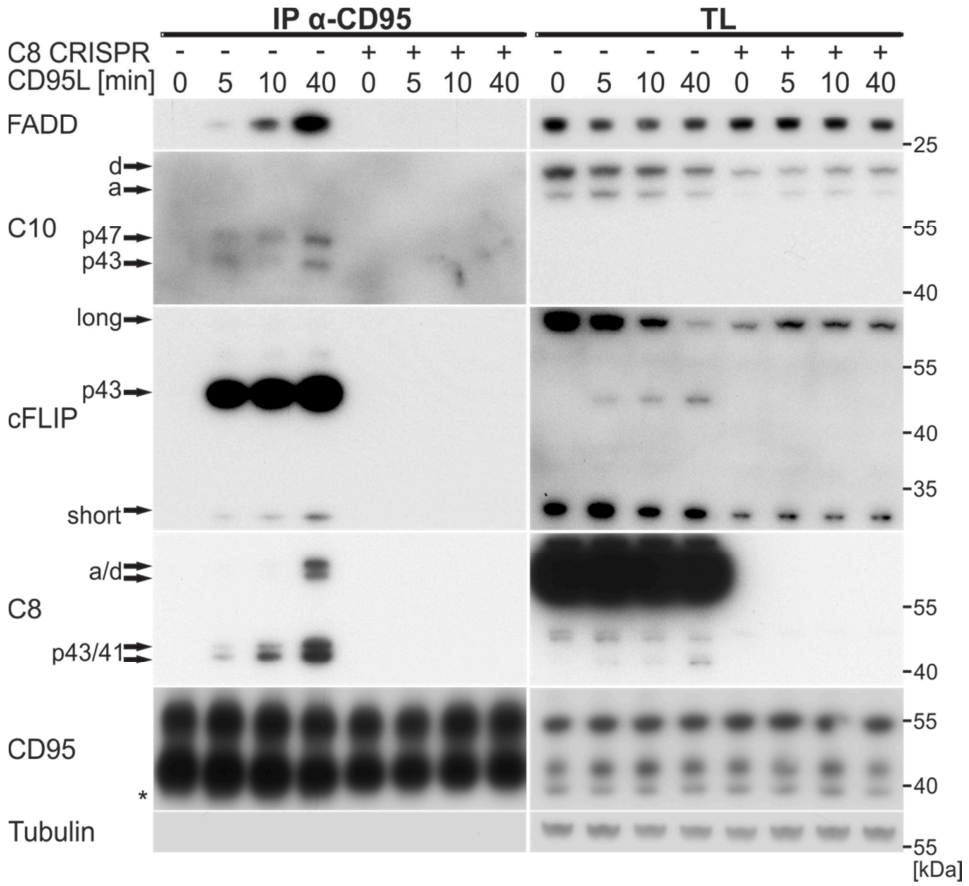


Figure 16 CD95-DISC does not form in the absence of caspase-8
CD95-DISC formation was analysed in parental and caspase-8 deficient (C8 CRISPR) HeLa cells. CD95 was immunoprecipitated from total cell lysates (TL) after stimulation with 2 U/ml CD95L-Fc for the indicated time points. DISC-associated proteins were analysed by Western blotting. The asterisk marks an unspecific band.

However, for the generation of knockout cell lines *via* the CRISPR-Cas9 system, cells undergo clonal selection which potentially heavily alters the characteristics of the newly generated cell line compared to the parental origin. To exclude that this selection caused any artefacts which resulted in the inability to form the DISC, caspase-8a and its respective ASM were reconstituted in the C8 CRISPR cells by inducible overexpression. To generate an enzymatic inactive caspase-8 construct, the cysteine at amino acid position 360 was mutated to alanine (C360A). In contrast to the expression of the wild-type protein, the generated cell lines showed a high leakiness in the expression of the caspase-8a ASM (Figure 17A). However, we were unable to reach endogenous protein levels of caspase-8 by the re-expression without inducing cell death solely by the reconstitution of caspase-8a (data not shown). In contrast to wild-type caspase-8a, the ASM could be reconstituted to reach endogenous protein levels (Figure 17A). Importantly, even if the re-expression of caspase-8a was weak, it conferred sensitivity to CD95L stimulation (Figure 17A; dark grey columns). In addition, expression of the ASM fully prevented from cell death induction by CD95L. As a next step, formation of the DISC was analysed after reconstitution of caspase-8 in the C8 CRISPR cells. Intriguingly, reconstitution of caspase-8a protein restored the recruitment of caspase-10, cFLIP, and, importantly, FADD in the CD95-DISC (Figure 17B). Of note, the association of FADD with the receptor was diminished after the reconstitution compared to receptor-bound FADD levels in parental HeLa cells (Figure 17B, compare lane 3 with 9 and 12). However, the data show that the formation of the DISC occurred independent from the enzymatic activity of caspase-8. Interestingly, the ASM of caspase-8 fully protected the cleavage of caspase-10, cFLIP, and, of course, caspase-8 itself (Figure 17B, lanes 11 and 12). Indeed, the weak expression of enzymatically active caspase-8a resulted in a weaker association of caspase-10 and cFLIP with the complex (Figure 17B, lanes 7-9). However, these data strongly demonstrate that the formation and stability of the CD95-DISC highly depends on caspase-8. Caspase-8 has to be placed upstream of its two regulators, caspase-10 and cFLIP, in the DISC to stabilise the association of FADD with the receptor. FADD and caspase-8 represent a scaffold in the DISC which can be further regulated by caspase-10 as well as by cFLIP.

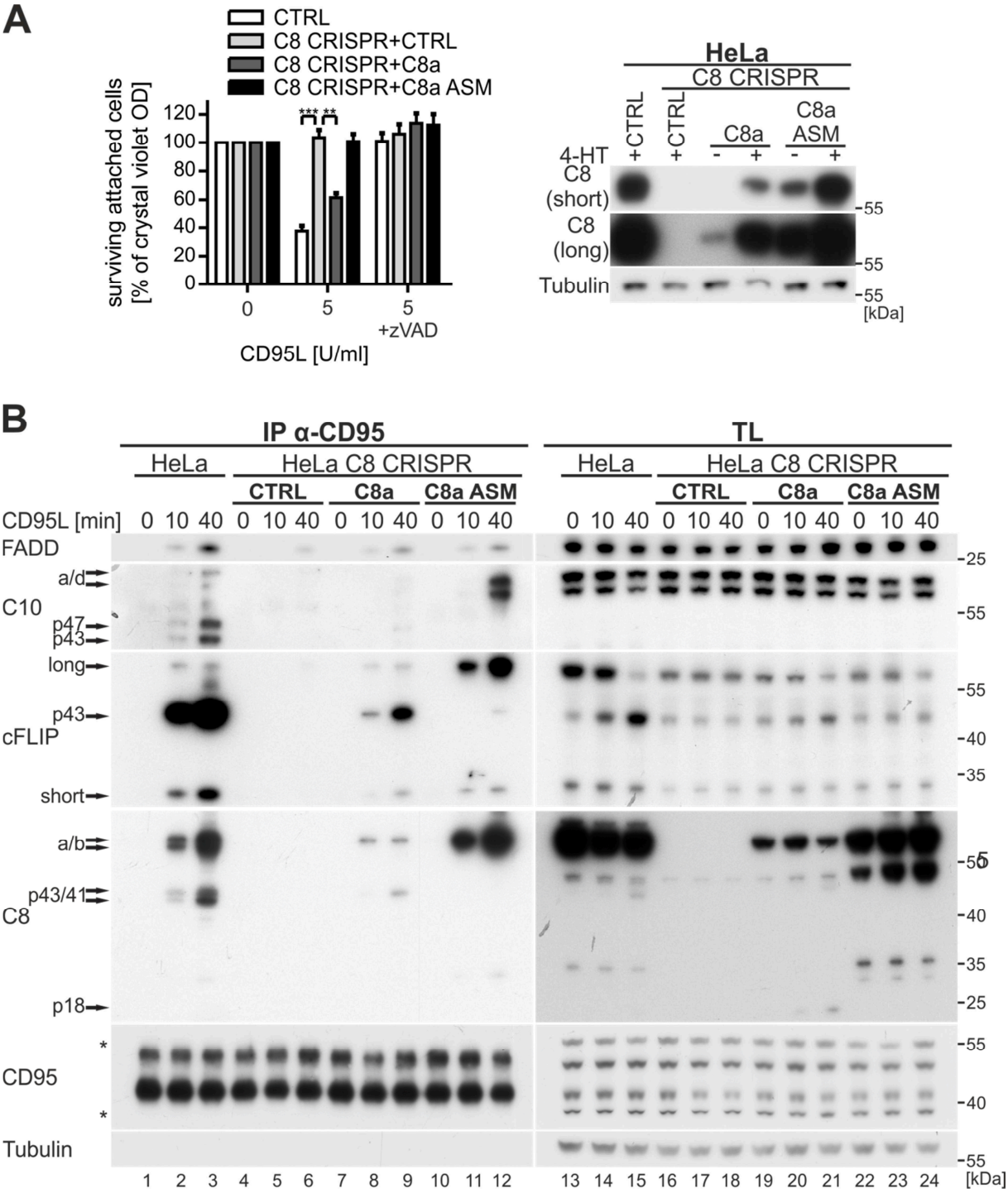


Figure 17 The scaffold function of caspase-8 is indispensable for DISC stability and recruitment of DED proteins

Caspase-8a (C8a) and its respective active site mutant (C8a ASM) were reconstituted in caspase-8 deficient (C8 CRISPR) HeLa cells. **A** Parental and C8 CRISPR HeLa cells either overexpressing the empty vector (CTRL), C8a, or C8a ASM were treated with 10 nM 4-hydroxytamoxifen (4-HT) for 6 h to induce the expression of the respective constructs. Cells were pre-stimulated with 10 µM zVAD-fmk for 1 h followed by stimulation with 5 U/ml CD95L-Fc for 3 h. Cell viability was analysed in triplicates by crystal violet staining. Shown are mean values ± SEM of three independent experiments. Re-expression of C8a and C8a ASM and the leakiness of the inducible system were analysed by Western blotting.

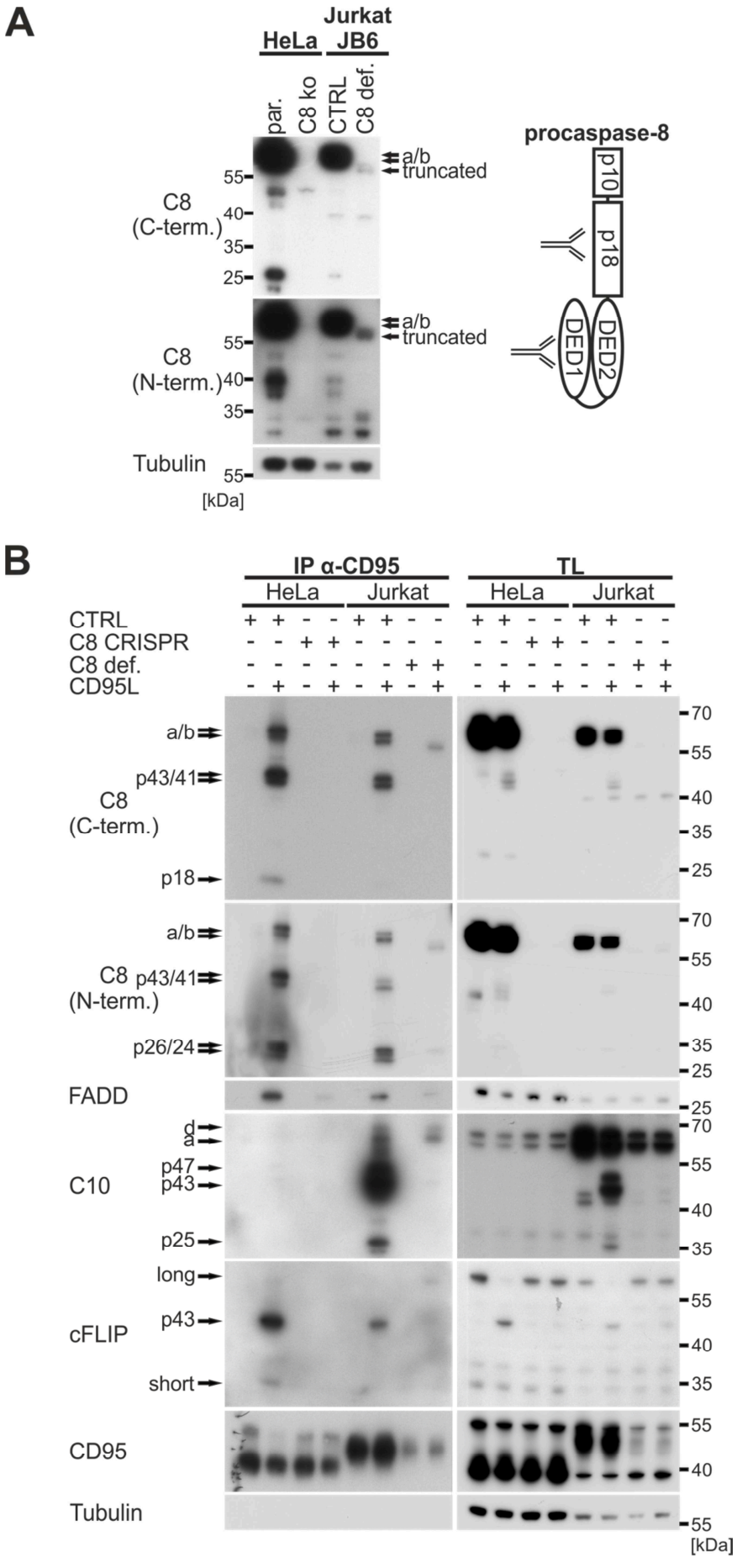
(legend continued on next page)

B As described in A, caspase-8a was reconstituted in caspase-8 deficient HeLa cells for 6 h by 4-HT. Cell lines were stimulated with 2 U/ml CD95L-Fc for the indicated time points. CD95-DISC was immunoprecipitated from total cell lysates (TL) and analysed for DISC-associated proteins. Asterisks mark unspecific bands. Significance level (p value) was measured by Student´s *t*-test. Asterisks indicate the p values in comparison to the respective reference (** $p<0.01$; *** $p<0.001$).

VIII.5.1. Caspase-8 deficient Jurkat cells express a truncated form of caspase-8

These finding is in contrast to a previous study in which Sprick and colleagues showed that caspase-10 is recruited to the DISC in the absence of caspase-8 [146]. Based on these controversial results, we aimed to compare our caspase-8 knockout HeLa cells to their cellular model. They used caspase-8 deficient Jurkat JB6 cell lines in their study which were generated by the use of mutagens [156]. To compare both cell lines, the expression of caspase-8 between both cell lines was analysed. Therefore, caspase-8 was detected by immunoblotting using two specific antibodies either directed against the C- or the N-terminus of caspase-8 (Figure 18A). Interestingly, Jurkat cells which were supposed to be caspase-8 deficient expressed a truncated version of caspase-8 that could be detected with both antibodies. Indeed, expression of the truncated form was much weaker compared to endogenous protein levels in the control cells. To prove if this truncated form of caspase-8 is functional and might explain the controversial results, the CD95-DISC from caspase-8 knockout HeLa was compared to caspase-8 deficient Jurkat cells. As hypothesised, the truncated form of caspase-8 was recruited to the DISC in Jurkat cells (Figure 18B). Furthermore, full-length caspase-10 as well as weak levels of $cFLIP_L$ were associated with the complex. Of note, caspase-10 expression in Jurkat cells is much higher compared to HeLa cells and we were unable to detect caspase-10 in the DISC in HeLa when loaded next to Jurkat. However, Jurkat cells also showed a repression in the association of FADD with the receptor, but these cells also diminished their expression of CD95 (Figure 18B, total lysate). Therefore, the amount of precipitated complex is much weaker in caspase-8 deficient Jurkat cells and does not allow any comparison to the control cells.

Summing up, this experiment shows that Jurkat cells used in the studies from Sprick and colleagues are not deficient in caspase-8 as previously described. These cells express a truncated form of caspase-8 which is able to fulfil the scaffold function of caspase-8 in the DISC to recruit other proteins into the complex. The wrong assumption of caspase-8 deficiency led to false positive results regarding DISC formation in the absence of caspase-8. These cells have further deregulations in apoptosis-relevant genes as exemplified for CD95 and thus have to be critically considered as a suitable tool to study caspase-8-mediated cell death.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Jurkat
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JB6
par.
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procaspase-8
p10
p18
DED1
DED2
a/b
truncated
C8
(C-term.)
C8
(N-term.)
Tubulin
55
40
35
25
[kDa]
B
IP α-CD95
TL
HeLa
Jurkat
CTRL
C8 CRISPR
C8 def.
CD95L
a/b
p43/41
p18
p26/24
FADD
d
a
p47
p43
p25
C10
long
short
cFLIP
CD95
Tubulin
70
55
40
35
25
[kDa]

Figure 18 Caspase-8 deficient Jurkat JB6 cells express a truncated and functional form of caspase-8
A Parental and caspase-8 knockout (C8 ko) HeLa cells as well as caspase-8 deficient (C8 def.) Jurkat JB6 and the respective control cells were analysed by Western blotting for their deficiency in caspase-8 expression. Therefore, C-terminal and N-terminal antibodies against caspase-8 were used for immunoblotting. **B** Caspase-8 knockout HeLa, caspase-8 deficient Jurkat, and the respective control (CTRL) cell lines were treated for 30 min with 2 U/ml CD95L-Fc. CD95-DISC was immunoprecipitated from total cell lysates (TL) and analysed for DISC-associated proteins.

VIII.6. Caspase-10 promotes CD95L-mediated gene induction

Thus far, the impact of caspase-10 in death receptor-induced cell death signalling has been explored. Even if the data strongly reveal a regulatory instead of a pro-apoptotic function of caspase-10 in the DISC, it remains to be analysed which function the enzymatic activity of caspase-10 has in this process. We hypothesised that caspase-10 might have an additional function in DISC signalling which could be related to the recently discovered gene-inductive properties exerted by CD95 stimulation [97], [132], [134]. To prove the hypothesis, the impact of caspase-10 on death receptor-mediated gene induction was characterised by microarray analysis in HeLa cells. Therefore, cells were stimulated with CD95L and significant up-regulated genes were investigated that had a log2 fold change higher than 1 upon stimulation. A typical pattern of NF-κB-induced cytokines such as TNF, interleukins, different chemokines, and others were detected to be induced after CD95L treatment (Table 12). Interestingly, a subset of these genes were partially induced to lesser extend when caspase-10 was knocked down (compare log2 fold changes from shCTRL and shC10 in Table 12).

To further verify these data, selected genes were analysed by alternative methods at the mRNA and protein level. Interleukin-8 (IL-8) is a typical target of NF-κB [157], [158] and was therefore studied in detail. It was found that HeLa cells secreted high levels of IL-8 following CD95L stimulation (Figure 19A, left panel). Confirming the microarray data at the protein level, IL-8 secretion upon death receptor stimulation was drastically diminished after the knockdown of caspase-10 (Figure 19A, left panel). The overall sensitisation to the death ligand was thereby still detectable (Figure 19A, right panel). In addition, specific analysis on the mRNA induction of *IL-8* upon CD95L treatment by qPCR confirmed the microarray results, too. After the knockdown of caspase-10, less *IL-8* mRNA was expressed compared to control cells (Figure 19B). Interestingly, when caspase activity was blocked by zVAD-fmk, gene induction following CD95L stimulation was highly increased compared to death ligand treatment alone (Figure 19B). However, caspase-10 promoted *IL-8* induction independent of

the use of zVAD-fmk. Furthermore, CD95L-mediated induction of *TNF* mRNA was also analysed by qPCR and revealed to be decreased after the knockdown of caspase-10 (Figure 19C). Therefore, the microarray data and the specific analysis on IL-8 and TNF demonstrate that caspase-10 promotes DISC-mediated gene induction.

Table 12 CD95L-mediated gene induction analysis after the knockdown of caspase-10 by microarray

Gene symbol	log 2 fold change		p-values		Full name
	shCTRL	shC10	shCTRL	shC10	
CSF1	1.00	0.99	0.005	0.005	colony stimulating factor 1
IER5	1.01	0.69	0.002	0.020	immediate early response 5
SOD2	1.02	1.11	< 0.001	< 0.001	superoxide dismutase 2, mitochondrial
KDM6B	1.03	0.93	0.003	0.007	lysine (K)-specific demethylase 6B
NR4A2	1.04	0.76	< 0.001	0.002	nuclear receptor subfamily 4, group A
NUAK2	1.06	1.05	< 0.001	< 0.001	NUAK family, SNF1-like kinase, 2
DUSP5	1.08	0.91	< 0.001	< 0.001	dual specificity phosphatase 5
OLR1	1.12	1.21	< 0.001	< 0.001	oxidized low density lipoprotein
TNF	1.16	0.61	0.001	0.031	tumor necrosis factor
NFKBIE	1.16	0.79	< 0.001	0.002	nuclear factor of kappa light polypeptid
ICAM1	1.36	1.26	0.001	0.001	intercellular adhesion molecule 1
IRF1	1.46	1.45	< 0.001	< 0.001	interferon regulatory factor 1
EFNA1	1.51	1.59	< 0.001	< 0.001	ephrin-A1
JUN	1.60	1.12	< 0.001	0.001	jun proto-oncogene
NFKBIA	1.77	1.46	< 0.001	< 0.001	nuclear factor of kappa light polypeptid
IL1A	1.85	1.53	0.001	0.005	interleukin 1, alpha
TNFAIP3	1.96	1.56	< 0.001	< 0.001	tumor necrosis factor, alpha-induced pro
EGR1	1.99	1.17	< 0.001	< 0.001	early growth response 1
CXCL3	2.03	1.76	0.012	0.022	chemokine (C-X-C motif) ligand 3
IL6	2.07	1.71	< 0.001	0.001	interleukin 6 (interferon, beta 2)
CCL2	2.09	1.80	0.015	0.031	chemokine (C-C motif) ligand 2
PTX3	2.69	2.41	< 0.001	< 0.001	pentraxin 3, long
IL8	4.57	4.17	< 0.001	< 0.001	interleukin 8
CCL20	4.65	4.75	< 0.001	< 0.001	chemokine (C-C motif) ligand 20

Previously, our group reported that cFLIP inhibits death receptor-mediated gene induction [134]. Thus, cFLIP was depleted by CHX in combination with a caspase-10 knockdown and analysed for *IL-8* mRNA induction after CD95L treatment. Confirming the previous data, depletion of cFLIP by CHX resulted in a strong induction of *IL-8* (Figure 19D). When combined with caspase-10 knockdown, *IL-8* induction was diminished compared to CHX treatment alone. These data reveal that caspase-10 and cFLIP cooperate their function in regulating caspase-8-mediated cell death, but both proteins have differential abilities in gene induction.

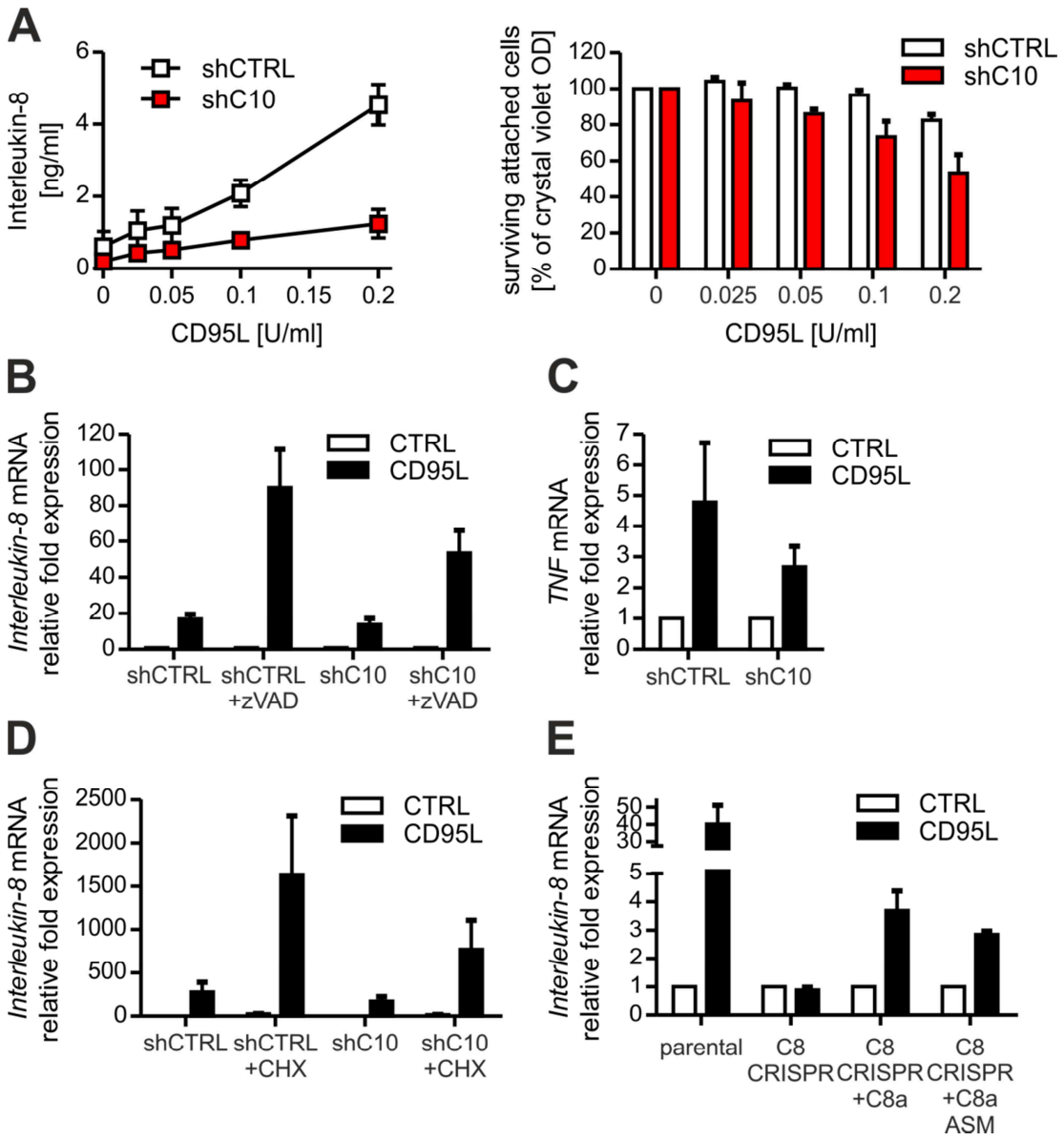

Figure 19 Caspase-10 promotes CD95L-mediated gene induction
HeLa cells expressing caspase-10 (shC10) or control (shCTRL) shRNA were treated 72 h with 0.5 µg/ml doxycycline (A-D). **A** Doublets were stimulated in media containing 0.5 % FBS with the indicated concentrations of CD95L-Fc for 24 h. Supernatants were analysed for secreted Interleukin-8 by ELISA. Cell viability was assayed using crystal violet staining. Shown are mean values ± SEM of three independent experiments. **B** HeLa ± shC10 cells were pre-starved for 4 h in media containing 0.5 % FBS followed by treatment with 10 µM zVAD-fmk (zVAD) for 1 h. Cells were stimulated with 0.1 U/ml CD95L-Fc for 3 h. RNA was isolated, reverse transcribed to cDNA and mRNA expression levels of *Interleukin-8* were analysed by qPCR. **C** *TNF* mRNA expression from zVAD treated cells from B were analysed by qPCR. **D** HeLa ± shC10 cells were treated with 5 µg/ml cycloheximide (CHX) for 2 h during starvation and further treated as described in B. DISC-mediated gene-induction was analysed in the presence of zVAD. **E** Parental and caspase-8 deficient (C8 CRISPR) HeLa cells were treated with 10 nM 4-hydroxytamoxifen for 6 h in media containing 0.5 % FBS to induce the expression of either control plasmid, caspase-8a (C8a), or the respective active site mutant (C8a ASM). Cells were stimulated with zVAD and CD95L-Fc as described in B and analysed for *Interleukin-8* mRNA expression by qPCR. Shown are mean values ± SEM of three independent experiments.

Next, the finding for the indispensable function of caspase-8 in placing upstream of caspase-10 and cFLIP in the DISC should be corroborated in light of DISC-mediated gene induction. Therefore, caspase-8a and its ASM were reconstituted in C8 CRISPR HeLa cells and analysed for their *IL-8* mRNA induction upon CD95L treatment. Gene induction was fully absent in caspase-8 knockout cells, whereas reconstitution with caspase-8a allowed for *IL-8* induction (Figure 19E). Of note, *IL-8* was induced to a lesser extent after the reconstitution compared to parental cells. Interestingly, re-expression of the caspase-8a ASM also induced *IL-8* expression after CD95L treatment, albeit the induction was very weak (Figure 19E).

As described for TNF-R-signalling, CD95L-mediated gene induction is driven by multiple protein kinases such as the IKK complex, JNK, or p38 MAP kinases [132], [159]. Cullen and colleagues found that NF-κB activation after DISC formation is mainly driven by IκBα. To characterise if caspase-10 promotes the activation of NF-κB through IκBα, the phosphorylation-dependent degradation of IκBα was analysed upon CD95L stimulation. When caspase-10 was knocked down by shRNA, DISC-induced degradation and phosphorylation of IκBα were delayed (Figure 20A). The quantification of relative IκBα degradation in control and caspase-10 knockdown cells from three independent experiments revealed the significance of this finding (Figure 20B). A20/TNFAIP3 inhibits TNF-mediated NF-κB activation and apoptosis and is potently induced upon NF-κB activation [160]. The microarray analysis showed that A20 gene induction is promoted by caspase-10 (Table 12). To confirm these data at the protein level, A20 expression was investigated under the same conditions as the IκBα degradation. In line with the microarray, CD95L-induced A20 expression was promoted by caspase-10 when compared to unstimulated expression levels (Figure 20A). However, the phosphorylation status of JNK as well as p38 was additionally analysed under conditions of CD95L stimulation and caspase-10 knockdown. Both proteins were phosphorylated following DISC stimulation, but caspase-10 was not affecting this process (Figure 20C). The transcription factor c-Jun was also found to be differentially regulated by caspase-10 in the microarray screen (Table 12), but these data could not be confirmed at the protein level (Figure 20C).

Taken together, caspase-10 is a negative regulator of caspase-8-mediated cell death, but it promotes DISC-mediated gene induction. Caspase-10 favours at least the degradation of IκBα resulting in enhanced activation of NF-κB and subsequent gene induction.

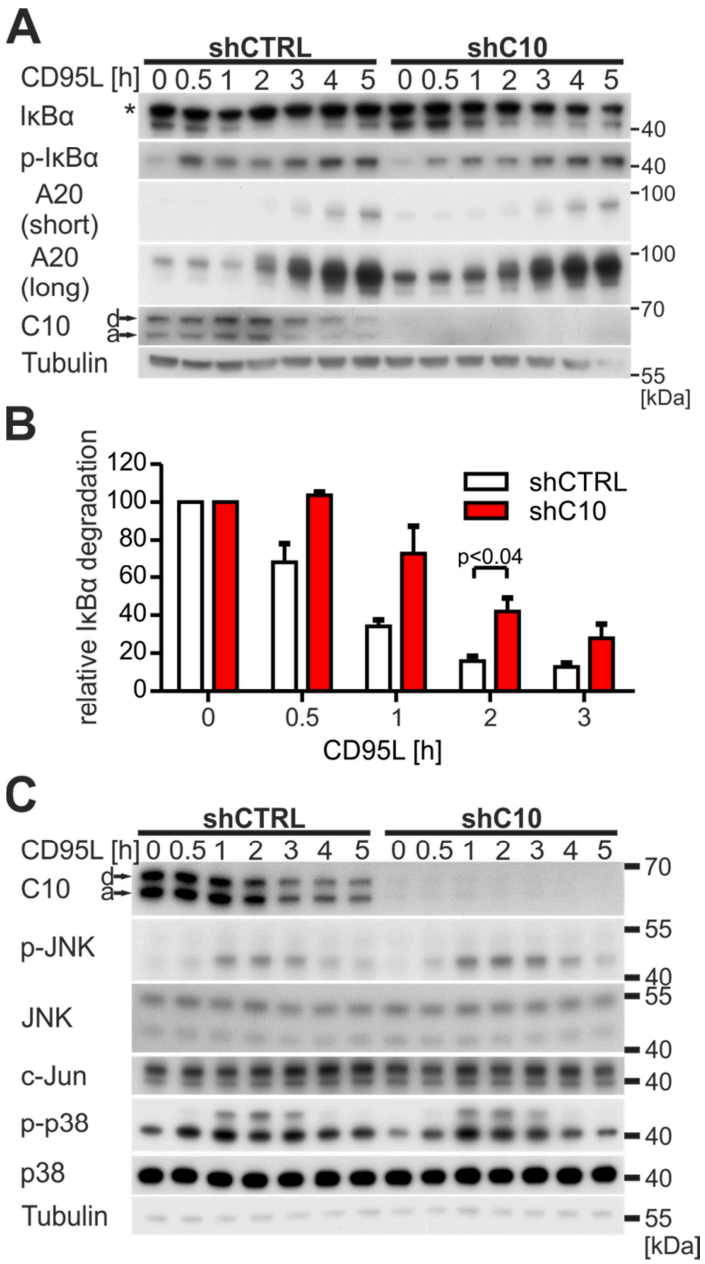

Figure 20 Caspase-10 promotes IκBα phosphorylation/degradation
A HeLa cells expressing caspase-10 (shC10) or control (shCTRL) shRNA were treated 72 h with 0.5 µg/ml doxycycline. Cells were pre-starved for 4 h in media containing 0.5 % FBS followed by treatment with 10 µM zVAD-fmk for 1 h and stimulated with 0.1 U/ml CD95L-Fc for the indicated time points. Caspase-10 knockdown efficiency, A20 expression, and IκBα phosphorylation as well as degradation were analysed by Western blotting. **B** Relative degradation of IκBα in respect to untreated cells was quantified. Shown are mean values ± SEM of three independent experiments. **C** Cells treated as described in A were analysed by Western blotting for the phosphorylation of JNK (p-JNK) and p38 (p-p38) as well as the expression of c-Jun after the knockdown of caspase-10. Significance level (p value) was measured by Student´s *t*-test.

VIII.6.1. Caspase-10 prevents RIPK1 from phosphorylation at serine166

The signalling pathway connecting DISC formation and IκBα degradation remains elusive. However, we analysed the impact of caspase-10 on DISC bound RIPK1 and on the soluble complex II which forms upon DISC stimulation. Therefore, the CD95-DISC was first precipitated following knockdown of caspase-10 and CD95L treatment and then a second immunoprecipitation enriching soluble caspase-8 was performed. The knockdown of caspase-10 had no influence on the association of RIPK1 to either the DISC or complex II (Figure 21). But strikingly, the phosphorylation of complex-bound RIPK1 at serine166 was drastically enriched in the absence of caspase-10. This effect was seen both in the DISC and in complex II (Figure 21, lanes 13-15 and 30-32). Indeed, as previously reported, the inhibition of caspase activity enhanced the assembly of RIPK1 to the complexes and solely $cFLIP_L$ was found within complex II [132], [133]. Furthermore, caspase-8 was again enriched in the DISC in the absence of caspase-10 at the early time point (Figure 21, compare lanes 3+4 and 10+11), whereas FADD and cFLIP were unaffected by caspase-10 in the DISC as well as in complex II. However, this experiment showed for the first time, that caspase-10 assembles with the soluble complex II upon CD95L stimulation (Figure 21, lanes 23-25).

Summing up, in contrast to previous thoughts, caspase-10 negatively regulates caspase-8-mediated cell death signalling. Caspase-10 and cFLIP act independently, but cooperate to block the recruitment of caspase-8 to the DISC and thereby delay its activation. Caspase-10 and cFLIP do not compete with caspase-8 in binding *via* FADD to the receptor. Caspase-8 represents the scaffold which is modulated by caspase-10 and cFLIP for further signalling. Caspase-10 rewires cell survival to NF-κB activation and gene induction by at least enhancing the degradation of IκBα. Even if the molecular mechanisms are elusive, caspase-10 enhances the dephosphorylation of RIPK1 in the DISC as well as in complex II.

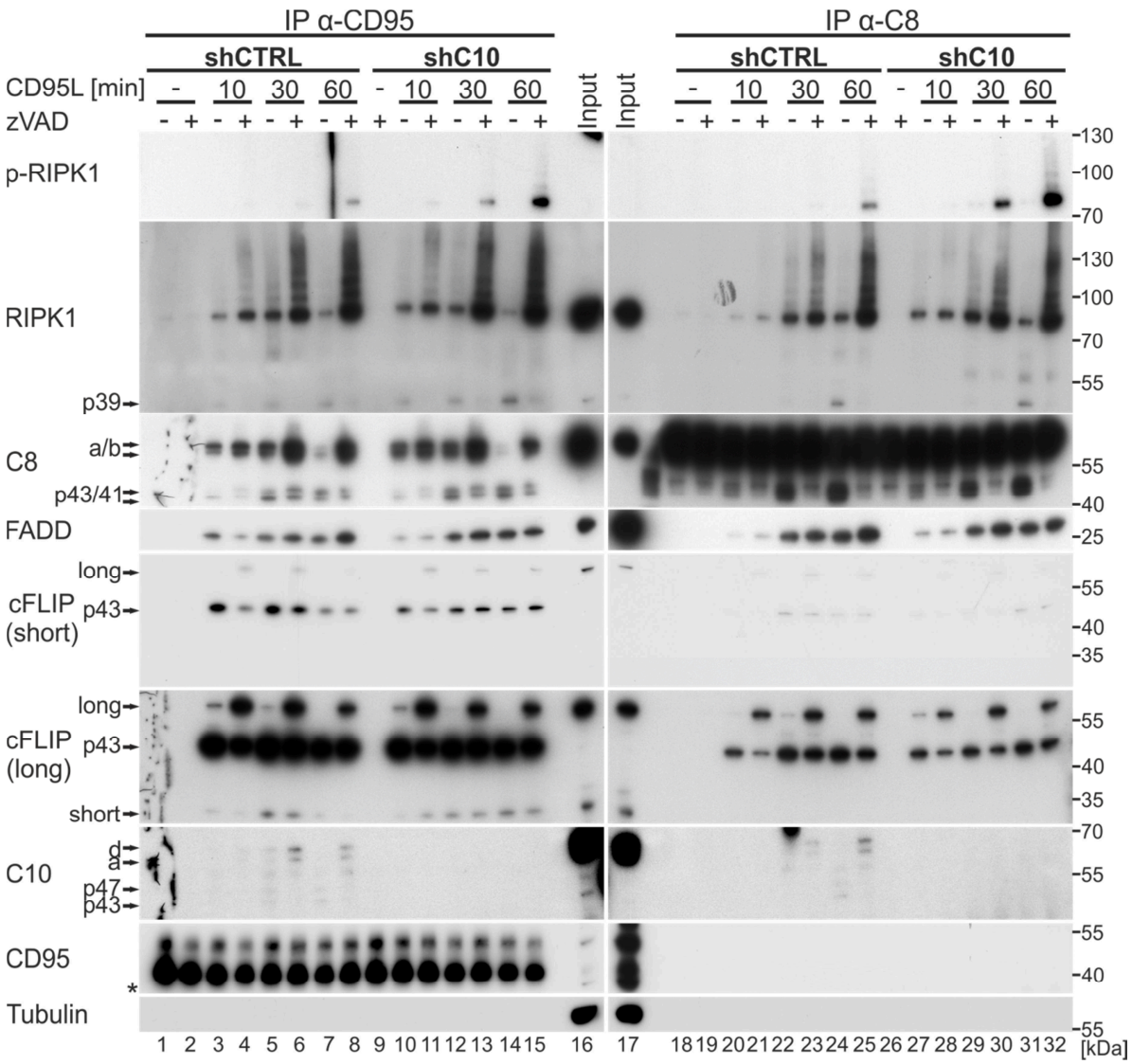

Figure 21 Caspase-10 inhibits RIPK1 phosphorylation
Caspase-10 (shC10) or control (shCTRL) shRNA expression was induced for 72 h in HeLa cells by the addition of 0.5 µg/ml doxycycline. Cells were pre-treated with 10 µM zVAD-fmk (zVAD) for 1 h followed by stimulation with 2 U/ml CD95L-Fc for the indicated time points. CD95-DISC was immunoprecipitated from total cell lysates. After the depletion of CD95, cell lysates were immunoprecipitated against caspase-8. Coprecipitated proteins bound either to the DISC or caspase-8 were analysed by Western blotting. Total lysates of untreated HeLa+shCTRL cells (input) served as loading control. The asterisk marks an unspecific band.

IX. Discussion

IX.1. Caspase-10 inhibits DISC-mediated cell death signalling

Research on the extrinsic apoptotic signalling pathway made substantial progress in the last years. Since its discovery, a lot of knowledge was gained about the DISC and its key players. However, studies analysing the DISC and its signalling pathways have mainly focussed on caspase-8 and its regulator cFLIP. In contrast, the function of caspase-10 is more controversial and less understood to date. Indeed, the absence of caspase-10 in rodents restricts the possibilities to study it; nevertheless, this thesis clearly demonstrates the requirement to study caspase-10 simultaneously with the other tandem DED proteins, caspase-8 and cFLIP.

The above presented results reveal a tight connection in the genetic regulation of caspase-10, cFLIP, and caspase-8. Modifying the expression of one of these proteins by either knockdown or knockout approaches frequently resulted in the counter regulation of at least one of the other tandem DED proteins. For example, knockout of caspase-8 led to down-regulation of cFLIP in HeLa cells (Figure 15B, 16, 17B). This effect was additionally observed when caspase-8 was knocked down by siRNA as well as shRNA (data not shown). Indeed, it appears to be logical that cells respond with a reduction of cFLIP as a consequence to loss of caspase-8. When the caspase is absent, its inhibitors are redundant. Furthermore, HaCaT cells counter-regulated the loss of caspase-10 by an up-regulation of cFLIP (Figure 11A+B) and caspase-8 deficient Jurkat cells expressed less caspase-10, as previously shown by Sprick and colleagues [146] and in figure 18B. These phenomena occur remarkably fast during the cultivation of the cells and demonstrate how closely these proteins are interdependent and critical for cell survival. Interestingly, genes encoding for caspase-10, caspase-8, and cFLIP are located on the same chromosome 2q33-q34. It can solely be speculated if there is a correlation between the close interdependence and the genetic localisation of these proteins, but genetic studies revealed that co-regulated genes tend to be clustered within the same genetic neighbourhood [161].

Moreover, the initial analysis on overexpressed caspase-10 shows that the approach to study DISC signalling might drastically determines the experimental results. In line with previous publications, caspase-10 seemed to be an pro-apoptotic initiator of CD95L-induced cell death (Figure 5) [139], [162]. When overexpressed, caspase-10 increases sensitivity to death receptor stimulation. However, only overexpression which was far apart from physiological expression levels promoted cell death (Figure 5). If caspase-10 was up-

regulated by solely the leakiness of the inducible vector system, this, still strong, overexpression had no effect on cell death response to CD95L stimulation (Figure 5). As further studies using siRNA as well as shRNA-mediated knockdown of caspase-10 show, caspase-10 is an inhibitor rather than a promoter of DISC-induced cell death (Figure 6+7). Therefore, the experimental approach is absolutely critical to study the DISC and its components.

An interesting finding is the spontaneous cell death induction upon overexpression of enzymatically inactive caspase-10 (indicated in Figure 5A). It is remarkable that a dead protease induces cell death whereas overexpression of caspase-10c has no effects on cell survival. DED proteins have been reported to assemble in DED filaments upon overexpression which induce apoptosis [163]. In contrast, only the overexpression of caspase-10c has been published to form filaments whereas other caspase-10 isoforms did not show this phenotype [164]. However, our observations cannot confirm this report as the overexpression of caspase-10c did not result in cell death induction (indicated in Figure 5A). Thus a potential formation of DED filaments by the overexpression of caspase-10a/d ASMs which might lead to cell death will not explain the observed spontaneous cell death induction. It remains an open question how the ASM of caspase-10 triggers cell death, but as this finding again based on overexpression experiments, we decided not to analyse the molecular mechanisms inducing spontaneous cell death under this conditions to avoid highly artificial results.

In this study, the focus was put on the function of caspase-10 by knockdown and knockout approaches to extrapolate the role of endogenous caspase-10 in cell death signalling. The data strikingly demonstrate that caspase-10 is a negative regulator of DISC-mediated cell death (Figure 6+7). Thereby, knockdown of caspase-10 sensitises against both death ligands, CD95L as well as TRAIL (Figure 6A+B). As both receptors have redundant mechanisms in the initiation of cell death, the mechanistic analyses of caspase-10 were proceeded solely in the CD95-DISC. Furthermore, the presented data reveal the specificity of the observed phenotype. The use of four different siRNAs plus a shRNA confirmed the anti-apoptotic function of caspase-10 and ruled out any off-target effect of RNA interference (Figure 6C+7A). In addition, the inhibitory function of caspase-10 was demonstrated by the use of three different apoptosis assays (Figure 7). Indeed, knockdown of caspase-10 did not sensitise each cell line analysed against the death ligand (Figure 9), but as these cell lines were not protected from cell death by the knockdown, the data exclude any pro-apoptotic function of caspase-10. All cell lines unaffected by the knockdown of caspase-10 expressed relatively high levels of caspase-10. Thereby, protein levels remaining after the knockdown were comparable to endogenous protein levels in cell lines which were sensitised by the loss

of caspase-10 (Figure 9). Therefore, we initially hypothesised that the protein levels remaining after successful knockdown might be sufficient to prevent from the observed phenotype. In line with this assumption, DISC-associated caspase-10 in HaCaT cells following knockdown was comparable to endogenous levels in HeLa cells (Figure 10). Since RNA interference will not fully deplete the gene of interest, caspase-10 has to be knocked out by CRISPR-Cas9-mediated recombination in these high expressing cell lines to prove this hypothesis. However, a thorough analysis of the CD95-DISC stoichiometry in caspase-10 knockdown HaCaT cells revealed that the loss of caspase-10 is compensated in these cells by an up-regulation of cFLIP (Figure 11A+B). When the knockdown of caspase-10 was combined with cFLIP knockdown, HaCaT cells were further sensitised against the death ligand compared to the single cFLIP knockdown (Figure 11C). How HaCaT cells circumvent the loss of caspase-10 by an up-regulation of cFLIP remains elusive; but, as these cells express only weak levels of cFLIP, caspase-10 seems to be the main regulator of cell death in these cells. Therefore, loss of caspase-10 would have drastic effects on the control of cell death. Thus, cFLIP might be up-regulated in HaCaT cells to guarantee cell survival. This finding once more emphasises the necessity to study all tandem DED proteins simultaneously.

The anti-apoptotic function of caspase-10 in the initiation of cell death is indeed very surprising and against current scientific knowledge. However, a more recent study has proposed a pro-survival function of caspase-10, as well. In line with our data, Lamy and colleagues described a more complex role of caspase-10 in controlling cell survival. They showed that caspase-10 inhibits autophagic cell death in multiple myeloma cell lines [145]. Simply the knockdown of caspase-10 favoured the induction of autophagy after six to nine days in their study. To prove that the induction of autophagic cell death is not interfering with our analysis, we screened HeLa cells for autophagy markers after the knockdown of caspase-10. The conditions used for the knockdown in our study did not induce any autophagy pathway (data not shown). However, the control of autophagy together with our finding demonstrates that the previous assumptions postulating a pro-apoptotic function of caspase-10 are not contemporary anymore.

When the impact of caspase-10 on DISC signalling was mechanistically studied, it ruled out that caspase-10 is blocking the recruitment of caspase-8 to the complex (Figure 12). As a result, less caspase-8 is processed and the overall cell death response is diminished (Figure 12A). Previous mass spectrometry analysis of the native CD95- as well as TRAIL-DISCs revealed that a single FADD molecule is able to recruit a multitude of caspase-8 molecules to the complex. These findings led to novel model in which caspase-8 elongates in DED chains in the DISC [77], [78]. In contrast to caspase-8, the respective ratio of

caspase-10 or cFLIP to FADD in the DISC is much lower, but this has not been examined in detail to date. A recent study suggested that short DED proteins, other than caspase-10 or cFLIP, do not modify chaining of caspase-8 [88]. Based on our data, it is a likely scenario that caspase-10 disturbs chain formation of caspase-8, which would explain why caspase-8 is enriched in the DISC in the absence of caspase-10. However, to prove this hypothesis, further quantitative mass spectrometry analyses are required to address the impact of caspase-10 on caspase-8 chaining. Another possibility for caspase-10 in inhibiting DISC-mediated cell death could be that caspase-10 forms inactive heterodimers with caspase-8 upon DISC stimulation. Heterodimerisation between caspase-8/-10 and cFLIP has been reported [91], [92], but it is highly controversial if the resulting heterodimer has pro- or anti-apoptotic functions [reviewed in 165]. However, it is difficult to address this hypothesis, because recombinant caspase-10 as well as caspase-8 undergo autoproteolytic cleavage during purification of the precursor forms. Thus, it is intricate to analyse heterodimer formation and its enzymatic activity.

IX.2. Caspase-10 and cFLIP cooperate to inhibit caspase-8

The regulation of cell death by caspase-10 and cFLIP is tightly linked. As discussed above, HaCaT cells counter regulate the knockdown of caspase-10 by an up-regulation of cFLIP. When both inhibitors of caspase-8 were studied in HeLa cells which express in contrast to HaCaT cells adequate levels of cFLIP (Figure 9D), it showed that cFLIP blocks cell death signalling much more efficient as compared to caspase-10 in these cells (Figure 13A). Of course, as the molarities of these proteins are unknown in HeLa cells, it cannot be ruled out who is the stronger inhibitor of caspase-8. However, both proteins independently inhibit CD95-mediated cell death signalling (Figure 13A). Furthermore, cFLIP and caspase-10 interfere with the recruitment of caspase-8 to the DISC and its activation in the complex (Figure 14). As described above, it can solely be speculated if cFLIP and caspase-10 block chain elongation of caspase-8 and it would need further mass spectrometry analysis to prove this hypothesis. Interestingly, DISC-bound caspase-10 is strongly enriched when cFLIP is down-regulated, whereas the *vice versa* effect does not occur (Figure 12A and 14). This indicates that the affinity of cFLIP to the DISC is higher as compared to caspase-10. In a model in which cFLIP and caspase-10 cooperate in modulating caspase-8 chaining in the DISC, cFLIP might be faster recruited to the complex and competes with caspase-8 in chain elongation.

An obvious question rises at this point: What is the differential function of cFLIP and caspase-10 if both act as inhibitors of caspase-8 with different affinities? In contrast to the

short half-life of cFLIP, the stability of caspase-10 protein is unaffected by translational inhibitors such as CHX for up to five hours (Figure 13B). It is therefore a likely hypothesis that in a physiological setting in which protein translation is mostly inhibited by stress signals, e.g. DNA damage [166], caspase-10 could serve as a cellular backup for cFLIP. Thus, it potentially prevents from excessive caspase-8 activation and cell death even in the absence of cFLIP.

cFLIP is absolutely essential in the regulation of cell fate. cFLIP knockout mice are embryonic lethal [85] and even the knockdown of cFLIP in cellular systems induces spontaneous cell death [86], [87]. We observed the same phenomenon when we introduced the knockdown of cFLIP in HaCaT and HeLa cells (data not shown). By drastically decreasing the molarity of cFLIP siRNA the knockdown was accomplished without inducing spontaneous cell death (Figure 13A). To mechanistically study the impact of cFLIP in the DISC, CHX was used to decrease cellular protein levels. CHX has obviously further effects on cell death signalling than solely the depletion of cFLIP (Figure 13D). However, CHX-induced depletion of cFLIP confirmed the data collected by knockdown approaches (Figure 13C) and we reasoned that the impact of CHX on the formation of the DISC is mainly limited to cFLIP. As all other key players in the DISC were unaffected by CHX treatment, we supposed it as a suitable tool to study the function of cFLIP in the DISC.

IX.3. Caspase-10 and cFLIP do not compete with caspase-8 for DISC recruitment

It is currently assumed that cFLIP competes with caspase-8 for binding *via* FADD to the death receptor [81], [167], [168]. Thereby, less caspase-8 is activated in the DISC resulting in diminished cell death induction. The above presented data now lead to the conclusion that the same function is linked to caspase-10. However, the data collected by the use of caspase-8 knockout cells demonstrate that caspase-8 is absolutely critical for the formation of the DISC (Figure 16+17). They furthermore show that caspase-8 has to bind upstream of caspase-10 and cFLIP to the DISC and it is relevant for the formation and stability of the complex. Thus, the DISC is not forming in the absence of caspase-8 (Figure 16). Re-expression of wild type as well as mutant caspase-8 restored the recruitment of caspase-10 and cFLIP. Most importantly, even receptor-bound FADD levels were recovered after the re-expression (Figure 17). Interestingly, the enzymatic activity of caspase-8 is dispensable in this process. Independent if the cells express wild type or mutant caspase-8, both variants enabled DISC formation. This leads to the conclusion that caspase-8 has a scaffold function in the DISC which is necessary and critical for DISC formation and/or stability.

These findings underline the elusive and in part controversial role of caspase-10 and its homologue. In contrast to caspase-8, caspase-10 is not essential for DISC signalling. When caspase-8 is absent, death receptor-induced cell death is fully blocked (Figure 15B). Thus, endogenous caspase-10 cannot substitute for caspase-8. The presented data demonstrate that caspase-10 and cFLIP do not compete with caspase-8 in binding to FADD. They support a new model in which caspase-8 binding *via* FADD primes the DISC and caspase-10 as well as cFLIP negatively regulate caspase-8-mediated cell death signalling downstream of caspase-8 but within the DISC. Another fact supports this model as well. Interestingly, caspase-10 and cFLIP are mainly found as processed fragments in the DISC whereas full length caspase-8 is usually present (Figure 12A, 14, 16, and 18B). This indicates that full length caspase-8 is able to linger in the DISC whereas the regulators, caspase-10 and cFLIP, are rapidly processed. All these findings strengthen the hypothesis that caspase-10 and cFLIP control DED chain elongation of caspase-8.

In the past, caspase-8 deficient Jurkat cell lines have been a fundamental tool to study DISC-mediated apoptosis and gene induction. In contrast to the here presented data, a previous report using these cells reported that caspase-10 binds to the DISC in the absence of caspase-8 [146]. The screening of caspase-8 deficient Jurkat cells revealed that they are not deficient in caspase-8 (Figure 18A). Cell death signalling might be heavily impaired in these cells, but they do express a truncated version of caspase-8 which can still be recruited to the DISC (Figure 18B). Therefore, the DED structure seems to be intact and thus allows further recruitment of caspase-10 and cFLIP to the complex. Indeed, nor caspase-10 neither cFLIP are processed in the DISC, but it is highly important that even the weak expression of the truncated caspase-8 version allows for DISC formation and stability.

Based on the data, the collected knowledge from caspase-8 deficient Jurkat cells should be critically re-evaluated. These cells do express caspase-8 and they repressed their expression levels of CD95 (Figure 18B). As this dramatically impacts cell death signalling, it is likely that some data have been misinterpreted. The finding that caspase-8 deficient Jurkat cells form a DISC, even if it is weak, might explain why others observed that these cells died upon heavy stimulations with CD95L [146], [147], [169]. It can solely be speculated what the relevant cell death trigger is, but interestingly, caspase inhibition by zVAD-fmk does not prevent it [169]. As Holler and colleagues suggested, RIPK1 might potentially be activated upon CD95L stimulation in these cells and induces necroptotic cell death. But in contrast to what they suggested, DISC-induced and RIPK1-mediated cell death depends on caspase-8. Without caspase-8, the DISC would not form and RIPK1 could not been activated.

However, the most striking finding in light of caspase-8 is that the association of FADD to the death receptor seems to be dependent on caspase-8 (Figure 16+17B). FADD dissociates

from the receptor in the absence of caspase-8. Thus, caspase-8 might stabilise the binding of FADD with its death domain to the receptor. This highly important and in part bizarre finding needs further analysis to clarify the molecular mechanisms underlying the stabilisation of FADD by caspase-8. Previously, it has been reported that FADD interaction with the DISC is regulated by ubiquitination [170]. The E3 ubiquitin-protein ligase makorin-1 (MKRN1) mediates ubiquitination of FADD, thus marking it for proteasomal degradation. Depletion of MKRN1 resulted in the stabilisation of DISC-associated FADD and enhanced cell death signalling. It is a likely scenario that caspase-8 binding prevents FADD from ubiquitination by e.g. blocking the binding site of MKRN1 and therefore ensures the stability of the DISC.

Taken together, the presented data support a model in which caspase-8 binding to the DISC is an indispensable upstream event in DISC signalling. Subsequently, caspase-10 and cFLIP independently bind to caspase-8 and both negatively regulate further DED chain elongation of caspase-8.

IX.4. Caspase-10 promotes DISC-mediated gene induction

Apart from the necroptotic signalling pathway which can solely be activated when caspase activity is actively inhibited, two major DISC-induced signalling pathways have been studied in the last years. DISC-mediated gene induction is the second important pathway which can be activated either concomitant to apoptotic cell death (Figure 19A and [132]) or independent of apoptotic caspase activation, e.g. by the use of caspase inhibitors [134], [171], [172]. Interestingly, the blockade of caspase activity highly increases the gene inductive properties of the DISC (Figure 19B).

When the impact of caspase-10 on DISC-mediated gene induction was studied, the data demonstrated for the first time that caspase-10 promotes cytokine expression (Table 12 and Figure 19). Of note, knockdown of caspase-10 did not fully abolish gene induction and only a subset of CD95L-induced genes were affected by the depletion of caspase-10, but even small changes in gene regulation might have dramatic effects on protein expression and cell signalling. For instance, the repression of *IL-8* mRNA detected by microarray analysis was very small following knockdown of caspase-10 (Table 12) and only slightly stronger when the same conditions were analysed by qPCR (Figure 19B). However, the secretion of IL-8 protein was up to five times less in the absence of caspase-10 (Figure 19A). Further investigations on the impact of caspase-10 on DISC-mediated cytokine production at the

mRNA (*TNF*, Figure 19C) and protein (A20, Figure 20A) level confirmed the gene inducing properties of caspase-10.

Our group previously demonstrated that cFLIP blocks death receptor-mediated gene induction in epithelial cells [134]. This report was confirmed when cFLIP was depleted by the use of CHX (Figure 19D). Importantly, knockdown of caspase-10 compensated the hyperactivation of cytokine production following depletion of cFLIP (Figure 19D). Thus, in contrast to cFLIP, caspase-10 promotes gene induction. As observed in DISC-induced apoptotic cell death, caspase-10 and cFLIP act independent in cytokine regulation. Confirming our DISC model for the critical upstream binding of caspase-8 to FADD, caspase-8 knockout cells fully lost their gene-inductive properties (Figure 19E). However, when caspase-8 was reconstituted in these cells, they were again able to induce the expression of *IL-8* mRNA, albeit to a lesser extend (Figure 19E). The inability to re-express caspase-8 to the same extent when compared to parental cells (Figure 17) might be a potential explanation for the reduced *IL-8* induction. Interestingly, an impaired *IL-8* induction was also observed when the enzymatic inactive mutant of caspase-8a was re-expressed in the knockout cells (Figure 19E). Caspase-8 protein levels in this cells reached endogenous levels (Figure 17), thus indicating that the scaffold function of caspase-8 is necessary for DISC-mediated gene induction, but its enzymatic activity is required for full activation of the NF-κB activation pathway.

Similar to the detailed TNF signalling pathway, TRAIL and CD95L induce cytokine expression *via* the activation of different protein complexes such as IKK/IκBα, p38 MAPK, or MEK/ERK [159]. A more recent study reported that p38, JNK, as well as ERK have no or just little impact on CD95-mediated gene induction, whereas the knockdown of the NF-κB p65 subunit profoundly attenuated cytokine production [132]. The here presented data confirm these findings. Caspase-10 promoted gene induction by enhancing the degradation of IκBα (Figure 20A and B). In contrast, other signal transducer such as JNK or p38 were unaffected by the knockdown of caspase-10 (Figure 20C). The activation of JNK, p38, or ERK is potentially a secondary event during DISC-induced cytokine production and thus is not or just slightly influenced after DISC stimulation. The microarray screen showed that a number of immediate early response genes such as IER5, EGR1, or JUN are upregulated following CD95L treatment (Table 12). They potentially induce a feedback loop in which JNK, p38, or ERK are activated.

Cullen and colleagues showed that RIPK1 interconnects DISC formation and cytokine production analogous to TNF signalling [132]. They further reported that RIPK1 assembles in

a soluble complex II with caspase-8, $cFLIP_L$, and FADD after DISC stimulation. It is unknown if RIPK1 is activated in the DISC or in complex II, but its kinase activity seems to be redundant in this pathway. Therefore, RIPK1 revealed to act as a scaffold to promote gene induction [132]. The here presented data now demonstrate that caspase-10 is also recruited to complex II (Figure 21). Besides its function in the DISC, caspase-10 has no impact on the composition of complex II. It does not interfere with the association of RIPK1 to the DISC or complex II, but it efficiently inhibits phosphorylation of RIPK1 at serine166 in both complexes (Figure 21). It is thus far unknown which consequences the phosphorylation of RIKP1 has at this position. It was shown that phosphorylation of RIPK1 and RIPK3 stabilises their association with the ripoptosome and activates pro-necroptotic kinase activity [173]. If RIPK1 comprises a scaffold function in DISC-mediated gene induction, as Cullen and Co. suggested, it might be of advantage to keep RIPK1 dephosphorylated. Thus its necroptotic kinase activity would be reduced and the activation of NF-κB might be enhanced. In contrast, it has been reported that phosphorylation of RIPK1 by IKKα/β prevents it from entering the cell death complexes in TNF signalling [174]. However, it remains to be elucidated how caspase-10 interferes with RIPK1 and how it is involved in either promoting the dephosphorylation or preventing the phosphorylation of RIPK1.

Taken together, the data presented in this thesis are consistent with a DISC model (Figure 22) in which caspase-8 binding *via* FADD to the receptor is an indispensable initiation step in DISC formation. Thereby, caspase-8 stabilises the association of FADD with the receptor and allows further recruitment of other caspase-8 molecules which assemble in DED chains in the DISC. Next, caspase-10 and cFLIP are recruited *via* caspase-8 to the complex and both negatively modulate the activation of caspase-8 in the DISC. In contrast to cFLIP, caspase-10 promotes DISC-mediated gene induction by interfering with the phosphorylation status of RIPK1, enhancing the degradation of IκBα, and thereby the activation of NF-κB.

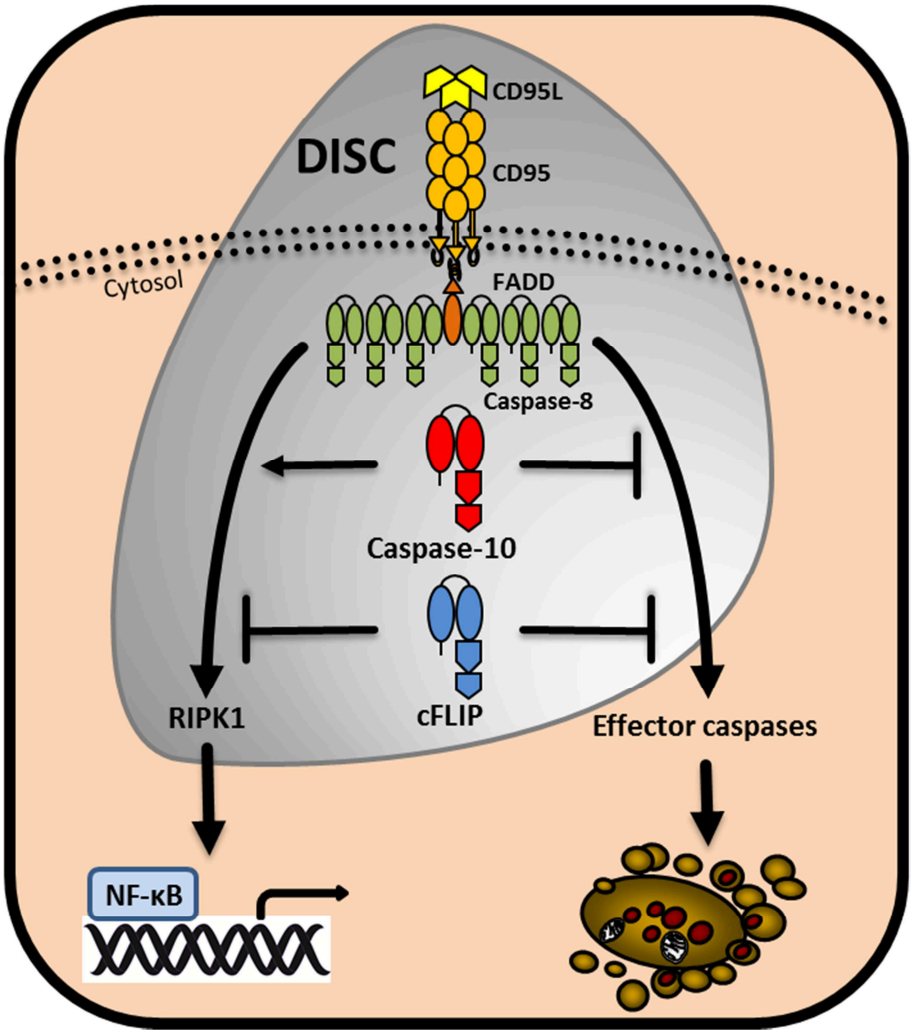

Figure 22 Proposed working model of caspase-10 function in the DISC

The newly described function of caspase-10 as an inhibitor of caspase-8-mediated cell death and a facilitator of DISC-mediated gene induction separates the function of cFLIP and caspase-10 in the DISC. The presented data clearly delineates that caspase-10 is not an initiator of DISC-induced apoptosis as previously believed [81], [167], [168]. These findings change our current understanding of the extrinsic apoptotic signalling pathway and open new possibilities in studying other tandem DED protein containing complexes. The ripoptosome as well as TNF complex II have been reported to contain caspase-8, cFLIP, and caspase-10 [98], [99], [175]. In light of the here presented findings, this complexes can be studied for the differential functions of the different tandem DED proteins. If caspase-10 interferes with the phosphorylation of RIPK1 in the DISC, does this happen in the ripoptosome as well and has this an influence on cell death response? Furthermore, this study hint at a possibility to modulate the expression levels of caspase-10 as a therapeutic oncological target. As inflammatory gene expression has been shown to favour tumour formation and progression [176], specific inhibitors of caspase-10 could be used to block cytokine expression.

Moreover, the indispensable function of caspase-8 in DISC signalling may facilitate future therapies for the treatment of cancer. Currently developed treatments of death receptor agonist may require robust expression of caspase-8 to allow killing of tumour cells.

X. References

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XI. Abbreviations

%	Percent
°C	Degree celcius
µ	Micro
4-HT	4-hydroxytamoxifen
A	Alanine
ALPS	Autoimmune lymphoproliferative syndrome
APAF1	Apoptotic protease-activating factor 1
ASM	Active site mutant
Bak	Bcl-2 antagonist or killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology 3
Bid	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
C	Cysteine
C10	Caspase-10
C8	Caspase-8
CAD	Caspase-activated DNase
CD40L	Cluster of differentiation 40 ligand
CD95L	Cluster of differentiation 95 ligand
cFLIP	Cellular FLICE-like inhibitory protein
CHX	Cycloheximide
cIAPs	Cellular inhibitor of apoptosis proteins
CRD	Cysteine-rich domain
CTRL	Control
d	Days
DD	Death domain
DED	Death effector domain
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Doxy	Doxycycline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
FADD	Fas-associated protein with death domain
FCS	Fetal calf serum
FLICE	FADD-like IL-1β-converting enzyme

g	Gramm
GAPDH	Glyceraldehyde 3-phosophate dehydrogenase
h	Hour
Hepes	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1L interacting protein
IKK	IκB kinase
IL-8	Interleukin-8
IMS	Intermembrane space
IκB	Inhibitor of NF-κB
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
l	Liter
LB	Lysogeny broth
m	Milli
M	Molarity
MAPK	Mitogen-activated protein kinases
min	Minute
MKRN1	E3 ubiquitin-protein ligase makorin-1
MLKL	Mixed lineage kinase domain-like
MOMP	Mitochondrial outer membrane permeabilization
n	Nano
n.s.	Not significant
Nec	Necrostatin-1
NEMO	NF-κB-essential modulator
NF-κB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells
NIK	NF-κB-inducing kinase
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PLAD	Pre-ligand-binding assembly domain
PVDF	Polyvinylidene difluoride
RIPK	Receptor-interacting protein kinase
RNA	Ribunucleic acid
RT	Room temperature
S	Serine
sh	Short hairpin
SHARPIN	Shank-associated RH domain-interacting protein
si	Small interfering
SMAC	Second mitochondria-derived activator of caspases

TAB1	TAK1 binding protein 1
TAK1	Transforming growth factor β-activated kinase 1
tBid	Truncated Bid
TL	Total cell lysate
TLR	Toll-like receptor
T_m	Melting temperature
TNF	Tumor necrosis factor
TNFAIP3/A20	TNFα-induced protein 3
TNF-R2	TNF receptor 2
TRADD	TNF receptor type 1-associated death domain protein
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R	TRAIL receptor
TWEAK	TNF-related weak inducer of apotosis
UbcH5	Ubiquitin conjugating enzyme E2 D1
UV	Ultra violet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
x g	Gravitational acceleration
XIAP	X-linked inhibitor of apoptosis protein
zVAD-fmk	z-Val-Ala-DL-Asp-fluoromethylketone

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