Molecular analysis of death receptors mediated apoptotic and non-apoptotic signalling pathways in human keratinocytes

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Abstract

Death receptors such as CD95 and TRAIL-R1/R2 induce apoptosis in many cells, but can also activate non-apoptotic signalling pathways (NF-□B as well as mitogen-activated protein kinases (JNK, p38). Different isoforms of FLIP ($cFLIP_S$ and $cFLIP_L$) inhibit different steps in death receptor (DR)-associated activation and maturation of procaspase-8. We reasoned that the cleavage of cFLIP, in turn, could differentially influence nonapoptotic DR signals. Thus, we established stable HaCaT cells expressing different cFLIP isoforms ($cFLIP_S$, $cFLIP_L$) or mutants of $cFLIP_L$ that are either uncleavable by capase-8 ($cFLIP_{D376N}$) or generated after stimulation by DISC-associated caspase-8-mediated cleavage ($cFLIP_{p43}$). All isoforms/mutants of $cFLIP_L$ blocked death ligand (DL)-mediated apoptosis, whereas a distinct cleavage pattern of caspase-8 was detected in the DISC. Only cells expressing full length $cFLIP_L$ (irrespective of cFLIP cleavage) sufficiently induced proteolysis of caspase-8 to its p43/41 fragments. In contrast, $cFLIP_S$ or $cFLIP_{p43}$ blocked procaspase-8 cleavage.

Furthermore, We examined DR-induced non-apoptotic signals. TRAIL or CD95L activated JNK within 15 minutes. MAPK p38 was induced in a biphasic manner. Interestingly, all cFLIP isoforms/mutants completely inhibited the late DL-induced activation of p38 or JNK. Moreover, cFLIP isoforms or mutants blocked DL-mediated IκBα phosphorylation, NF-κB activation, and induction of the target gene IL-8.

Conversely knockdown of cFLIP isoforms in primary human keratinocytes not only resulted in increased apoptotic cell death but also enhanced DL-induced NF-κB activation and also its target gene IL-8 induction underscoring the physiological relevance of cFLIP for these DL-induced signals.

In summary, cFLIP isoforms are not only potent inhibitors of DL-induced apoptosis, but also block DL-triggered activation of NF-κB. The inhibition of non-apoptotic signalling by CD95 and the TRAIL death receptors by FLIP proteins might be of crucial importance during tumorigenesis of keratinocyte skin cancer in order to avoid activation of innate or adaptive immune responses in tumor cells acquiring apoptosis resistance

Zusammenfassung

Todesrezeptoren, wie CD95 und TRAIL-R1/R2, können nicht nur Apoptose in vielen Zellen induzieren, sondern aktivieren auch nicht-apoptotische Signalwege (wie NF-κB und Mitogen-aktivierte Proteinkinasen (JNK, p38)). Die FLIP Isoformen ($cFLIP_S$ und $cFLIP_L$) inhibieren verschiedene Schritte der Todesrezeptor (TR)-assoziierten Aktivierung und Prozessierung von Procaspase-8. Wir glauben, dass die Spaltung von cFLIP, die nicht-apoptotische TR-Signalgebung differentiell und bedeutend beeinflusst. Aus diesem Grund wurden HaCaT Zellen etabliert, die verschiedene cFLIP-Isoformen ($cFLIP_S$, $cFLIP_L$) oder Mutanten von $cFLIP_L$ stabil exprimieren. Insbesondere wurde die $cFLIP_{D376}$-Mutante, welche von Caspase-8 nicht prozessiert werden kann sowie das DISC-assoziierte Spaltprodukt von $cFLIP_L$ ($cFLIP_{p43}$) stabil in HaCaT Zellen integriert.
Sowohl die Isoformen als auch die Mutanten von $cFLIP_L$ inhibieren die Todesligand (TL)-vermittelte Apoptose, wobei ein distinktes Spaltmuster von Caspase-8 im DISC detektiert wurde. Lediglich die Zellen, die die $cFLIP_L$-Isoform exprimieren (unabhängig von der cFLIP-Spaltung) induzieren substantiell die Proteolyse von Caspase-8 zum entsprechenden p41/p43 Spaltprodukt. Im Gegensatz dazu inhibieren $cFLIP_S$ oder $cFLIP_{p43}$ die Procaspase-8-Spaltung. Im nächsten Schritt analysierten wir die TR-induzierten nicht-apoptotischen Signalgebungen. Beide TL, TRAIL und CD95L, aktivieren JNK innerhalb von 15 Minuten. Die MAPK p38 wird in biphasischen Schritten aktiviert. Interessanterweise inhibieren alle cFLIP-Isoformen und -Mutanten vollständig die späte TL-induzierte Aktivierung von p38 und JNK. Des Weiteren inhibieren die cFLIP Isoformen und Mutanten die TL-vermittelte IκBα-Phosphorylierung, die NF-κB-Aktivierung und die Induktion des Zielgens IL-8.
Zusammenfassend zeigt diese Studie, dass die cFLIP Isoformen nicht nur potente Inhibitoren der TL-vermittelten Apoptose sind, sondern auch die TL-vermittelte nicht-apoptotische Signalgebung, wie NF-κB oder MAPK (JNK oder p38), inhibieren. Diese Daten zeigen, dass die Spaltung von $cFLIP_L$ oder Caspase-8 im DISC weder mit einer verstärkten NF-κB Signalgebung assoziiert

ist noch für die inhibitorische Funktion der cFLIP Isoformen in der TR-induzierten NF-κB oder MAPK Aktivierung notwendig ist. Weiterhin lassen die Daten dieser Studie vermuten, dass die cFLIP-Isoformen eine bedeutende Funktion für die Inhibition der TR-induzierten nicht-apoptotischen Signale übernehmen. Dieser Mechanismus könnte damit für die Tumorigenese von keratinozytären Hautkrebs von Bedeutung sein und auch eine Erklärung liefern warum maligne Krebsformen die Eliminierung durch die angeborene oder adaptive Immunantwort umgehen können.

Acknowledgements

It is an honour for me to acknowledge everyone involved in making this day come true in my life. I am deeply grateful to Professor Dr. Martin Leverkus, who accepted me as his PhD student and gave me the opportunity to work on such an exciting topic of death receptor signalling pathways. Had it not been for his constant belief and encouragement, I would not have successfully steered through every phase of my doctoral work. I shall always stay indebted to him for his valuable critical inputs from the initial to the final level of my PhD study. This association enabled me to develop a better understanding and appreciation for the subject. Suffice to say, that he truly has been my friend, philosopher and guide during all these years of my study. Thank you for everything, Martin.

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I.INTRODUCTION

1. Skin function and role in apoptosis

1.1 Skin function and homeostasis

The skin is the largest organ in the body with multiple functions, occupying 12-15% of body weight and having a surface area of 1-2 meters. There are two distinct layers that make up the skin: the dermis and the epidermis. The skin cells are constantly renewed by their respective progenitor cells. Epidermis is a thick keratinised and stratified squamous epithelium consisting of four distinct cell types that include keratinocytes, melanocytes, langerhans cells and merkel cells. Notably keratinocytes are the major cell type of the epidermis and contain keratin which is a fibrous protein responsible for protection of the epidermis. Melanocytes are specialized cells located at the base of the epidermis and synthesize the pigment melanin. Melanin shown to protect the cell nucleus from the destructive effects of UV-radiation. The dermis is a connective tissue layer under the epidermis, and contains nerve endings, sensory receptors, capillaries, and elastic fibers [1;2].

The integumentary system (the organ system that protects the body from damage and comprises the skin and its appendages) has multiple roles in skin homeostasis including protection, temperature regulation, sensory perception, biochemical synthesis, and absorption. All body systems work in co-ordination to maintain the internal conditions essential to the proper functioning of the body. Being the largest organ in the human body with diverse roles, the skin functions mainly in protecting the body against foreign pathogens. The skin is also involved in providing thermal insulation and in temperature regulation, touch and sensation and synthesis of vitamins D and B.

Homeostasis implies a balance between cell growth and cell death. This balance is essential for the development and maintenance of multi cellular organisms. Homeostasis is controlled by several mechanisms including apoptosis, a process by which cells condemned to death are completely eliminated [1].

Keratinocytes are the major cell type of the epidermis constituting 95% of all cells found on the outer skin. They express most of the important components of the apoptosis machinery and can activate this mechanism following exposure to various signals. Pathological modulation of apoptosis signalling in the skin may therefore lead to disorders such as psoriasis, alopecia areata, or skin cancer. Several death receptors are expressed in the keratinocytes and a function has been attributed to TNF-R1, CD95, TRAIL-R1, or TRAIL-R2 [3].

TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) was shown to overcome the relative resistance of senescent keratinocytes to apoptosis, and it was suggested that TRAL may play an important role in epidermal homeostasis. Human keratinocytes undergo apoptosis following treatment with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) via surface-expressed TRAIL receptors 1 and 2. In addition, TRAIL triggers non-apoptotic signalling pathways such as activation of the transcription factor NF-κB, particularly when TRAIL-induced apoptosis is blocked. The intracellular protein $cFLIP_L$ shown to interfere with TRAIL-induced apoptosis at the Death-inducing signalling complex (DISC) in many cell types, including keratinocytes [4;5].

1.2 Apoptosis resistance in the skin leads to skin cancer and other diseases

Several pathological and patho-physiological factors disturb the integrity of molecules involved in crucial cellular processes such as proliferation, survival and programmed cell death (apoptosis). Such alterations in the tumor cells and their surrounding stroma impact the cellular homeostasis, ultimately leading to the manifestation of (potentially metastatic) keratinocyte-derived skin tumor. The role of apoptosis resistance has now been clearly established as an important necessity for tumor development [6]. Apoptosis also represents an important cancer defence mechanism as keratinocytes that may have accumulated mutations or sustained other genetic damage as a consequence of exposure to

UV radiation or oxidative damage are eliminated by this process [2]. Resistance to apoptosis can be acquired by cancer cells through a variety of strategies; the most common being the loss of a pro-apoptotic regulator through mutation involving the p53 tumor suppressor gene. The resulting functional inactivation of p53 protein is observed in more than 50% of human cancers thereby leading to the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade. This often leads to impaired apoptotic machinery where the p53 tumor suppressor gene function is lost.

Tumor progression may require the upregulation of different anti-apoptotic regulators such as cFLIP, Bcl-x_L and various IAP molecules (cIAP1, cIAP2, XIAP and Livin) or conversely the loss of function of several pro-apoptotic proteins (see Figure 1). Intriguingly, a shift in the balance between these pro-and anti-apoptotic proteins may finally be sufficient to avoid tumor progression by apoptosis induction or may conversely promote tumor progression at the interface between tumor and the surrounding stroma [7].

Skin diseases	Apoptosis	Mechanism	Potential therapies
Sunburn	Increased	UVB-induced apoptosis Cytokine secretion	-
Toxic epidermal Necrolysis (TEN)	Increased	Upregulation of Fas ligand TNF production	Antibody blocking (anti-Fas, anti-TNF-intravenous Ig')
Graft-versus-host Disease (GVHD)	Increased	Fas-mediated lymphocyte Killing TNF-production	Antibody blocking (anti-Fas, anti-TNF)
Psoriasis	Decreased	Keratinocyte senescence Increased TNF (paradoxical) IL-15. Survivin Bcl-x_L expression	TNF inhibitors Anti IL-15 antibody
Skin cancer (Basal-cell and squamousCell carcinoma)	Decreased	p53 mutation or deletion Decreased death receptors, Stat3 activation. Survivin, Bcl-2, Bcl-x_L	Introduction of p53 TRIL Bortezomib Stat3 decoy

Table-1 Summary of skin diseases with deregulated apoptosis [2].

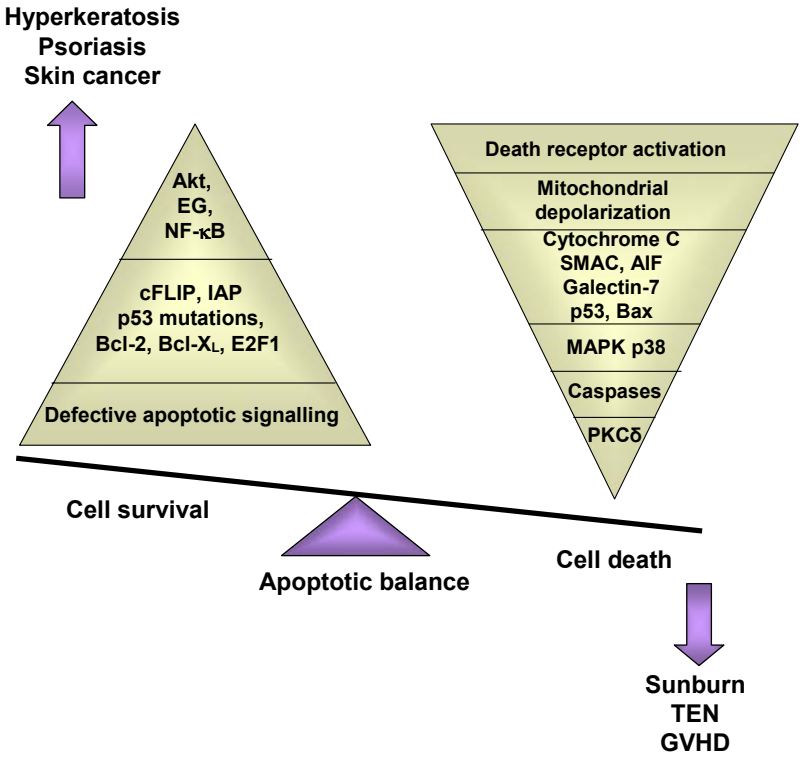


Figure-1 Apoptotic balance in keratinocytes is mediated by multiple factors at multiple levels.

1.3 Apoptosis – functions and mechanisms (Extrinsic and Intrinsic)

Apoptosis is a form of cell death that plays an important role in the regulation of growth in normal adult tissues and in early development and disease. The term "Apoptosis" is derived from a Greek word which means "falling off", suggesting it might play a role opposite to that of mitosis. Apoptosis is regulated by different physiological stimuli and occurs in many species and tissues. Besides apoptosis there is another form of cell death, termed as necrosis, which is a result of acute injury to the tissue [7].

Notably, apoptosis may not be harmful to the host and is also necessary for normal physiological functions. The term apoptosis is sometimes considered synonymous with programmed cell death which somehow implies a lethal genetic

program. It is a widely accepted notion that derangement of apoptotic regulation in development could result in structural and functional abnormalities while a lack

Sequence of events in cells undergoing apoptosis [7;8].

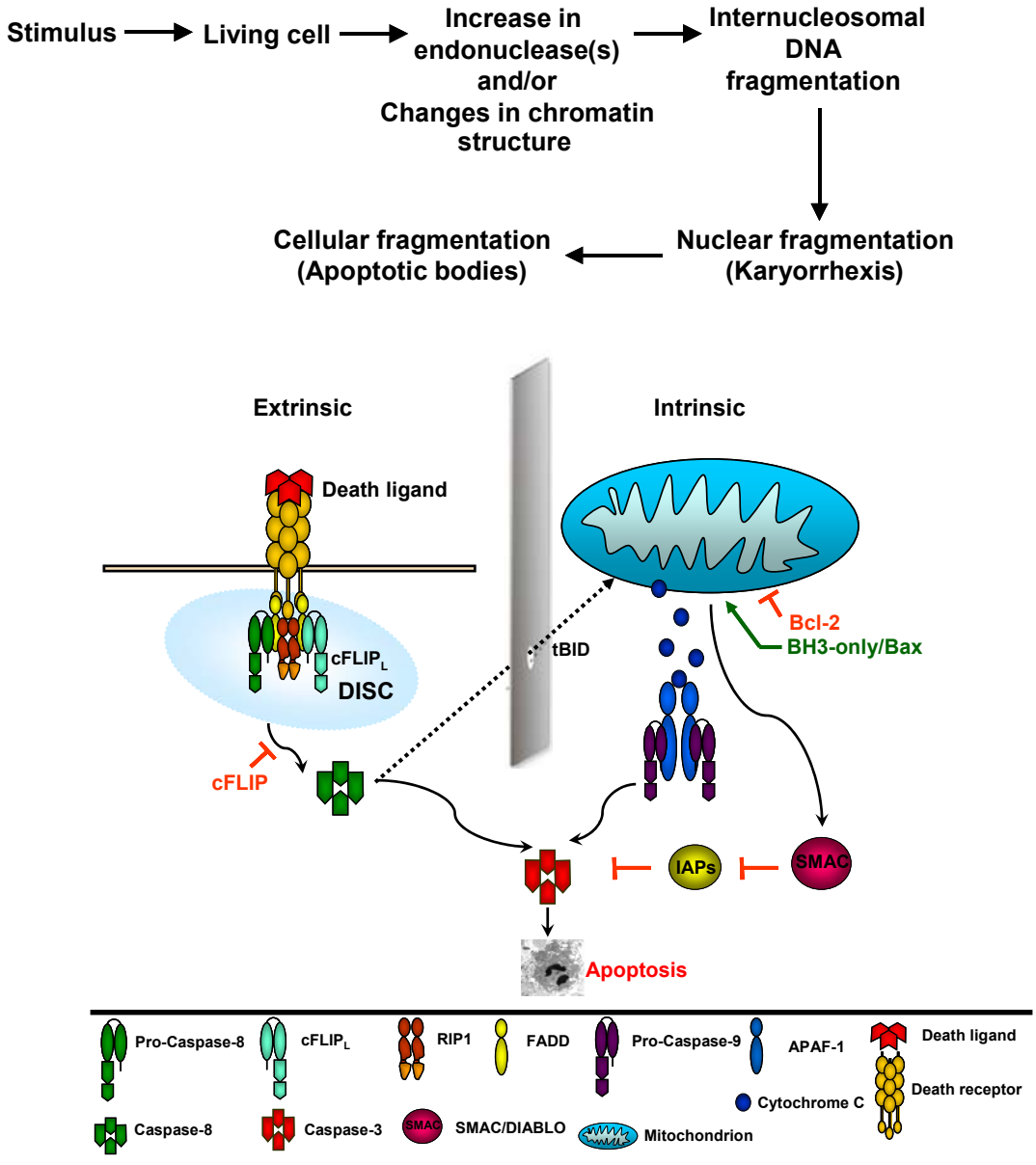


Figure-2 Extrinsic and intrinsic cell death signaling pathways.

of its tight regulation in growth could result in the formation of tumors. Defects in apoptosis strengthen both tumorigenesis and drug resistance, and often leads to minimal success of chemotherapeutic treatment of tumors. On the other hand, the fact that apoptosis is present in tumors suggests that its induction could be used as a mode of therapy [7].

The hallmarks of apoptosis are a series of typical biochemical and morphological features, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighboring cells. Furthermore, activation of a class of cysteine proteases called caspases, determines the phenotype of cell death and plays a major role in the execution of apoptosis [7].

Apoptotic cell suicide can be initiated by a plethora of stimuli that generally belongs to one of the two known cell death signalling pathways.

The main components of apoptotic pathways are as follows,

1. Apoptosis triggered by internal signals - the intrinsic or mitochondrial pathway
2. Apoptosis triggered by external signals - the extrinsic or death receptor pathway

The majority of proteolytic cleavage events that manifest the apoptotic phenotype are mediated by ‘effector’ caspases, such as caspase-3 and caspase-7, which become fully activated when the large and small subunits that are harbored within the dormant pro-enzyme are liberated after endoproteolysis by upstream ‘initiator’ caspases, such as caspase-8, caspase-9 or caspase-10. These initiator caspases themselves are activated by autoproteolytic mechanisms after facilitated oligomerization. In the extrinsic pathway, this event occurs as a consequence of ligand binding to ‘death receptor’ complexes, which leads to the recruitment of procaspase-8 via the adapter molecule FADD/Mort1 that forms a complex called Death inducing signalling complex (DISC) (see Figure 3). Interestingly, this pathway is modulated by the availability of molecular components (putative type I and type II cells differ in this regard) and dominant-

negative regulators such as decoy receptors and cFLIP or IAPs. Notably for the majority of cell death stimuli, the intrinsic death signal is communicated through the mitochondrion by less described mechanism, which leads to several changes in the organelle, including the release of polypeptide agents, such as cytochrome C and second mitochondria-derived activator of caspases SMAC/DIABLO. This pathway is highly dependent on the stoichiometry of anti versus pro-apoptotic Bcl-2 family members. When enabled, caspase-9 activation occurs via the oligomerization mediator APAF-1, which requires cytochrome C for the appropriate conformational change. Furthermore, SMAC/DIABLO protein helps to cross another apoptosis checkpoint by sequestering the inhibitors of apoptosis proteins (IAPs), which would otherwise block the actions of downstream effector caspases even in the presence of proteolytic maturation [8] (see Figure 2). Furthermore, signals from the extrinsic pathway may require the assistance of the intrinsic pathway, for example, when the signal strength is weak or when the IAP barrier is high and the actions of SMAC/DIABLO then become necessary [9;10].

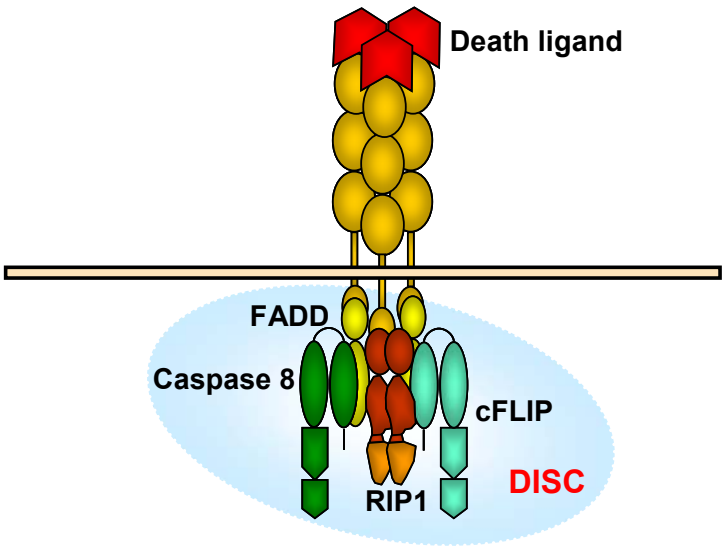


Figure-3 Extrinsic pathway is initiated by death ligands such as TRAIL/CD95L and upon stimulation, death receptors recruits DISC-associated proteins at DISC.

1.4 HaCaT keratinocytes (primary and transformed keratinocytes)

The HaCaT keratinocyte cell line is derived from the spontaneous transformation of the adult human keratinocytes and is shown to be associated with sequential chromosomal alterations *in vitro*. However, it is not necessarily linked to major defects in differentiation. The HaCaT cell line is derived from adult skin and maintains a complete epidermis differentiation capacity. This cell line has been shown to be immortal (>140 passages), has a transformed phenotype *in vitro* (clonogenic on plastic and in agar) but remains non-tumorigenic. Notably different keratin proteins (Nos. 1 and 10) and other known keratinocyte markers (involucrin and filaggrin) are commonly found and expressed in HaCaT keratinocytes. Thus, HaCaT cell line is the first permanent epithelial cell line derived from an adult human skin that exhibits normal differentiation properties and serve as a promising tool to study regulation of keratinization in human skin cells [1]. We chose HaCaT keratinocytes as our cellular system because it expresses all the necessary death receptors (TRAIL R1/2 and CD-95R). Its ideal cellular system to study the role of intracellular regulators (cFLIP isoforms and its cleavage products) and their role in DR-mediated signalling pathways.

On an interesting note, TRAIL induces apoptosis in primary keratinocytes (PK) and transformed keratinocytes (TK) in a dose dependent manner. Previous studies have shown that despite the increased surface expression of TRAIL R1/R2 in PK rather than TK, PK and TK exhibit significant differences in sensitivity to TRAIL with as much as 5-fold higher concentrations of TRAIL required to kill PK compared to TK [11]. These studies clearly indicate that it is mainly the intracellular regulators which are responsible for the observed relative resistance of PK to TRAIL. Furthermore, ectopic expression of $cFLIP_L$ leads to TRAIL resistance of TK. Taken together, these data suggest an important role for $cFLIP_L$ in determining differential sensitivity of PK versus TK to TRAIL. This mechanism of resistance to TRAIL-mediated apoptosis may indeed important in many different cellular systems [11]. Notably, proteasome inhibitors enhance TRAIL sensitization without interfering with the NF-κB activation in primary human

keratinocytes. This sensitization was not mediated at the receptor-proximal level of TRAIL DISC formation or caspase-8 activation but was shown to be effective further downstream [5].

2. Pro-apoptotic properties of death receptors

2.1 Death receptors (TRAIL-R1/2 and CD95-R system)

The extrinsic apoptotic pathway is initiated by the ligation of death receptors (TNFR1/2, CD95-R and TRAIL-R1/2) with their respective ligands (TNF, CD95L, and TNF-related apoptosis inducing ligand or TRAIL) all of which are members of the TNF super family. Three different subclasses of death receptors are recently described (see Figure 4). The first subgroup includes TRAILR1/2, CD95-R, which are apoptosis promoting cell surface receptors having an N-terminal cysteine rich domain and a C-terminal death domain. The other subgroups include two decoy receptors (TRAIL-R3/R4), which lack the cytosolic domain and are unable to induce apoptosis**.** Interestingly, the known apoptosis-inducing members of the TNF family, CD95L and TNF are detrimental upon systemic administration. However, the property of TRAIL to kill tumor cells more efficiently than normal cells prompted many research groups to test the anti tumor potential of TRAIL *in vivo*. Notably, recent reports suggest that TRAIL can be used to inhibit the tumor growth *in vivo* with out any toxicity problems [10].

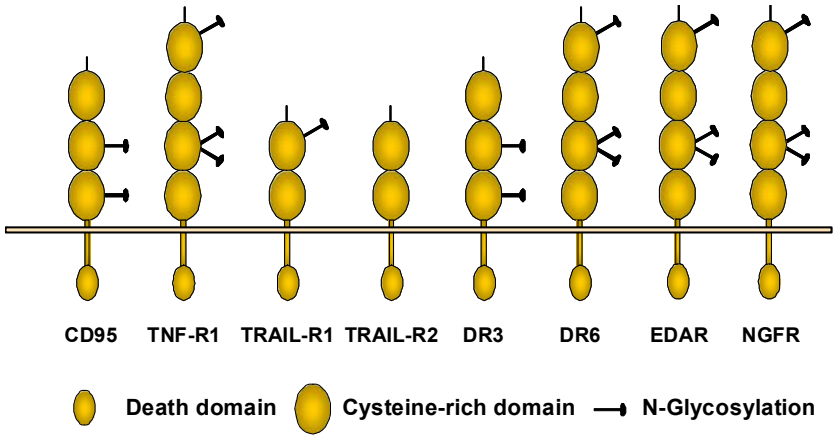


Figure-4 Structure of death receptors and members of TNF superfamily.

Furthermore, these data also suggest a novel alternative anti-tumor strategy by using TRAIL against Bcl_2 or Bcl-x_L overexpressing tumors, preferably one that combines the cytotoxic potential of TRAIL with chemo- and/or radiotherapy. Therapeutic targeting of tumor cells from two different angles could likely diminish the chances of the tumor from developing into therapy resistance variants. Interestingly, such a combinatorial therapy with TRAIL and chemotherapeutic agents has shown to act synergistically via distinct apoptotic pathways. Notably TRAIL preferentially makes use of the direct caspase pathway bypassing the mitochondrial pathway while most of the chemotherapeutic and radiation agents exert their apoptotic potential primarily via the mitochondrial apoptotic pathway. Thus, the treatment of cancer by direct induction of apoptosis in tumor cells, with anti-APO-1 antibody may soon become a reality as clinical trials with TRAIL/APO-2L are proposed to get underway soon [10].

2.2 DISC components and mode of DISC assembly

The interaction of death receptors with their cognate ligands results in receptor trimerization and clustering of the death receptors, which in turn facilitates the recruitment of effector molecules that include the adaptor protein FADD (Fas-associated death domain–containing protein). FADD enables the recruitment of the pro-form of the initiator caspase-8 to the death receptors and leads to the formation of a multi-molecular signalling complex known as DISC (see Figure 3). The interplay between the DISC components (see Figure 5) are mediated by conserved protein motifs which interact in a homotypic mode. Two prominent domains involved in these interactions are the death domain (DD) and the death effector domain (DED). Interestingly the formation of the DISC allows the dimerization of the pro-caspase-8 molecules, leading to the stimulation of its proteolytic activity, cleavage and release of the active caspase-8 molecules into the cytosol. Furthermore, this active enzyme then initiates apoptosis in death receptor-sensitive cells by cleaving substrates such as Bid, a pro-apoptotic member of the Bcl_2 family, or caspase-3 [4].

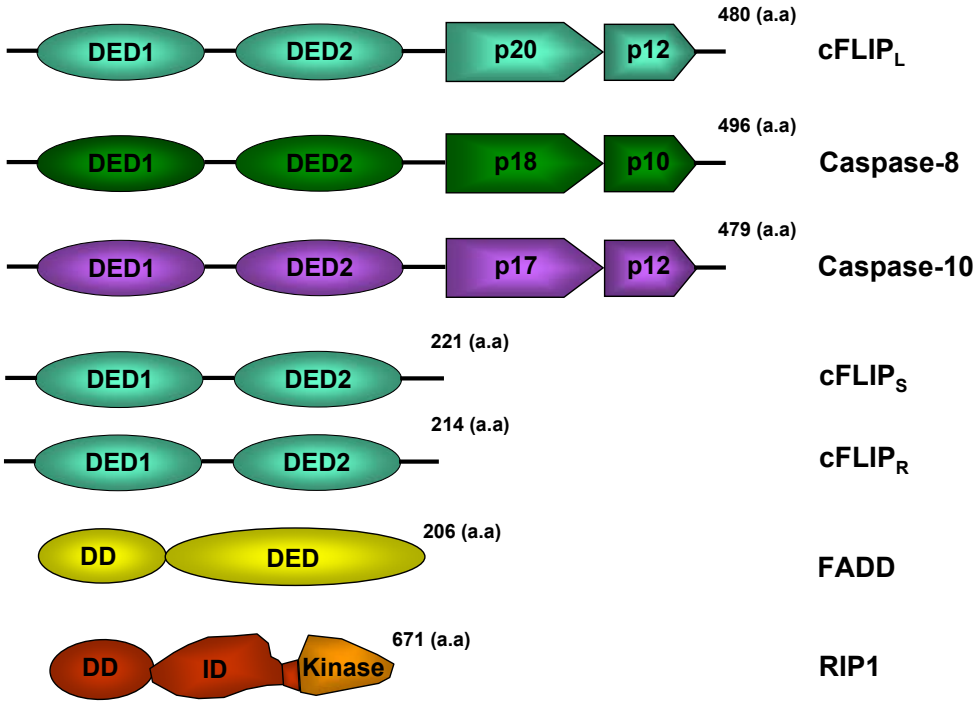


Figure-5 Overview of the domain structure of DISC associated proteins.

3. Negative regulators of apoptosis

3.1 Decoy receptors and Inhibitor of apoptosis proteins (IAPs)

Decoy receptors

Besides two pro-apoptotic receptors (TRAIL-R1/R2), TRAIL can also bind to three different anti-apoptotic receptors which include TRAIL-R3 and TRAIL-R4, which are membrane bound receptors known to suppress TRAIL-induced apoptosis. Osteoprotegerin (OPG), which is a secreted protein known to bind TRAIL and inhibit TRAIL induced apoptosis. These three decoy receptors (TRAIL-R3/R4, OPG) bind to TRAIL but do not mediate signalling. Normal tissues usually express all four of the TRAIL receptors, and this balance prevents TRAIL-induced apoptosis. Interestingly, cancer cells on the other hand often lack the expression of the decoy receptors (DcRs). Furthermore this imbalance favors the increased pro-apoptotic receptor induced sensitivity to TRAIL in cancer cells. On the contrary specific expression of decoy receptors in normal tissues possibly explains their resistance to TRAIL-induced apoptosis [12].

Furthermore decoy TRAIL receptors DcR1, DcR2 (TRAIL-R3/R4) were shown to be down regulated specifically in tumor cell lines. To study the possible hypothesis for tumor-specific down-regulation of decoy TRAIL receptors, many research groups extended their studies to promoter regions and methylation changes in the CpG islands of these receptors. All normal tissues were found to be completely un-methylated, whereas in the tumor cell lines, the promoter regions for decoy receptors DcR1, DcR2 (TRAIL R3/R4) were frequently hypermethylated. The modifications such as hypermethylation pattern in the promoter regions of decoy receptors (TRAIL-R3/R4) are shown to be prerequisite for downregulation of these receptors in most of the tumor cells [13].

Inhibitor of apoptosis proteins (IAPs)

The IAP family of proteins, including XIAP, cIAP1, cIAP2, neuronal apoptosis inhibitor protein (NAIP), and survivin are highly conserved through evolution. These proteins are characterized by the presence of baculoviral IAP repeat (BIR)-binding domains and RING zinc finger domain. XIAP, cIAP1, and cIAP2 have been shown to bind specifically to the terminal effector cell death proteases such as caspases-3 and -7, but not to the known proximal proteases, caspases-8, -1, or –6. Notably, various other reports suggest that IAP family proteins including XIAP, cIAP1, cIAP2 did not prevent caspase-8-induced proteolytic activation of pro caspase-8. However, they subsequently inhibit caspase-3, -6, -7 directly by blocking cytochrome C induced activation of pro-caspase-9 [14] and subsequently stop downstream apoptotic events such as further activation of caspases. These findings demonstrate that IAPs can suppress different apoptotic pathways by inhibiting distinct caspases. Interestingly these reports also identify pro–caspase-9 as a new target for IAP-mediated inhibition of apoptosis [15;16].

3.2 cFLIP isoforms/mutants and structure and mechanism of action

The human homolog of vFLIP is also called cFLIP/FLAME-1/IFLICE/ Casper/CASH/MRIT/CLARP/Usurpin. Several reports suggest that many cFLIP splice variants are expressed at the mRNA level although at the protein level only

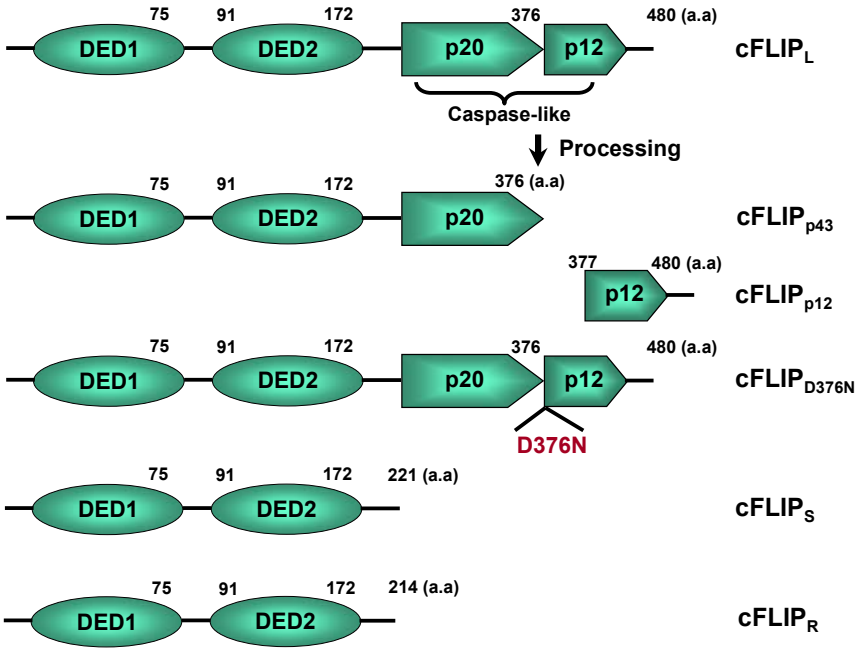


Figure-6 Schematic structure of known cFLIP isoforms and its cleavage products.

three endogenous forms, $cFLIP_L$ and $cFLIP_S$, and $cFLIP_R$ could be detected so far [17] (see Figure 6). $cFLIP_L$ is structurally similar to procaspase-8, since it contains two death effector domains and a caspase-like domain. However, this domain lacks residues that are important for the catalytic activity of caspase-8, most notably the cysteine within the active site. The short form of cFLIP, $cFLIP_S$, structurally resembles vFLIP. Despite the functional analysis of mice deficient for cFLIP, in which the role of cFLIP in cardiac development was elucidated but the definitive physiological role of this molecule still remains controversial [4;18]. Recent reports suggest that high expression of cFLIP promotes tumor growth and facilitates immune escape by tumors. In addition to these studies, it has also been shown that mouse embryonic fibroblasts deficient of cFLIP clearly display an increased sensitivity toward death receptor mediated apoptosis. Several other reports suggest an involvement of cFLIP in the modulation of the immune

response. Interestingly, when present in high amounts $cFLIP_L$ and $cFLIP_S$ have blocked death receptor mediated apoptosis by interfering with caspase activation in the DISC in many cellular systems [4;18-22].

3.3 cFLIP regulates DR mediated apoptosis

Upon stimulation, death receptors CD95-R (APO-1/Fas), TRAIL-R1 recruit the adaptor molecule FADD/MORT1, procaspase-8, and the cellular FLICE-inhibitory proteins (cFLIP), RIP1 (Receptor interacting protein1) into the DISC. According to the proposed induced proximity model, procaspase-8 is activated in the DISC in an autoproteolytic manner by two subsequent cleavage steps. cFLIP proteins exist as long ($cFLIP_L$) and short ($cFLIP_S$) splice variant forms are both capable of protecting cells from death receptor mediated apoptosis. In ectopically expressed immune cells (BJAB cells), both $cFLIP_S$ and $cFLIP_L$ block procaspase-8 activation at the DISC. However, cleavage is blocked at different steps; $cFLIP_L$ allows the first cleavage step of procaspase-8, leading to the generation of the p10 subunit. In contrast, $cFLIP_S$ completely inhibit cleavage of procaspase-8. Interestingly, the cleavage product $cFLIP_{p43}$ (lacking the p12 subunit) also prevents cleavage of procaspase-8. In contrast, a non-processable mutant, $cFLIP_{D376N}$ allows the first cleavage of procaspase-8. In conclusion, both cFLIP proteins prevent caspase-8 activation at different levels of procaspase-8 processing at the DISC [20]. These results indicate that $cFLIP_L$ induces a conformational change in procaspase-8 that allows partial but not complete proteolytic processing, whereas in contrast $FLIP_S$ even prevents partial procaspase-8 activation at the DISC [4;20;23].

3.4 Physiological and pathological functions of cFLIP

The generation of cFLIP knockout mice has provided interesting insights into the physiological roles of this protein. Notably cFLIP knockout mice die *in utero* (E10.5–11.5) from cardiac failure associated with severely impaired heart development. Furthermore, studies on cFLIP deficient mice revealed that cFLIP has two distinct physiological roles. Firstly, cFLIP is required for the proper

development of embryos, playing a particularly important role in the development of the heart. Secondly, cFLIP is essential for regulating death receptor–mediated apoptosis induced by death ligands (CD95L, TRAIL) engagement [24]. These results provide insights into the definitive protective role of cFLIP against death receptor-induced apoptosis. Intriguingly, these studies clearly demonstrate that cFLIP is yet another death receptor signal transducer like FADD and caspase-8 which were previously reported to have an important function in heart development [24].

Moreover, elevated expression of cFLIP has recently been linked to the escape of tumors from immune-surveillance and their resistance to early activated T-cells and differentiating macrophages. These observations suggest that death receptor signalling pathways are important biological processes and the modulation of cFLIP expression and death receptor signalling could be therapeutically beneficial for patients with cancers or immune system disorders. In cFLIP deficient cells, the “off” switch for apoptotic signalling is lost, thereby making these cells a valuable tool for investigating death receptor signalling [6;24].

3.5 Inhibitors of anti-apoptotic factors can be used as therapeutic agents

Its known that inappropriate regulation of apoptosis contributes to many human diseases, including cancer, autoimmune and neurodegenerative disorders. Over the past decade, suppression of apoptosis has been recognized as a key element in cancer progression, and therapies designed to relieve the apoptosis blockade in cancer cells are currently undergoing clinical trials [25].

Cancer cells negatively regulate caspases and thereby suppress apoptosis by three known fundamental mechanisms. Firstly, they prevent activation of caspase zymogens (proenzymes), after which they neutralize active caspases (active enzymes) and finally suppress expression of genes encoding caspases or caspase-activating proteins. Moreover, endogenous suppressors of caspases include the inhibitor of apoptosis proteins (IAPs), of which eight are reported to be

expressed in humans. It is known that all the 10 IAPs are an evolutionarily conserved family of proteins that directly bind to active caspases. They either suppress their protease activity or target them for destruction by ubiquitination and subsequent proteasome- mediated degradation.

Subsequent studies identified various inhibitory agents that suppress the expression of anti-apoptotic proteins such as cFLIP, IAPs (XIAP, cIAP1, cIAP2), anti-apoptotic Bcl_2 family members and also known anti apoptotic transcriptional factor NF-κB and Akt. Interestingly, these inhibitors sensitize many tumor cells to death ligand (CD95L, TRAIL) induced apoptosis [26]. This led researchers to identify a series of synthetic compounds, which could block or inhibit the anti-apoptotic property of some potential proteins [26].

In an attempt to design synthetic compounds which inhibit IAPs, Bertrand et.al, investigated the role of an IAP antagonist termed as AEG40730. This inhibitor was shown to bind BIR3 domains of IAPs with nM affinity and was revealed to have profound apoptotic sensitization to death ligands. Strikingly, the IAP antagonist was shown to induce caspase-8 dependent apoptosis in a subset of cancer cell lines through activation of a TNF-α autocrine loop. Moreover, reducing cellular levels of cIAP1 and cIAP2 using AEG40730 or siRNA resulted in a dramatic reduction in RIP1 ubiquitination and caused RIP1 to switch from functioning as a prosurvival scaffold molecule to a pro-apoptotic adaptor protein [27]. Recent reports suggests that loss of cIAP results dramatic sensitization to CD95 and interestingly this form of cell death can only be blocked with a combination of RIP1 kinase and caspase inhibitors. These findings further demonstrate the fundamental role of CD95 signalling and provide support for a physiological role of caspase independent DR-cell death [16].

4. Death receptors induced non-apoptotic signalling pathways

An increasing number of reports suggest the possible role of death ligands such as CD95L, TRAIL in apoptosis independent functions. These functions include induction of proliferation in T-cells and fibroblasts, liver regeneration, chemokine

production, differentiation of dendritic cells and neurons as well as recently reported invasiveness promoting capacities. Accordingly, it has been found that CD95L and TRAIL can induce non-apoptotic signalling pathways, such as the various MAP kinase cascades, PKC and the NF−κB. The activation of NF-κB is counteracted by apoptotic caspases and is strongly enhanced in cells, which are protected from apoptosis by caspase inhibitors or expression of anti-apoptotic members of the Bcl2 family. The NF-κB activation can be triggered by direct stimulation of death receptors CD95 and TRAIL[4;28;29]. In addition, there is growing evidence that suggests a physiologic role for CD95 in regulating neuronal development, growth, differentiation, and regeneration in the CNS. Interestingly, CD95 is not only highly expressed but also shown to mediate non-apoptotic signalling pathways in other tissues such as heart, pancreas and colon. All these above lines of evidences highlight the significant role of death receptors (TRAIL/CD95-R) in non-apoptotic signalling events [21].

4.1 Death receptors activate NF-κB activation

The nuclear factor-kappa B (NF-κB)/Rel family of proteins are composed of a group of dimeric transcription factors that have an outstanding role in the regulation of inflammation and immunity. The control of transcription by NF-κB proteins can be quite relevant to the death receptor functions in three ways. First, induction of anti-apoptotic NF-κB dependent genes critically determines cellular susceptibility toward apoptosis induction by TRAIL-R1/R2 and other death receptors. Each of the multiple known NF-κB inducers therefore has the potential to interfere with death receptors induced cell death. Second, TRAIL and some of its receptors are inducible by NF-κB, disclosing the possibility of autoamplifying TRAIL signalling loops. Third, the death receptors (TRAIL/CD95-R) can activate the NF-κB signalling pathway directly.

The phylogenetically conserved family of nuclear factor-kappaB (NF-κB) transcription factors in mammals are composed of more than 10 defined homo-

and heterodimers of the c-Rel, RelA (p65), RelB, p50 (NF-κB1), and p52 (NF-κB2) proteins. The NF-κB transcription factors can be activated by a variety of extracellular stimuli and physical stresses and they regulate a large number of genes involved in inflammation, differentiation, and apoptosis control. Notably the proinflammatory cytokines are known to induce NF-κB activation mainly by two pathways, the canonical and the non-canonical pathways.

The IκBs (inhibitors of NF-κB) are the centerpiece of the canonical (classical) pathway. In unstimulated cells, NF-κB is complexed with the IκBs and thereby locked into the cytoplasm. The current view of this process is that upon stimulation of a cell, multiple intracellular signalling pathways are activated that converge at the IκB kinase (IKK) complex. The most common form of this complex consists of two functionally non-redundant kinases, IKKα (IKK1) and IKKβ (IKK2), as well as a regulatory subunit, IKKγ also called NEMO (NF-κB essential modulator). Upon activation, the IKK complex phosphorylates the IκBs at specific amino acid residues. The phosphorylation of IκBα at Ser-32 and Ser-36 is predominantly mediated by IKKβ. This site-specific phosphorylation was shown to be a prerequisite for subsequent posttranslational modifications such as ubiquitination of IκBα, which tags the NF-κB inhibitor for degradation in the 26S proteasome unit. Furthermore NF-κB subunits are now reported to translocate into the nucleus as a homo or heterodimers, where they regulate the expression of genes involved in fundamental physiological and patho-physiological cellular processes such as control of the immune system, especially of the innate immune response, as well as the regulation of inflammation and apoptosis.

In non-canonical (alternative) signalling pathway, activation of NF-κB is reported to involve the processing of the p52 precursor p100. This pathway is completely independent of IKKγ or NEMO, whereas IKKα and the NF-κB-inducing kinase (NIK) are essential. Moreover here NIK is positioned immediately upstream of the IKKα homodimers. Strikingly, cytokines such as lymphotoxin β (LT-β) and B cell-activating factor, CD40 ligand, as well as viruses such as the human T cell

leukemia virus 1 (HTLV-1) or the Epstein-Barr virus (EBV), are among the few select stimuli shown to activate the non-canonical pathway. The phosphorylation of p100 at two specific C-terminal serine residues by IKKα homodimers is reported to be a key event in this pathway. Interestingly, site-specific phosphorylation is essential for polyubiquitination and proteasomal degradation. However, the entire molecule is not degraded rather only the C terminus of p100 is destroyed. Furthermore, the RelB/p52 heterodimers are the main NF-κB factors generated by p100 processing [28;30-33].

4.2 Sustained activation of JNK in response to TNF-family members (TNF-α, TRAIL) in NF-κB deleted HaCaT keratinocytes

Cell death induction by apoptosis and necrosis, NF-κB activation and stimulation of JNK cascade are the most prominent cellular responses of TNFR1 signalling. Notably, the pathways that mediate these responses do not act independently in a parallel manner but are shown to interconnect to TNFR1 signalling network through various mechanisms. Recent findings suggests that apoptosis induction is inhibited by several targets genes of the NF-κB pathway including those encoding cFLIP, cIAP2, TRAF1, Bcl_2, $Bclx_L$ and XIAP. In recent years, there is growing evidence which suggests that the balance between NF-κB activation and apoptosis induction in the context of TNFR1 signalling is regulated by the JNK pathway. Notably the role of various JNKs and their major targets the AP1/jun family of transcriptional factors in the TNF-induced signaling network is complex and depends on the cellular context. TNFR1 induced JNK activation is generally very rapid and transient in viable cells, where as in NF-κB inhibited cells, which are primed to undergo cell death after TNFR1 stimulation. The JNKs are persistently activated. *In vitro* experiments suggest that expression of the NF-κB target genes A20, GADD45β and XIAP inhibits TNF-induced JNK activation and thus prevent prolonged JNK signalling after TNFR1 stimulation in normal cells.

Interestingly, sustained JNK activity in TNF signalling was speculated to result from reactive oxygen species (ROS) mediated oxidative inhibition of JNK inactivating phosphatases. Furthermore, NF-κB pathway not only blocks apoptosis but also ROS production and necrosis by inducing ROS-detoxifying enzymes such as manganese superoxide dismutase and the ferritin heavy chain. It is therefore tempting to speculate that ROS generation, oxidative inhibition of phosphatases and apoptosis signalling kinase activation are of special relevance for sustained JNK activation in cells undergoing TNF-induced necrosis [34].

4.3 Death receptors induces MAPkinases

Several mitogen-activated protein kinase (MAPK) signal transduction pathways have been identified in mammals, including extracellular signal-regulated protein kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK. Each of these groups of MAPK is activated by dual phosphorylation on Thr and Tyr within a tripeptide motif (Thr-Xaa-Tyr) located within the activation loop of the MAPK. This phosphorylation is mediated by seven MAPK kinases (MAPKKs) that have specificity for individual MAPK isoforms. The JNK is activated by MKK4 and MKK7, and p38 MAPK is activated by MKK3 and MKK6 [35;36]. These MAPKKs and MAPKs can function as independent signalling modules that may work in parallel. A large body of evidence suggests that the specificity of MAPKKs to activate individual MAPK isoforms is mediated, in part, by an interaction between an N-terminal region located on the MAPKK and a docking site located on the MAPK [37;38]. It is of interest to know that both the up stream activators of JNK- MKK4 and MKK7 are shown to have non-redundant functions. Furthermore, JNK is phosphorylated preferentially on Tyr by MKK4, whereas MKK7 phosphorylates JNK on Thr [39;40]. Interestingly, many other reports describe that dual phosphorylation on Thr and Tyr is required for full activation of JNK [41]. These studies suggest that MKK4 and MKK7 may cooperate to activate JNK and also MKK4 and MKK7 gene disruption strongly supports these conclusions. In addition, ultraviolet (UV) radiation causes activation of both MKK4 and MKK7 [42],

and loss-of-function mutations in either MKK4 or MKK7 cause reduced UV-stimulated JNK activation [39;40;43].

Many previous reports have identified the isoforms MKK3 and MKK6 as specific activators of p38 MAPK [44;45]. It has also been reported that some cell surface receptors, including tumor necrosis factor (TNF), may activate p38 MAPK by a MAPKK-independent mechanism. Interestingly, the effect of loss-of-function mutations in the murine MKK3, MKK4, and MKK6 genes revealed that all three MAPKK isoforms can contribute to p38 MAPK activation and that the repertoire of MAPKK isoforms that results in p38MAPK activation *in vivo* depends on the specific stimulus that is studied. The loss of p38 MAPK regulation in the mutant cells causes defects in growth arrest and increased tumorigenesis (See review [46]).

Preliminary preclinical data suggest that inhibitors that target JNK, p38 MAPK cascades exhibit anti-inflammatory activity. In addition, inhibition of ERK is a promising means of reducing production of TNF-α, although no direct inhibitors have been identified so far. However, it is difficult to extrapolate from gene knockout studies, in which the activity of a given kinase is completely abolished. For drug therapy partial inhibitors may be sufficient to achieve the desired therapeutic effect. Further experimentation with actual JNK, p38 inhibitors is needed (See review [47]).

4.4 cFLIP isoforms mediated non-apoptotic signalling pathways upon DR-treatment

Emerging evidence suggests that cFLIP not only regulates apoptosis but also modulate non-apoptotic signals, which in turn may be important for tumor promoting functions such as proliferation, migration, inflammation or metastasis [6;18]. It has been suggested that NF-κB activation is independent of caspase activation and that the inhibition of caspases leads to a complete blockade of apoptosis and a concomitant increase in the activation of NF-κB and its target gene IL-8. These data indicate that gene induction is a distinct apoptosis-

independent event, elicited by TRAIL/Fas receptors in keratinocytes [5]. Previous findings suggest that $cFLIP_L$ acts as an inhibitor of TRAIL-mediated NF-κB activation by directly interfering with RIP1 (Receptor interacting protein1) recruitment to the DISC [4;28]. The cFLIP isoforms include $cFLIP_s$ and $cFLIP_L$. Upon recruitment to the DISC, $cFLIP_L$ is cleaved to a p43 fragment that is retained within the DISC. Based on overexpression studies in 293T cells, it was suggested that $cFLIP_L$ has differential interaction partners that might explain its distinct signalling pattern (for review see [48]).

There is growing evidence that cFLIP can act as a tumor progression factor. For example, cFLIP expression correlates with resistance against death receptor-induced apoptosis in a variety of B-cell lymphomas, and cFLIP transfected tumor cell lines develop into more aggressive tumors *in vivo* [49]. Conversely, administering chemotherapeutic drugs to sensitize cells that are resistant to death receptor–induced apoptosis often correlates with decreased expression of cFLIP. Additionally, cFLIP is a target of the major anti-apoptotic pathways involved in carcinogenesis, namely the NF-κB, Akt/PKB, and MAPK pathways [50]. The particular relevance of cFLIP for apoptosis-resistance has been pinpointed in recent reports showing that decreased expression of cFLIP is sufficient to confer sensitivity against death receptor induced apoptosis as shown in human melanoma cells [23]. Moreover, cFLIP antisense oligonucleotides have been successfully used in three other studies to sensitize resistant prostate cancer cells, multiple myeloma cells and chronic lymphocytic leukemia cells to death receptor-induced apoptosis [23;51].

4.5 Regulation of cFLIP expression in tumor cells

Inhibition of cFLIP expression in tumor cells might be of particular importance for TNF-related apoptosis-inducing ligand (TRAIL)-based cancer therapies. TRAIL is the ligand of two death receptors, TRAIL-R1 and TRAIL-R2, and has attracted considerable attention in recent years as a potential anti-cancer molecule

because of its ability to induce apoptosis preferentially in tumor cells [50]. It is worth noting that TRAIL requires co-treatment with conventional chemotherapeutic drugs to sensitize tumor cells, although unfortunately some normal healthy cells also undergo death receptor induced apoptosis. Chemotherapy has often-pleiotropic effects, including the inhibition of anti-apoptotic pathways that typically regulate a whole battery of effector molecules. For example, the NF-κB pathway induces in a cell-type specific way, almost a dozen anti-apoptotic proteins including cFLIP [32;33]. Therefore, it seems conceivable that chemotherapeutic drugs sensitize normal and cancer cells by targeting different cell-type specific effector proteins. It has also been recently shown that proteasome inhibitors sensitize primary keratinocytes to TRAIL induced apoptosis by blocking the maturation and activity of caspase-3, most likely by interfering with the function of the inhibitory XIAP protein, an E3 ligase that drives the proteasomal degradation of caspase-3 and SMAC/DIABLO [5]. However, proteasome inhibitors can also sensitize tumor cells to death receptor-induced apoptosis by blocking NF-κB–dependent increased expression of cFLIP [32;33]. These above examples illustrate that selective sensitizers of apoptosis might broaden the applicability of anti-cancer strategies related to death-receptor activation. Future studies must show if a selective decrease of cFLIP expression allows for differential sensitization of tumor cells and normal cells to death receptor-induced apoptosis.

5. Aim of my study

Death receptors (DRs) such as CD95 and TRAIL-R1/R2 induce not only apoptosis but also stimulate non-apoptotic signalling pathways such as NF-κB and mitogen-activated protein kinase (MAPK) activation. The inhibition of DR-activated maturation of procaspase-8 is achieved by the action of distinct isoforms of cellular FLIP proteins ($cFLIP_S$, $cFLIP_L$) in various cellular systems. However, recent studies suggest more complex physiological roles of the cFLIP proteins beyond apoptosis protection, involving the regulation of several DR-associated pathways, which might ultimately manifest in phenotypes associated with skin patho-physiology such as tumorigenesis or inflammatory diseases. These evidences thus led us to investigate and elucidate the following objectives in the present PhD study, using human primary and transformed (HaCaT) keratinocytes as the model of study.

1. Is cFLIP cleavage a pre-requisite for the activation of DR-associated non-apoptotic pathways such as NF-κB and MAPK signalling? (see Figure 7)
2. If so, which cFLIP variants (isoforms/mutants) are involved in this process and what are their significant roles in regulating the DR-mediated activation of these proliferative/inflammatory signalling pathways?
3. What are the precise cellular mechanisms by which these cFLIP variants modulate keratinocytes cell fate from different death receptors in various physio- and pathophysiological situations?
4. Do other important regulators of cell differentiation, survival and apoptosis, such as members of the TNF super family (TNF, TRAIL) and JNKs, also play a role in transducing cell death mechanisms in the HaCaT cells? If so, what is the precise role for activation of the MAPK JNK in TNF-mediated apoptosis, when NF-κB signalling is blocked in these cells?
5. Further, would the cFLIP variants also differentially influence the death ligand-mediated activation and phosphorylation of JNK and the transactivation of its downstream targets such as AP-1 in HaCaT cells? If

so, through which mechanisms would the cFLIP isoforms/mutants achieve this modulation?

6. How would the cFLIP variants mechanistically impact the DR-induced activation of other MAPKs, such as the p38 MAPK and its downstream target Hsp27 to elicit non-apoptotic responses in the HaCaT cells?
7. Do the cFLIP variants also possibly act upon the DR-associated differential recruitment to CD95-R/TRAIL and further, the post-translational modifications of the death-inducing signalling complex (DISC) and its associated proteins such as FADD, caspase-8, RIP1, TRAF2 etc. to trigger the non-apoptotic signals in HaCaT keratinocytes? What would be the importance of such a modulation to the keratinocytes cells?
8. Finally, what is the crucial role of cFLIP and its variants in DR-induced activation of non-apoptotic signalling pathways, such as NF-κB, in primary human keratinocytes? Would specific targeting of cFLIP expression be of particular importance in TRAIL-based cancer therapies related to DR-activation? (see Figure-8)

Based on the afore-mentioned aims, the present PhD study thus endeavours to answer questions pertaining to the physiological relevance of cFLIP and its variants in death receptors (CD95 and TRAIL) mediated regulation of apoptotic and non-apoptotic signalling cascades such as NF-κB and MAPKs. These findings could thus be very important to understand mechanisms of keratinocyte skin cancer and the activation of innate and/or adaptive immune responses triggered by DR activation in the skin. This might led us to understand the less known biological functions of cFLIP and its variants in normal and disease states of the skin.

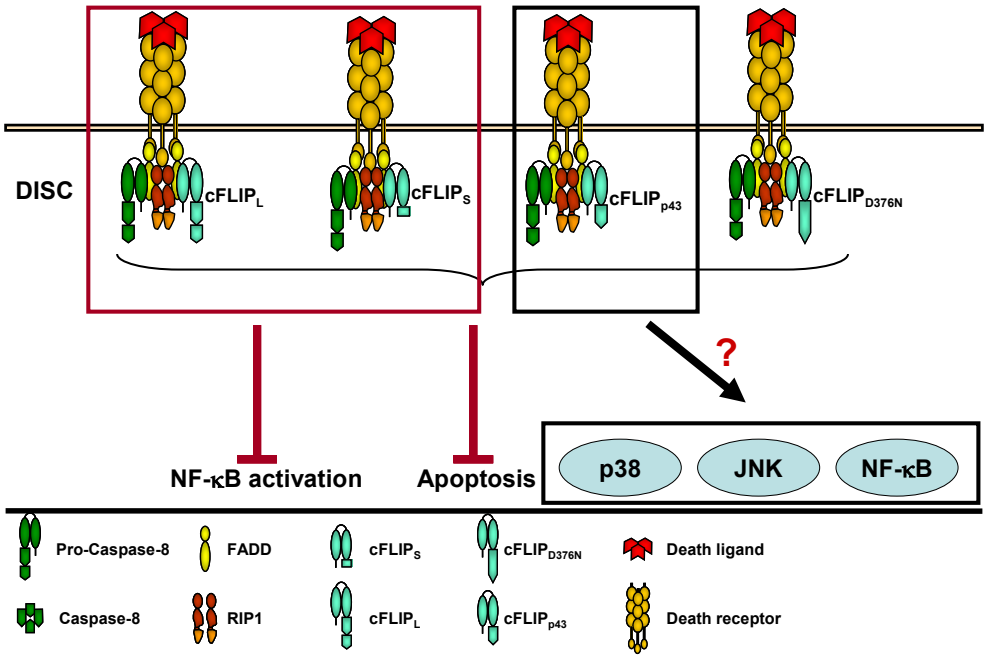


Figure-7 Does cleavage of cFLIP is essential for death ligand mediated non-apoptotic signalling pathways in human keratinocyte?

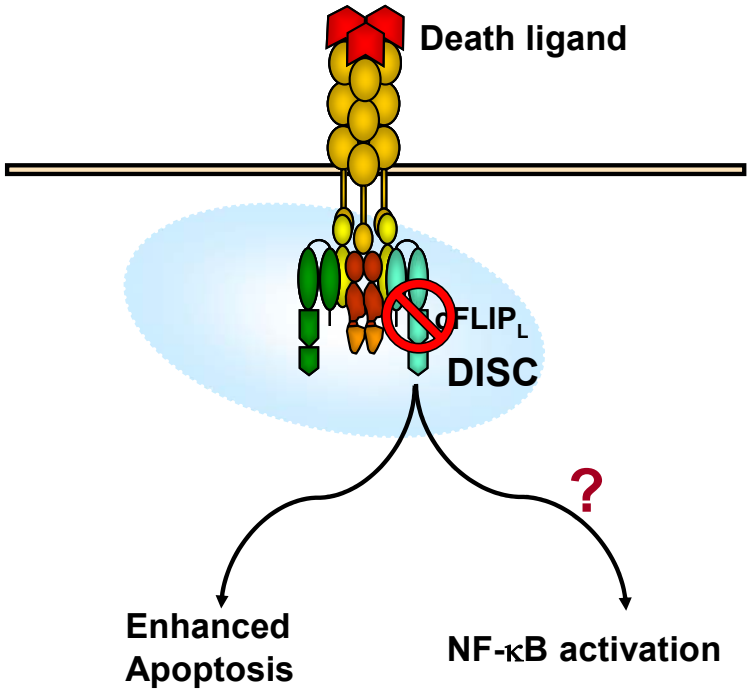


Figure-8 cFLIP isoforms modulates DR-induced NF-κB activation under physiological conditions?

II. MATERIALS AND METHODS

1. Materials

1.1 Lab instruments/Chemicals

Instruments	Company
Sterile work bench	Hera-safe (Heraeus), Thermo scientific
Centrifuges and Incubator	Eppendorf, Heraeus-biofuge, Multifuge Co_2-Auto Zero. Heraeus, Biosafe plus
Water bath	Medingen
Thermal block	Thermo mixer (Eppendorf)
Microscope	Zeiss
Balancing machine	Sartorius
Spectrophotometer	Perkin Elmer, Amersham
Western blot (Electrophoresis Instruments - chambers, blotting Apparatus)	Bio-Rad, and Invitrogen
Pipetboy	Falcon Express, Becton Dickinson
Pipettes	10µl, 20µl, 100µl, 200µl, 1000µl Eppendorf
Neubauer-Cell counter	Heiland Fachversand
Hypercassette Intensifying Screen	Amersham
Cell counter	Sarstedt
FACScan-instrument/FACS-Canto	Becton Dickinson/BD biosciences
Bacterial incubator	Binder
EMSA (electrophoresis units, glass plates)	Sigma
Gel dryer	Bio-Rad

1.2 Common buffers and reagents

Company	Product	Catlog No
Gibco, Darmstadt, Germany	DMEM (4,5g/L Glucose, L-Glutamine, 3,7g/L $NaHCO_3$ pyruvate)	P04-03590
	PBS Dulbecco´s Phospate Buffered Saline	14190-094
	Trypsin	25300-054
	FCS	26400-044
PAN Biotech GmbH, Heidenheim/Germany	Penicillin/Streptomycin (10000UPen/10mg Strep)	A-9164
Sigma, St. Louis/USA	APS (Ammonium Persulfate) (Final con. 10%)	A-3678
	DMSO	D-2650
	Lauryl Sulfat (Sodium dodecyl sulfate)	L-4509
	2-Mercaptoethanol	M-7522
	MOPS	M-5755
	Ponceau S Concentrate	P-7767
	Propidiumiodid	P-4170
	Sodium chloride	S-7653
	Sodium dioxoarsenate ($NaAsO_2$)	S-7400
	Sodium citrate	S-4641
Merck, Darmstadt, Germany	Triton X-100	T-9284
	Crystal violet	1159400025
	Phosphorsaure (H_3PO_4)	100573
	Tween 20	817072
BioRad Hercules,CA, USA	30% Acrylamide/ Bis Solution, 29:1	161-0154
	Coomassie Brilliant Blue G-250	161-0406
	TEMED	161-0801
	(N,N,N',N'-Tetra-methyl-ethylenediamine)	161-0800
Carl Roth, Karlsruhe, Germany	TRIS buffer p.a.	4855.2
	Milk powder	T145:3
ROCHE	Protease-Inhibitor cocktail	11206893001
	Sepharose-G beads	11134515001
Amersham	ECL Western-Blotting detection reagents	RPN 2106
	ECL +	RPN 3004
Pierce, Rockford, England	Stripping Buffer	21059
J.T.Baker, Deventer/NL.	Methanol.	8045

1.2.1 Western blot

Buffers	Composition
Tween/PBS	0,1% in 1000ml PBS
DISC-lysis-buffer	30mM TRIS (pH 7,5)-HCL, 120mM NaCl 10% Glycerol 1% Triton X 1 x Complete protease inhibitors cocktail 1mM Na-ortho-vanadate
TritonX-100 lysis buffer (MAPKinases lysis buffer)	20 mM Tris (pH 7.4) 137 mM NaCl 10% (v/v) glycerol 1% (v/v) TritonX-100 2 mM EDTA 50 mM sodium β-glycerophosphate 20 mM sodium pyrophosphate 1 mM ABSF 5 mg/ml aprotinine 5 mg/ml leupeptin 5 mM benzamidine and 1 mM sodium orthovanadate
5x lamellae buffer	2,5ml of 2M Tris-HCl pH 6,8, 2g SDS 100mg Bromphenoblau, 10ml Glycerol 1,542g DTT and make up the volume with H_2O.and store them –20°C.
TAE (Tris-Acetate-EDTA)	40 mM Tris Base 20 mM Acetic acid 10 mM EDTA
MOPS-SDS Running Buffer	50 mM MOPS 50 mM Tris-base 0.1% (w/v) SDS 1 mM EDTA pH = 7.7
MES-SDS Running Buffer	50 mM MES 50 mM Tris-base 0.1% (w/v) SDS 1 mM EDTA, pH = 7.3
Transfer buffer	25 mM Tris, 192 mM glycine, 10% methanol

1.2.2 FACS-Analysis

Nicoletti-buffer	Composition
Nicoletti buffer	2,5ml 20x Sodiumcitrate/Triton Stock solution (Dissolve 2g Sodiumcitrat in 98ml H_2O + 2ml of Triton X-100) 2,5ml 20x Propidiumjodid Stock solution (1mg/ml in 45ml H_2O).

1.2.3 IL-8 ELISA

Buffers	Composition
Coating buffer	0,1M Sodium carbonate, pH 9,5 8,4g $NaHCO_3$
Assay diluent	PBS with 10% FBS, pH 7,0 (BD Pharmingen™ assay diluent)
Wash buffer	PBS with 0,05% tween-20
Substrate solution	Tetramethylbenizidine (TMB) and Hydrogen Peroxide (BD Pharmingen™ TMB Substrate Reagent Set)
Stop solution	1 M Phosphorsaure (H_3PO_4)

1.3 Culture media

1.3.1 Media for culturing bacteria

Media for culturing bacteria (LB)	10 g/l Tryptone, 10 g/l NaCl, 5 g/l Yeast extract. LB-Agar plates were prepared by addition of 15g/l Bacto-Agar before autoclaving.

1.3.2 Media for culturing eukaryotic cells

Cell culture medium	Composition
HaCaT-Culturmedium:	DMEM 500ml 10% FCS (PAA) 1% Penicillin/Streptomycin
Phoenix culture medium	DMEM 500mL 10% FCS (PAA) d.h. 50mL 1% Penicillin/Streptomycin 1x aminoacids 1x HEPES 1x Sodium pyruvate
1xPBS	137 mM NaCl, 8,1 mMNa2HPO4 2,7 mM KCl, 1,5 mM KH2PO4 pH = 7.4.
Primary keratinocytes medium	Purchased from CellIntec Advanced Cell Systems

1.4 Biological Material

1.4.1 Bacterial strains

Strain designation	Purpose	References (Supplier)
XL-10	Cloning and propagation of retroviral plasmid constructs	Stratagene
DH5α	Propagation of plasmids	Clontech
BL21 (DE3) pLysS	Protein expression	Invitrogen

1.5 Antibodies

1.5.1 Primary antibodies for western blot

Antibody	Company	Cat.-Nr.	Sec.-Ab.	Working stock
ERK (C-14)	Santa Cruz Biotechnology, Santa Cruz, USA	Sc-154	DaR	1:1000 5% milk TBST
p-ERK	Santa Cruz Biotechnology, Santa Cruz, USA	SC-32577	IgG2a	1:1000 5% TBST
p-HSP-27	Cell signalling Hamburg,Germany	2401	DaR	1:1000 5% milk TBST
JNK	Cell Signaling, Hamburg,Germany	9252	DaR	1:1000 5% BSA TBST
p-JNK	Cell Signaling, Hamburg,Germany	9251	DaR	1:1000 5% BSA TBST
p38	Santa Cruz Biotechnology, Santa Cruz, USA	SC-535	DaR	1:1000 5% BSA TBST
p-p38	Cell signaling, Hamburg, Germany	9215	DaR	1:1000 5% BSA TBST
Anti-tubulin	Anti β-tubulin Clone Tub ascites fluid, 2.1 mouse Sigma	T4026	GaM IgG1	1:5000 5% milk TBST
cFLIP	Alexis, San Diego, USA	NF-6	GaM IgG1	1:1000 5% milk TBST
Caspase-8	Alexis, San Diego, USA	C-15	GaM IgG2b	1:1000 5% milk TBST
IκB-α (C-21)	Santa cruz Biotechnolgy USA	SC-371	DaR	1:1000 5% milk TBS
PIκB-α	Cell signaling Hamburg, Germany	9246	GaM IgG1	1:1000 5% milk TBST
Caspase-3	Merck Frosst (Cpp32)	MF-393	DaR	1:2000 5%milk

1.5.2 Secondary antibodies for western blot

Antibody	Company	Cat.-Nr.	Working solution
GaR (Goat Anti rabbit) IgG	Southern biotech Birmingham, USA	4030-05	1:5000 5% milk TBST
GaM (Goat Anti mouse) IgG1	Southern biotech Birmingham, USA	1070-05	1:5000 5% milk TBST
GaM (Goat Anti mouse) IgG2a	Southern biotech Birmingham, USA	1080-05	1:5000 5% milk TBST
GaM (Goat Anti mouse) IgG2b.	Southern biotech Birmingham, USA	1090-05	1:5000 5% milk TBST

1.6 TNF-Superfamily ligands

Ligands	Reference
His-Flag-TRAIL	[10]
His-Flag-TNF-α	[10]
FasL-Fc	[52]

1.7 Materials for molecular biology

1.7.1 Vectors

Cell line	Reference, supplier	Purpose
HaCaT	[1]	Parental HaCaT
HaCaT pCF-65.retro.Empty	[4]	Control cector
HaCaT pCF-65.retro.cFLIPshort	[28]	Eukaryotic expression of cFLIPshort
HaCaT pCF-65.retro.cFLIPLong	[4]	Eukaryotic expression of cFLIPLong
HaCaT pCF-65.retro.cFLIPD376N	As shown in Fig-6	Eukaryotic expression of cFLIPD376N
HaCaT pCF-65.retro.cFLIPp43	As shown in Fig-6	Eukaryotic expression of cFLIPp43

1.7.2 Oligonucleotides

Name	Sequence (5'-3')
cFLIP$_L$.fwd (Bgl-II)	GGCCAGATCTACCATGTCTGCTGAAGTCATC
cFLIP$_L$.rev (Xba-I)	GGCCTCTAGATTATGTGTAGGAGAGGAT
cFLIP$_S$.rev (Xba-I)	GGCCTCTAGATCACATGGAACAATTTCC
cFLIP$_{p43}$.rev (Xba-I)	GGCCTCTAGATTAATCCACCTCCAAGAGGCT
cFLIP$_L$.fwd (Bgl-II-)	GGCCAGATCTACCATGTCTGCTGAAGTCATC
cFLIP$_L$.rev (Xba-I)	GGCCTCTAGATTATGTGTAGGAGAGGAT
IL-8.fwd	CACCCCAAATTTATCAAAGA
IL-8.rev	ACTGGCATCTTCACTGATTC
NF-κB (TcEda► c).fwd	AGCTTGACCAAGAGGGATTTCCCCTAAATC
NF-κB (TcEda► c).rev	TTGATTTAGGGGAAATCCCTCTTGGTC
GAPDH.fwd	CCTGGTATGACAACGAATTT
GAPDH.rev	GTGAGGGTCTCTCTCTTCCT
ACTB.fwd	AGAAAATCTGGCACCACACC
ACTB.rev	GGGGTGTTGAAGGTCTCAAA

1.7.3 Enzymes and kits

Enzymes and kits	Kit supplier
Pfu Turbo DNA Polymerase	Promega
Taq DNA Polymerase	Promega
Restriction enzymes	Promega
DNA Ligase	Promega

1.7.4 Pharmacological inhibitors

Name of Inhibitior	Proteins	Company	Cat. -Nr.	References
ZVAD-fmk	Caspases	Bachem Weil am Rhein. Germany	1560	[53]
Aprotinin	Protease-Inhibitor	Sigma Missoouri USA	A-6103	[54]
Leupeptin	Protease-Inhibitor	Sigma Missoouri USA	L-2884	[54]
ABSF	Protease Inhibitor	Applichem Darmstadt Germany	A1421	[54]
Benzamidine	Protease Inhibitor	Fluka Missoouri USA	12072	[54]
Na-Ortho-vanadate	Phoshpatase Inhibitor	Sigma Missoouri USA	S-6508	[54]
β-Glycerophosphoate	Phosphotase Inhibitor	Sigma Missoouri USA	G6376	[54]
Na-Pyrophsphoate.	Phosphotase Inhibitor.	Sigma Missoouri USA.	S6422	[54]

2. Methods

2.1 Cell biological methods

2.1.1 Cell culture

The HaCaT Cells were grown in medium consisting of DMEM supplemented with 10% fetal calf serum and 1% Penicillin and streptomycin at 5% CO_2 in a humidified atmosphere at 37°C. HaCaT keratinocytes were always trypsinized when the cells were around 90% confluent to maintain the cellular integrity.

2.1.2 Transfections of cFLIP variants in phoenix cells

The amphotrophic phoenix cells were seeded at a density of $2x10^6$ to have 60%-70% confluency the following day for the transfections. The transfections were carried out as follows, 10-20µg of each pCF-65.cFLIP variant retroviral constructs were used to which 29.6µl of 2.5M $CaCl_2$ was added and the volume was made upto 600µl with sterile water. Subsequently, transfer the DNA+$CaCl_2$ mixture was transfered to a different falcon tube which already contained equal volume of 2xHBS drop by drop with gently swirling and leave the reaction mixture was left for 30mins at room temperature. Meanwhile, old medium of phoenix cells was replaced with fresh DMEM medium containing 25µM chloroquine and incubate for atleast for 30mins before the DNA precipitate were added drop by drop to the phoenix cells. After 12 hours of incubation under standard conditions, the medium including the precipitate was removed from the phoenix cells. Cells were washed carefully with 1x PBS and 6 ml of fresh medium were added to collect the first virus supernatant after 24 hours. The recombinant retroviral supernatants were harvested by filter sterilization (With a 0,45µM filter). Recombinant supernatants were either quick frozen (using liquid nitrogen) and stored at -80°C or used directly for the transduction on the same day.

2.1.3 Transduction of retroviral supernatants in HaCaT keratinocytes

Cell culture supernatants containing viral particles were generated by incubation of producer cells with HaCaT medium (Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum) overnight as described earlier [4]. Following

filtration, culture supernatants were were added to HaCaT cells seeded in 6-well plates 24h earlier in the presence of 5µg/ml Polybrene. These HaCaT cells were centrifuged for 1h 30mins at 21 °C, and the viral particle containing supernatant were subsequently replaced by fresh medium. After 10–14 day recovery, the bulk infected cultures were selected with eukaryotic selection marker zeocin for two weeks at a concentration of 150µg/ml of zeocin. Successfully selected polyclonal populations were chosen for further expansion and later on confirmed for the GFP expression by FACS analysis and also overexpression by western blot analysis.

2.1.4 Stable expression of cFLIP siRNA.

We used stable expression of shRNA containing the cFLIP targeting sequence as published recently [23]. The cFLIP targeting vector or a hyper random sequence (HRS) expressing vector which served as an internal control were transfected into the amphotrophic producer cell line exactly as outlined above. The retrovirus-containing supernatant was then used to infect primary human keratinocytes, and later the infected cells were selected with puromycin (1µg/ml) for 3-7 days in order to obtain puromycin-resistant bulk infected cultures for further analysis. Aliquots of cells were subsequently used for cytotoxicity assays and biochemical characterization between passage 2-5 following selection.

2.2 Molecular biological methods

2.2.1 Cloning of cFLIP variants in retroviral eukaryotic expression vector

All the sub-clones generated in this study contain fragments ($cFLIP_{short,}$ $cFLIP_{Long}$, $cFLIP_{D376N}$ mutant, $cFLIP_{p43}$) that were PCR-amplified from pcDNA clones (generated by cloning an ORF obtained from human cFLIP isoforms/mutants in the pcDNA vector - gift by Peter Krammer). Since the *Pfu* polymerase enzyme has 3'-5' exonuclease proof reading activity that reduces the errors in nucleotide incorporation during PCR amplification, it was hence used for generating all fragments for DNA cloning. All PCR products were first cloned into the pJET blunt vector (Fermentas) at XbaI and BglII restriction sites and thereafter the

sequence of the clones were confirmed by sequencing using pJET.fwd and pJET.rev vector primers. The pJET clones ($cFLIP_{short,}$ $cFLIP_{Long}$, $cFLIP_{D376N}$ mutant, $cFLIP_{p43}$) were confirmed for the presence of the insert and sub cloned into eukaryotic retroviral expression vector (pCF.65.retro vector-gift by Dirk Linderman) in the right reading frame using XbaI and BglII restriction sites present in the pCF.65 vector. Authenticity of the clones was confirmed by restriction digestion analysis and also by sequencing.

2.3 Protein biochemical methods

2.3.1 Preparation of cell lysates

Cells were seeded at a density of $0{,}6x10^6$ in 5mm dishes to have a confluency of 50-70% for next day stimulations the next day. Cells were serum starved for 6h (for MAPKinases) and subsequently stimulations were performed with TRAIL 0,5µg/ml for the indicated time points. After stimulations, cells were washed twice with ice cold 1x PBS and lysed for 1h on ice in Triton X-100 lysis buffer buffer (20mM Tris (pH 7.4), 137mM NaCl, 10%(v/v) glycerol, 1%(v/v)Triton X-100, 2mM EDTA, 50mM sodium β-glycerophosphate,20 mM sodium pyrophosphate, 1mM ABSF, 5mg/ml aprotinin, 5mg/ml leupeptin, 5 mM benzamidine, and 1mM sodium ortho-vanadate). Cell debris was removed by centrifugation at 15,000xg for 10 min. The supernatants were collected and aliquoted before being frozen at -80°C for long term storage.

2.3.2 Preparation of cytosolic and nuclear extracts

Cells were harvested by centrifugation at 400xg for 5 min at 4°C, washed twice with ice cold 1x PBS and lysates were prepared by resuspending the resulting cell pellets in 200µl of buffer-A per 1.79 $x10^7$ cells (10mM Tris pH-7.9, 10mM KCl, 1.5mM $MgCl_{2,}$ 10% Glycerol, 10mM K_2HPO_4 supplemented with 1mM Sodium ortho-vanadate, 10mM NaF, 0.5mM DTT, 1mM ABSF, 1X-complete protease inhibitors (Roche Diagnostics). This was immediately followed by addition of 0.125% NP-40 (v/v) and incubation on ice for 5min. The supernatants were spun down at 1000xg for 10min at 4°C. The fresh cytosolic supernatants

were collected and the resulting pellets were washed again with 300µl of buffer-A at 1000xg for 10mins. The washed pellets were resuspended in buffer-C (20mM Tris pH-7.9, 0.42M NaCl, 1.5mM $MgCl_2$, 2mM EDTA, 10% Glycerol, 10mM K_2HPO_4) supplemented with 1mM Sodium ortho-vanadate, 10mM NaF, 0.5mM DTT, 1mM ABSF and 1X complete protease inhibitors. The resuspended pellets were centrifuged at 12,000xg at 4°C for 10mins. The fresh nuclear extracts were finally collected and store them at -80°C.

2.3.3 Western blot analysis

For Western blot analysis the resulting post cell lysates or DISC precipitates were supplemented with 4-fold concentrated laemmli buffer (4x laemmli). Subsequently, lysate containing 10 µg of protein as determined by the BCA method (Pierce) or proteins eluted from beads after ligand affinity immunoprecipitation were separated on a 4-12% NuPage Bis-Tris gradient gels (Novex) in MOPS buffer according to the manufacturer's instructions. After protein transfer onto a nitrocellulose membranes (Amersham) by electroblotting, membranes were blocked with 5% non-fat dry milk and 3%BSA in PBS/Tween (PBS containing 0,05% Tween-20) for at least 1h, washed with PBS/Tween, and incubated in PBS/Tween containing 3% non-fat dry milk (NFDM) and primary antibodies as required. After 5 washes for 10 min each in PBS/Tween the blots were incubated with HRP-conjugated isotype-specific secondary antibodies diluted 1:5000 in PBS for 60mins at RT. After washing 5 times for 3 min with PBS/Tween the blots were developed by enhanced chemiluminescence (ECL) using SuperSignal West Dura substrate following the manufacturer's protocol (Perbio Science). For stripping, the blots were incubated in 50 mM Glycine HCl 500 mM NaCl pH 2.3 for 20min at room temperature. Subsequently, blots were washed two times for 10 min in PBS/Tween and blocked again for further antibody incubation.

2.3.4 FACScan analysis. For surface staining of Death receptors (TRAIL-R1 to TRAIL-R4),APO-1 R cells were trypsinized and 2 x 10^5 cells were incubated with monoclonal Abs against TRAIL-R1 to TRAIL-R4 and APO-1 R, or isotype-

matched control IgG (1µg) for 60 min on ice. After washes with 2ml of 1x PBS, cells were incubated on ice with goat anti-mouse biotinylated secondary Abs for 60mins on ice. Further after the PBS washes, the cells were incubated with PCy5-Phycoerythrin-labeled streptavidin for 30mins and cells were left on ice (Caltag) as described previously [5]. For all experiments, 10^4 cells were analyzed by the FACScan (Dickinson & Co).

2.3.5 Quantification of apoptotic cell death by propidium iodide staining

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described earlier [55]. Briefly, $0{,}6x10^6$ cells were seeded in 5mm-well plates (Costar) with or without apoptotic stimuli (TRAIL or FasL) in 1ml medium at 37°C for 8h. The cells were harvested by centrifugation at 600xg for 10 min at 4°C, washed twice with PBS and then resuspended in 0,5ml of lysis buffer containing 0.1% (v/v) Triton-X-100, 0.1% (w/v) sodium citrate and 50 µg/ml propidium iodide (PI). Apoptosis was quantitatively determined after incubation at 4°C in the dark for 48h by flow cytometry as cells containing nuclei with subdiploid DNA content. Alternatively, apoptosis was determined by a drop in the forward scatter to sideward scatter (FSC/SSC) profile of apoptotic versus living cells. Percentage of specific cell death was calculated as follows: 100 x (% experimental cell death - % spontaneous cell death) / (100 - % spontaneous cell death).

2.3.6 Luciferase assays.

Cells were seeded in 48 well plates to attain a confluency of around 50% on the day of transfections. Transfections were carried out using 3x κB Luciferase (NF-κB promoter elements) or 5x TK (AP-1 promoter elements) with Renilla ubiquitin (internal control) using lipofectamine as a transfection reagent (according to manufacturer's recommendations-Invitrogen) The transfectants were left for 20-24h while for the post transfections, stimulations were performed for 6h. This was immediately followed by assaying the cells for luciferase and renilla activity (According to the manufacturer's recommendations-Promega).

2.3.7 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as explained earlier. Oligos (NF-κB and AP-1) were annealed (15µl of sense oligos of 100ng/µl and 15µl of anti sense oligos 100ng/µl) at 75°C for 15mins followed by gradual cooling (switching of the thermal block to allow gradual cooling) at RT overnight for annealing. Annealed oligonucleotides corresponding to either NF-κB binding sites was radio labeled by T4 polynucleotide kinase. Labelled oligonucleotides (1000 cpm) were purified from the gel. Radiolabeled oligonucleotides were added to 4µg of nuclear protein extract together with 1µg of poly(dI-dC) (Amersham Pharmacia) to prevent nonspecific binding and incubated for 5min on ice. After the incubation, the entire reaction mixtures were loaded into 4% native polyacrylamide gel and run for 3h at 200V. Dried gels were exposed to x-ray films for overnight.

2.3.8 Quantitative Real-time PCR (qPCR).

$0{,}5x10^6$ cells were seeded in 5mm dishes before stimulations were performed with TRAIL/CD95L for the indicated time points. After stimulations, cells were washed with ice cold PBS and total RNA extraction was performed using RNeasy Kit (Qiagen). The cDNA was synthesized in 20µl reaction volume using a mixture of oligo dT primers and random nonamers in a ratio of 1:10 and SuperScript II Reverse Transcriptase (Invitrogen). Primers were designed using Primer3 software (available at http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi). RT qPCR analyses for the genes encoding interleukin-8 (IL-8), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) were performed in a final volume of 25µl using IQ™ SYBR® Green (BioRad) in an iCycler (BioRad). Gene of interest and reference gene products were amplified individually, under equal cycling conditions. HotStart-Taq DNA Polymerase was activated by an initial denaturation at 15 min for 95°C followed by 42 cycles of one step (denaturation) at 94°C for 15 sec, one step (annealing) at 55°C for 30sec, and one step (extension) at 72°C for 30sec. The specific amplification of a single product of the expected size was confirmed by melting curve analysis. Consecutive dilutions of cDNA (1, 1:5, and 1:25) were amplified for the

construction of a standard curve (plotted as a logarithmic function of the cDNA dilution factor) and used for the calculation of the RT PCR efficiency using iCycler software. The relative quantification for IL-8 was calculated after dividing the standard curve value of IL-8 by that of the reference gene (GAPDH and ACTB) for each individual sample. Two reference genes were used for data normalization to account for possible variations as a result of DL treatment. The effects on IL-8 expression were calculated by analyzing mean values obtained from three independent experiments. In two independent experiments, RNA was three times reverse transcribed and the cDNA from the three independent reverse transcription reactions was assayed for RT qPCR in triplicates. The mean values obtained by the above explained procedure were compared for all different experimental conditions.

2.3.9 DISC analysis.

For the precipitation of the TRAIL or CD95L DISC, 5 x 10^6 HaCaT keratinocytes were used for each condition. Cells were washed once with DMEM medium at 37°C and subsequently incubated for the indicated time periods at 37°C in the presence of either 2,5 µg/ml Flag-TRAIL pre-complexed with 5 µg/ml anti-FLAG M2 (Sigma) or 10 units/ml of CD95L-Fc for 30 min, or, for the unstimulated control, in the absence of ligands. Receptor complex formation was stopped by washing the monolayer four times with ice-cold PBS. Cells were lysed on ice by addition of 2 ml lysis buffer (30 mM Tris-HCl pH 7.5 at 21°C, 120 mM NaCl, 50mM sodium β-Glycerophosphate, 20mM sodium pyrophosphate, 1mM sodium ortho-vanadate, 10% Glycerol, 1% Triton X-100, Complete® protease inhibitor cocktail (Roche). After lysing cells on ice for 30 min (Flag-TRAIL) or 2h (CD95L-Fc), lysates were centrifuged at 20,000xg for 5 min to remove cellular debris and later collect supernatants were collected in to fresh microfuge tube and were centrifuged again at 20,000xg for 30mins. A minor fraction of these clear lysates were used to control for the input of the respective proteins. Receptor complexes were precipitated from the lysates by co-incubation with 20µl protein G beads (Roche) for 24h on an end-over-end shaker at 4°C. For the precipitation of the

non-stimulated receptors, either 50ng of Flag-TRAIL precomplexed with anti-FLAG M2 antibody or 1µg of APO-1 antibody is precomplexed with 10 U/ml of Fc-CD95L per each IP condition were added to the lysates prepared from non-stimulated cells to control for protein association with non-stimulated receptor(s). Ligand affinity precipitates were washed 5 times with lysis buffer before the protein complexes were eluted from dried beads by addition of standard reducing sample buffer and boiled for 5mins at 95°C. Subsequently, proteins were separated by SDS-PAGE on 4-12% NuPAGE gradient gels (Invitrogen) before detection of DISC components by Western blot analysis.

2.3.10 Apoptosis and cytotoxicity assays.

Crystal violet staining of attached, living cells was performed 16 - 24h after stimulation with different concentrations of TRAIL (12–1000ng/ml) in 96 well plates as described previously [11]. Subdiploid DNA content was analyzed as described by Nicoletti et al [55]. Briefly, cells from a 35 mm dish were cultured till 70% confluency was attained and were subsequently stimulated with TRAIL for 3h. Cells were then detached, washed with ice cold PBS and resuspended in buffer (0.1% Sodium citrate) (w/v), Triton X 100 0,1% (v/v), 50µg/ml PI. Cells were kept in the dark at 4°C for 48h and then hypodiploidy was measured by FACScan analysis.

2.3.11 Determination of IL-8 secretion.

Pre-coated enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine interleukin-8 (IL-8) expression in keratinocyte cell culture supernatants. All samples were thawed only once at the time of testing and analyzed according to the procedures provided by the manufacturers (R&D Biosystems) as described previously [5].

2.3.12 In-vitro kinase assay

The activity JNK was analyzed in an *in vitro* kinase assay using 0.5 mg protein of total lysate incubated/immunoprecipitated with 1mg anti-JNK 1 (C-17; Santa Cruz Biotechnology) antibody/sample by permanent shaking for 3h at 4°C. Immune complexes were recovered using 50µl of a 50% protein A-sepharose bead

suspension (Amersham Biosciences) and incubated for 90min at 4°C in a permanent upside down shaker. Subsequently, immunoprecipitates were washed six times in the same lysis buffer and incubated with 1µg GST-c-jun in 25 µl kinase buffer (20 mM HEPES pH 7.6, 2 mM EGTA, 20 mM MgCl2, 1 mM Sodium ortho-vanadate, 1 mM DTT, 0.1% Triton X-100, 0.25 mM 32P-ATP) for 30 min at 30°C. GST-c jun was used as substrate in an *in vitro* kinase assay as described previously [56;57]. For further analysis, lysates were suspended in loading buffer, separated by SDS-PAGE, and transferred to PVDF membranes. The gels were dried and exposed to Amersham TM film (Amersham Pharmacia Biotech) at -70°C using an intensifying screen.

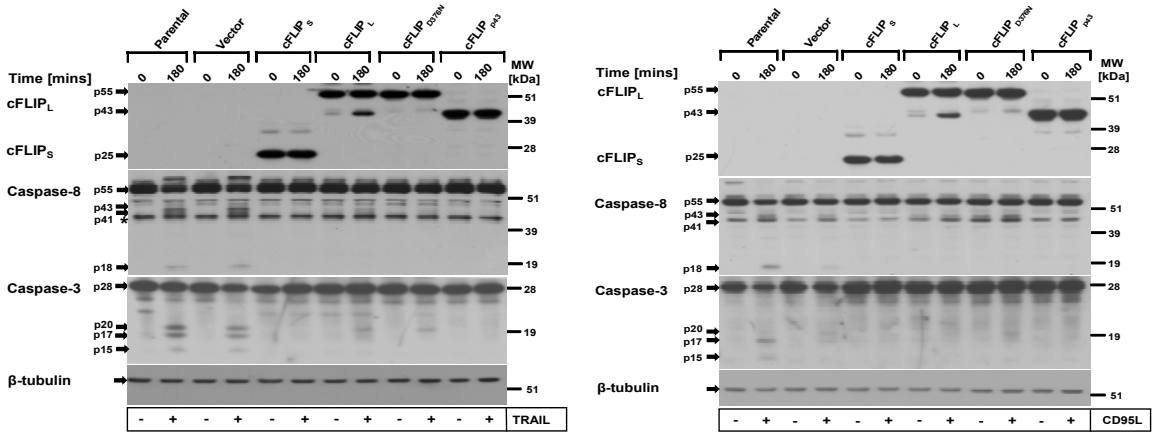
III. RESULTS

1. Biochemical characterization of cFLIP isoforms/mutants in HaCaT keratinocytes

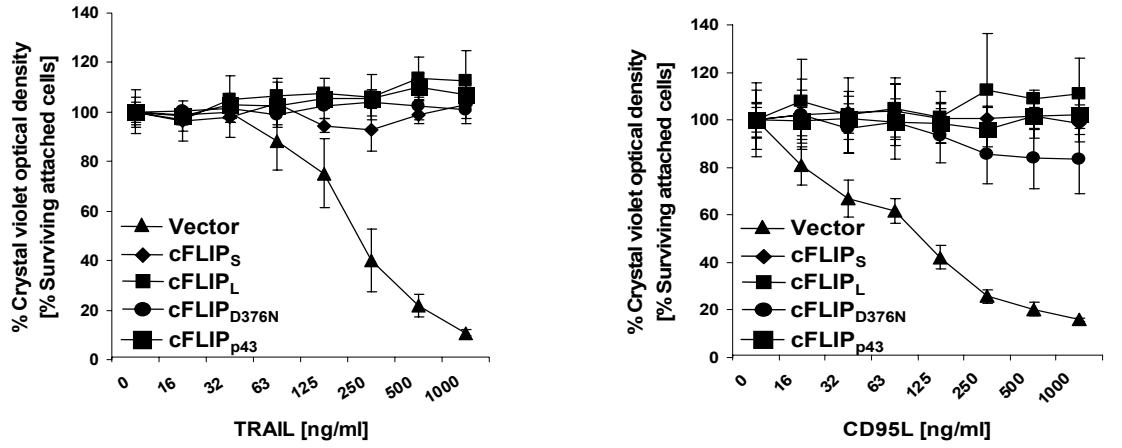
1.1 Different cFLIP isoforms protect keratinocytes from death ligand-mediated apoptosis

The role of cFLIP in death receptor-mediated signalling pathways has been a matter of intense debate over the past years [20;48;49;58]. It was suggested that cFLIP may activate proinflammatory signalling pathways in a DISC-independent manner [59]. In addition, overexpression studies using cleavage fragments such as p43 of $cFLIP_L$ indicated that cFLIP may differentially activate these signalling pathways by preferential binding to TRAF2, elicitating different signalling pathways depending upon the stoichiometry of DISC-associated components [60].

A)



B)



Since the spontaneously transformed keratinocyte cell line HaCaT expresses low levels of endogenous cFLIP, whereas the primary keratinocytes express high levels of cFLIP [11].

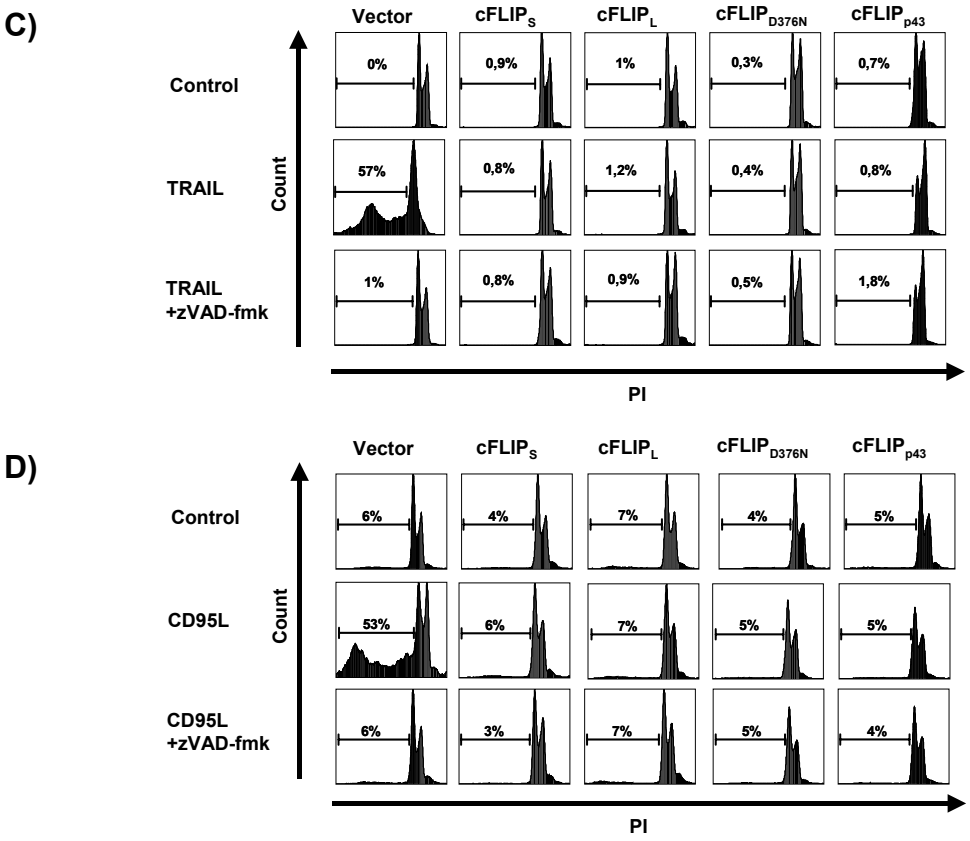


Figure-1.1 cFLIP$_L$, cFLIP$_S$, cFLIP$_{p43}$ and cFLIP$_{D376N}$ are equally effective in protecting the keratinocytes from death ligand-mediated apoptosis. A) HaCaT keratinocytes were retrovirally transduced with different forms of cFLIP or the respective mutants as described in materials and methods. Cells were untreated or incubated with TRAIL (0,5 µg/ml; left panel) or CD95L (0,5 µg/ml; right panel) for 3h. Subsequently, 3µg protein of total cellular lysates were analyzed by western blotting for cFLIP, caspase-8, and caspase-3. β-tubulin served as a control for comparable loading of protein. **B)** Cells were seeded in 96-well-plates in triplicates and stimulated with increasing concentrations of recombinant TRAIL (left panel) or CD95L (right panel) for 16-24h. Cellular viability was assessed using crystal violet assay as described in materials and methods. The percentage of living cells was normalized to mock-stimulated cells (~ 100%). Shown is mean +/- standard error of means (SEM) of a total of three independent experiments. **C)** Infected keratinocyte lines were either left untreated or stimulated with the indicated amounts of **(C)** TRAIL (0.5µg/ml) or **(D)** CD95 (0,5µg/ml) for 6h, harvested and examined for hypodiploidy by FACScan analysis.

The HaCaT keratinocytes are hence a prefect system for overexpression studies. Thus we chose to use this cellular model system for our experiments to investigate the different signalling capabilities of cFLIP variants, namely $cFLIP_L$, $cFLIP_S$, a cleavage-site mutated $cFLIP_L$ ($cFLIP_{D376N}$), and the caspase-8 cleaved fragment of $cFLIP_L$ ($cFLIP_{p43}$) in more detail. We first established a number of polyclonal cell lines expressing different isoforms or mutants of cFLIP using the retroviral vector system described elsewhere in detail. Briefly recombinant retroviral supernatants were harvested and these recombinant supernatants were transduced with HaCaT keratinocytes. Post transduction, these cell lines were selected with selection marker (zeocin) and analysed for the GFP expression as previously reported [5]. Expression of the different cFLIP isoforms or mutants was confirmed and proved to be comparable in different polyclonal cell lines (see Figure1.1A). When cells were treated with the death ligands TRAIL and CD95L, biochemical analysis showed that parental HaCaT as well as control-infected cells readily cleaved large amounts of the caspase-8 active cleavage product p18 within 3h. In contrast, $cFLIP_L$, $cFLIP_S$, $cFLIP_{D376N}$, as well as $cFLIP_{p43}$ fully blocked caspase-8 cleavage and the p18 fragment was not detected. We observed the release of partial cleavage product p43/p41 in $cFLIP_L$, $cFLIP_{D376N}$ overexpressing keratinocytes. On the contrary $cFLIP_S$, $cFLIP_{p43}$ HaCaT keratinocytes showed blockade of even the partial cleavage of caspase-8 (see Figure1.1A). As predicted, $cFLIP_L$ containing a cleavage site mutation ($cFLIP_{D376N}$) was fully protected against TRAIL- or CD95L-mediated $cFLIP_L$ cleavage (see Figure 1.1 A, left and right panel), in line with a previous report [20]. Finally, we also observed activation of caspase-3 in control infected keratinocytes which is blocked in the respective cFLIP isoforms/mutants expressing HaCaT cells. The different isoforms and mutants of $cFLIP_L$ were equally effective to protect HaCaT cells from TRAIL-or CD9SL-induced cell death, as indicated by crystal violet assays (see Figure 1.1B), or hypodiploidy analysis using propidium iodide staining, briefly stimulations were performed with death ligands (TRAIL or CD95L) for 6h and later the harvested pellets were

suspended in nicoletti buffer and the samples were stored in the dark for 48h. (see Figure 1.1C&1.1D). These data are in line with several other reports [20;28], including our own data for TRAIL-mediated cell death [4]. Importantly, our results suggest that cFLIP is able to protect against CD95L and TRAIL-mediated apoptosis independent of $cFLIP_L$ cleavage.

1.2 TRAIL-induced apoptosis is blocked by cFLIP isoforms or Caspase inhibitor or TRAIL-R-Fc in HaCaT keratinocytes

Our previous and current findings suggest that $cFLIP_S$ is recruited to the DISC and completely inhibits caspase-8 cleavage, but does not interfere with procaspase-8 recruitment to the DISC indicating that $cFLIP_S$ blocks procaspase-8 processing in the DISC. In the present study, we have shown that TRAIL activates caspase-8 by cleaving first cleavage p43/p41 and also releasing active p18 fragment from the receptor complex in to the cytosol thereby inducing apoptosis mediated via caspase-3. On the contrary, $cFLIP_S$ completely blocks partial cleavage of caspase-8 like the caspase inhibitor (ZVAD-fmk) which results in resistance to TRAIL mediated apoptosis. To investigate if TRAIL-mediated caspase-8 activation in HaCaT keratinocytes is very specific, we preincubated mock transfected and $cFLIP_S$ overexpressing HaCaT cells with caspase inhibitor (ZVAD-fmk) for 1h and subsequently stimulated with TRAIL for 3h. These lysates were analysed for caspase-8 activation (see Figure 1.2A-left) and also for TRAIL mediated cell death (see Figure 1.2A-right). $cFLIP_S$ completely suppressed. TRAIL mediated caspase-8 activation and also completely protects TRAIL mediated cell death as shown previously [28]. Stimulations were also performed by preincubating with TRAIL-R2-Fc or TNF-R-Fc for 1hr and later stimulated for 3h in mock transfected cells and assayed for caspase-8 activation. We observed that in the presence of TRAIL-R2-Fc, TRAIL mediated caspase-8 activation is completely inhibited (see Figure 1.2B). In addition TNF-R-Fc, which served as non-specific control, did not interfere with TRAIL mediated caspase-8 activation.

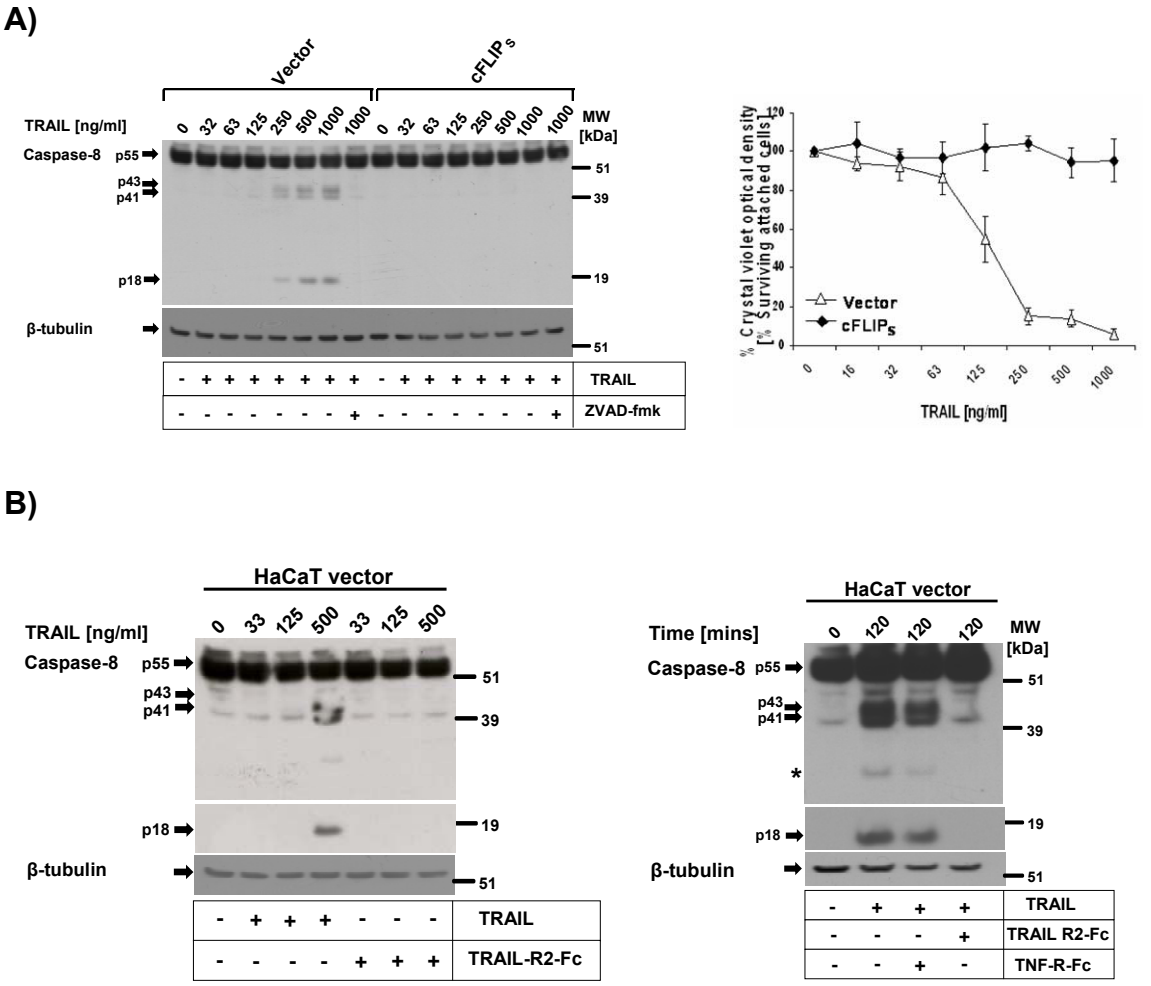


Figure-1.2 cFLIP$_S$ confers resistance to TRAIL- induced apoptosis and caspase-8 activation which is fully blocked by TRAIL-R2-Fc. A) Control cells or cFLIP$_S$-expressing HaCaT cells were preincubated with caspase inhibitor (ZVAD-fmk) and left untreated or treated with TRAIL (0,5 µg/ml; left panel) Subsequently, 3µg of total cellular lysates were analyzed by western blotting for caspase-8. β-tubulin served as a control for comparable loading of cellular protein. (Right panel), cells were seeded in 96-well-plates in triplicates and stimulated with increasing concentrations of recombinant TRAIL for 16-24 h. Cellular viability was assessed using crystal violet assay. The percentage of living cells was normalized to mock-stimulated cells. Shown is mean +/- standard error of means (SEM) of a total of two independent experiments. **B)** Mock transfected HaCaT keratinocytes were stimulated for 3h with 0,5µg/ml of TRAIL in the presence or absence of TRAIL-R2-Fc (left) or TNF-R-Fc or both (right) and subsequently, 3µg protein of total cellular lysates were then analyzed by western blotting for caspase-8, and β-tubulin served as a control for comparable loading of protein.

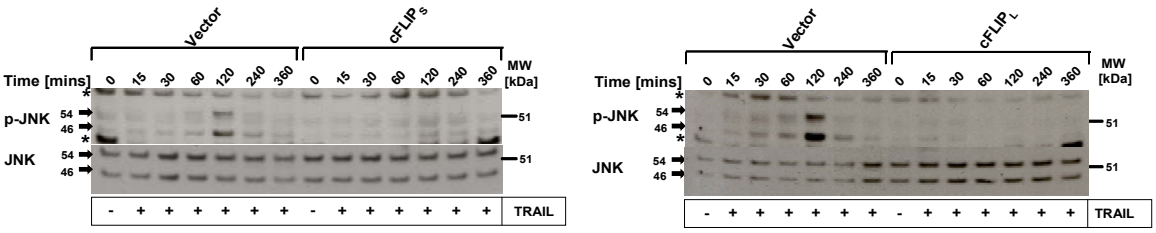
These results indicate that TRAIL mediated apoptotic signalling pathways are indeed specific in HaCaT keratinocytes.

2. Death ligands activate non-apoptotic signalling pathways in a cFLIP-dependent manner

2.1 cFLIP modulates DR-induced MAPK JNK independent of cFLIP cleavage

Over the past years, a number of reports have shown that death ligands such as CD95L or TRAIL may also activate non-apoptotic signalling pathways such as the MAPK JNK and its down stream target transcriptional factor AP-1 which eventually triggers pro-inflammatory or proliferative responses [21]. cFLIP, is not only a modulator of apoptosis but is also predicted to modulate pro-inflammatory or proliferative signals. We therefore next asked if the various cFLIP variants differentially regulate the activation of JNK in HaCaT keratinocytes. In order to address this question we preincubated the cells with caspase inhibitor (ZVAD-fmk) for 1h and also stimulated the control and also the respective cFLIP variants overexpressed HaCaT cells as indicated in figure legends. Interestingly, JNK is activated within 15 minutes of treatment with death ligands, with further increase up to 2h in control keratinocytes (see Figure 2.1A). This TRAIL-mediated induction was largely repressed by expression of $cFLIP_L$ (right panel) or $cFLIP_S$ (left panel). In order to test if the JNK activation is caspase dependent the pan-caspase inhibitor (ZVAD-fmk) known to completely protect keratinocytes against TRAIL-mediated apoptosis was used [5]. Remarkably, TRAIL-mediated activation of JNK was largely dependent upon the activation of caspases as indicated by blockade of JNK activation by caspase inhibition (see Figure 2.1B). In line with an activation of JNK downstream of caspase activation, the different isoforms of cFLIP or the respective mutants were equally effective in blocking TRAIL-induced JNK activation as determined by western blotting (see Figure 2.1B).

A)



B) **C)**

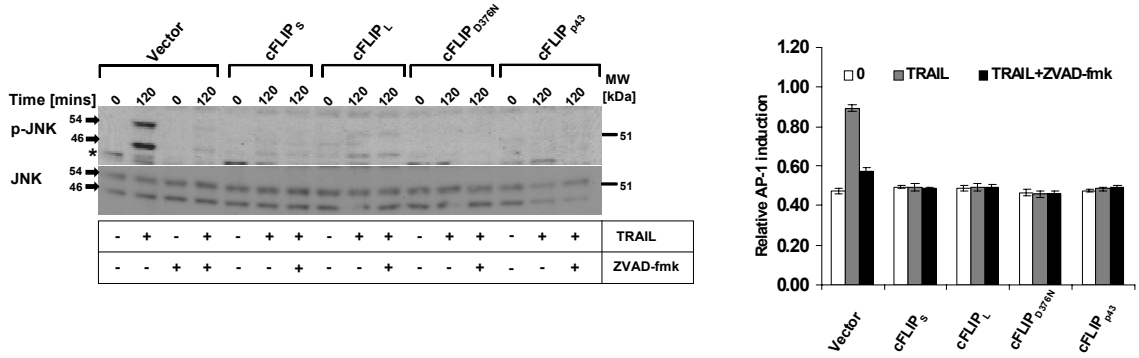


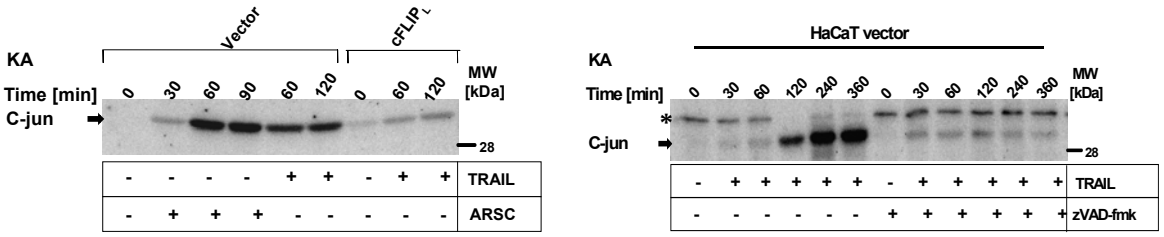
Figure-2.1 Death ligand-mediated phosphorylation of JNK and its downstream target transcriptional factor AP-1 is dependent upon active caspases and is blocked by different isoforms of cFLIP. A) Control keratinocytes and keratinocytes expressing $cFLIP_L$ or $cFLIP_S$ were stimulated with TRAIL (1µg/ml) for the indicated time points. Cellular lysates were subsequently analyzed for the phosphorylation of JNK using phospho-specific antibodies. Membranes were stripped and total levels of JNK were determined. **B)** HaCaT Keratinocytes expressing the different cFLIP isoforms as outlined in Figure 1 were preincubated for 1h in the presence or absence of ZVAD-fmk (20µM), and subsequently stimulated for 2h with TRAIL (1µg/ml). Total cellular lysates were characterized for JNK phosphorylation as indicated in A). **C)** cFLIP overexpressing HaCaT keratinocytes were seeded in a 96 well plate and transfected with 5x TK Firefly (AP-1 promoter elements) along with internal control Renilla ubiquitin as described in the method section. Post tranfectants were stimulated with TRAIL (0,5µg/ml) for 6h and assayed for transcriptional factor AP-1 activation as described in methods section. One of three independent experiments is shown representatively here.

Many previous reports suggest that AP-1 transcriptional factor is a downstream target of c-jun phosphorylation. We asked if inhibition of c-jun activation results in a reduction of AP-1 activation. To addressed this question, we co transfected AP-1 responsive reporter elements along with internal control reporter (Renilla ubquitin) and stimulated them for 6h and subsequently assayed for luciferase activity. In line with these findings, reporter assays using an AP-1 responsive reporter construct showed a 2-fold induction of AP-1 within 6h, whereas all isoforms or mutants of cFLIP fully blocked AP-1 induction (see Figure 2.1C). Collectively, these data indicate that cFLIP inhibits death ligand-mediated JNK activation independent of the caspase like-domain or its cleavage activity.

2.2 Death ligands mediated JNK activation is completely inhibited by $cFLIP_L$ or caspase inhibitor and TRAIL mediated JNK activation is completely blocked by TRAIL-R-Fc in HaCaT keratinocytes

Death receptors not only activate apoptosis but can also activate non-apoptotic signals such as MAPK JNK which eventually triggers pro-inflammatory responses. Our previous reports also suggest that, TRAIL induces MAPK JNK in HaCaT keratinocytes (Wachter.T, Felcht. M, 2007-unpublished reports). To investigate if $cFLIP_L$ or caspase inhibitor (ZVAD-fmk) modulates JNK activation, we addressed DR-induced JNK activation in control or $cFLIP_L$ HaCaT keratinocytes by In-vitro kinase assay. This assay was performed as described in materials and methods and is a very sensitive method to analyze activation of specific kinases. We preincubated the cells with caspase inhibitor (ZVAD-fmk) and stimulated them for indicated time points and performed the In-vitro kinase assay and later analyzed for c-jun phosphorylation. In the current study our results suggest that $cFLIP_L$ or caspase inhibitor (ZVAD-fmk) completely block TRAIL mediated JNK phosphorylation which was also reproduced by the In-vitro kinase assay. In order to address if TRAIL mediated JNK activation is specific to HaCaT cells we preincubated with TRAIL-R2-Fc or TNF-R-Fc or both for 1h and stimulated with TRAIL for indicated time points and analyzed for JNK activation.

A)



B)

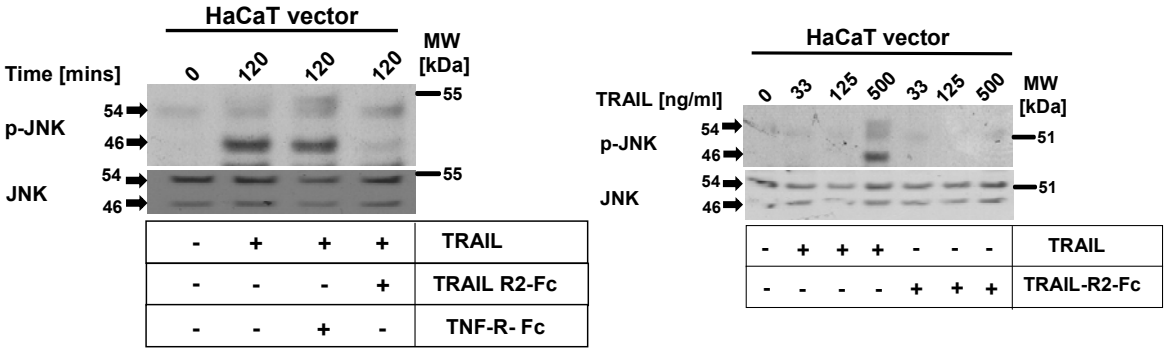


Figure-2.2 Death ligand-mediated JNK phosphorylation is dependent upon active caspases and blocked by cFLIP$_L$ or TRAIL-R2-Fc. A) Infected cFLIP$_L$ or control HaCaT keratinocytes were treated with TRAIL (0,5µg/ml) or Arsenic (81,5mg/ml) (left panel) or in the presence or absence of ZVAD-fmk (20µM) (right panel) for indicated time points and subsequently cell lysates were used in the In-vitro kinase assay as indicated in materials and methods and analyzed for c-Jun phosphorylation. **B)** Control HaCaT keratinocytes were treated with TRAIL (0,5µg/ml) for indicated time points in the presence or absence of TRAIL-R2-Fc (right) or TNF-R-Fc or both (left) and subsequently 3µg of total lysates were analyzed for the JNK phosphorylation.

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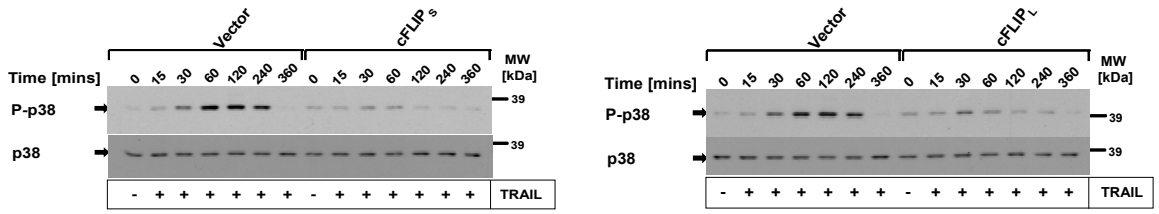
We noticed that TRAIL induced JNK phosphorylation is completely inhibited by TRAIL-R2-Fc but not with TNF-R2-Fc, indicating that TRAIL-induced JNK activation is TRAIL dependent (see Figure 2.2B). These data suggest that cFLIP is a central modulator of apoptotic and non apoptotic signalling pathways in HaCaT keratinocytes and TRAIL mediated non apoptotic signals are very specific to this cell type.

3. cFLIP mediates DR-induced MAPK p38 in HaCaT keratinocytes

3.1 Death ligands activate the MAPK p38 in a biphasic manner

We next investigated the activation of MAPK p38, highly relevant for the transcriptional as well as posttranscriptional regulation of gene expression by the death ligands [61].

A)



B) **C)**

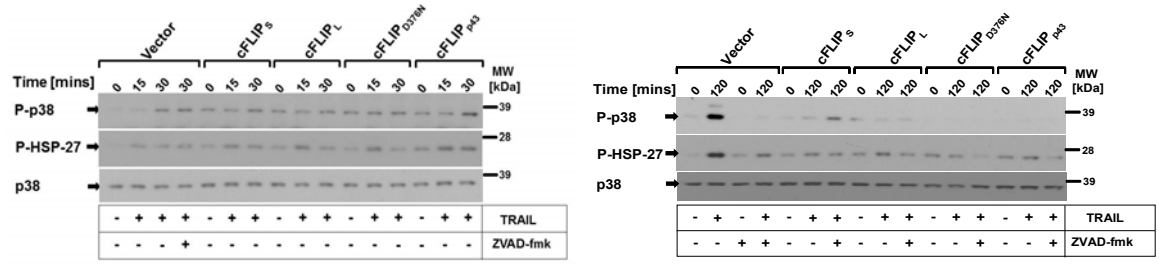


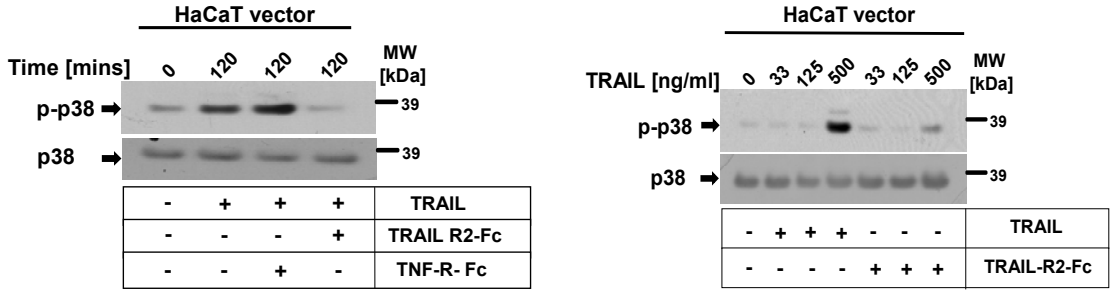
Figure-3.1 Death ligands rapidly activate MAPK p38 and its target gene Hsp-27 which is blocked by Caspase-inhibitor or cFLIP isoforms. A) Control keratinocytes and keratinocytes expressing $cFLIP_L$ or $cFLIP_S$ were stimulated with TRAIL (0,5 µg/ml) for the indicated time points. Cellular lysates were subsequently analyzed for the phosphorylation of MAPK p38, phosphorylation of Hsp-27 using phospho-specific antibodies. Membranes were stripped and total levels of p38 were determined to confirm even loading of proteins. **B)** Control cells or cells expressing the different cFLIP isoforms or mutants respectively, were preincubated for 1h in the presence or absence of ZVAD-fmk (20µM), and subsequently stimulated for 15 and 30 min **(B)** or 2h **(C)**, respectively with TRAIL (0,5 µg/ml). Total cellular lysates were characterized for p38 phosphorylation and Hsp-27 phosphorylation as indicated for A) One of three independent experiments is shown representatively here.

To investigate if cFLIP modulates DR-induced p38 activation and also known downstream target Hsp-27 in HaCaT keratinocytes we pre incubated the cells in the presence or absence of the caspase inhibitor (ZVAD-fmk) and stimulated with TRAIL for indicated time points and analyzed for the activation of p38. The death ligand TRAIL rapidly activated p38 within 15 minutes with a further increase up to 4h after stimulation. Interestingly, keratinocytes expressing different cFLIP isoforms demonstrated a complete inhibition of the late induction of death ligand-mediated p38 and its downstream target Hsp-27 activation between 1-4h (see Figure 3.1A), whereas the early phosphorylation of p38 was unchanged in the presence of either the pancaspase inhibitor or different cFLIP isoforms/mutants, respectively (see Figure 3.1 B&C). Collectively these data indicated that different cFLIP isoforms containing caspase-like domains or only Death-effector-domains (DED) block the death receptor-mediated p38 activation in a comparable fashion.

3.2 TRAIL mediated p38 activation is apoptosis dependent in HaCaT keratinocytes

MAPK p38 activation is involved in many cellular processes such as cell apoptosis, cell proliferation and in cell migration. Death receptors are known to induce p38 activation in many cell types. To investigate if TRAIL induced p38 activation is specific to HaCaT keratinocytes and also to analyze if it is caspase dependent, we preincubated the cells with caspase inhibitor (ZVAD-fmk) or TRAIL-R2-Fc or TNF-R-Fc or both and stimulated with TRAIL for indicated time points. These lysates were analyzed for the activation of p38. In the current study we have shown that TRAIL induces p38 MAPK in a biphasic manner and upon treatment with caspase inhibitor (ZVAD-fmk) the first peak is unchanged where as the second peak is completely inhibited (see Figure 3.2 A&B), indicating that it is apoptotic dependent. We have also shown here in our present study that TRAIL induced MAPK p38 activation is very specific to TRAIL as TRAIL-R2-Fc completely blocks TRAIL induced p38 activation.

A)



B)

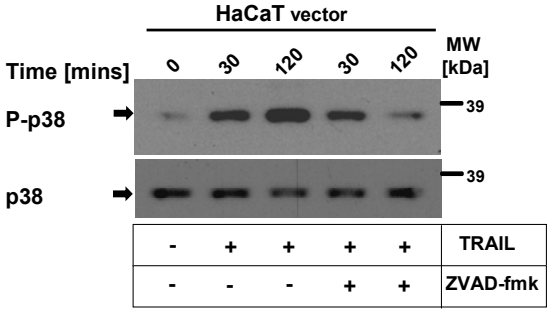


Figure-3.2 Death ligands rapidly activate MAPK p38 and that is blocked by Caspase-inhibitor or TRAIL-R2-Fc in HaCaT keratinocytes. A) Control HaCaT keratinocytes were treated with TRAIL (0,5µg/ml) for indicated time points (left) or 2h (right) in the presence or absence of TRAIL-R2-Fc (right) or TNF-R-Fc or both (left) and subsequently 3µg of total lysates were analysed for the p38 phosphorylation. **B)** HaCaT control keratinocytes were stimulated with TRAIL (0.5µg/ml) in the presence or absence of ZVAD-fmk (20µM) for indicated time points and subsequently 3µg of cell lysates were subjected to western blot analysis and p38 phosphorylation were analysed.

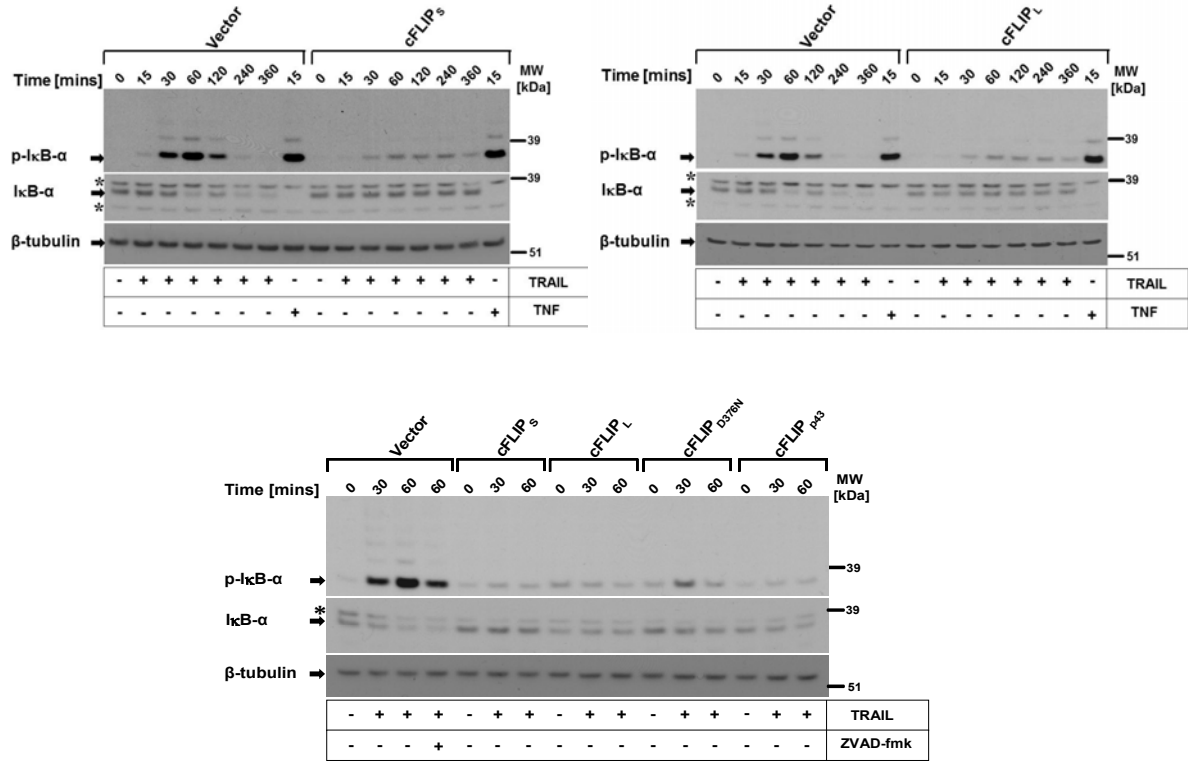
4. cFLIP isoforms are inhibitory for death receptor-induced NF-κB activation irrespective of caspase-8 or cFLIP processing

4.1 cFLIP isoforms are equally effective against CD95L- or TRAIL-mediated NF-κB activation

Death ligands such as TNF, TRAIL or CD95L induce a large array of proinflammatory NF-κB-regulated target genes in keratinocytes, in particular when apoptotic caspase activation is blocked by synthetic caspase inhibitors [5;62;63]. In contrast to TRAIL- and CD95L-induced delayed activation of JNK and

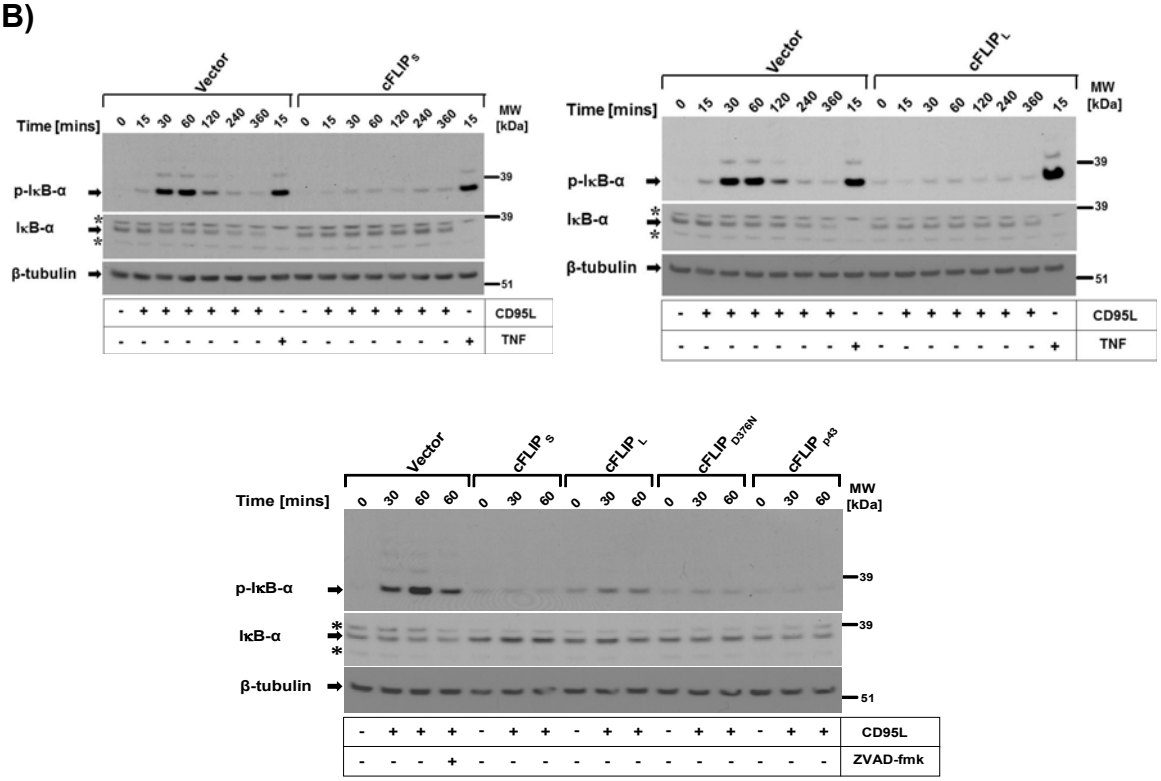
p38, NFκB activation was not blocked under chemical inhibition of caspases by ZVAD-fmk. Interestingly, expression of $cFLIP_L$ and $cFLIP_S$ interfered with TRAIL- and CD95L-induced NFκB activation, indicating a DISC-associated regulation of this signalling pathway [4;28]. We thus asked if different isoforms or distinct cleavage fragments of cFLIP may differentially regulate NFκB activation and its target gene IL-8 induction after DR-association. In these experiments, we used the closely related death ligand TNF as a putative control (another TNF family ligand). TRAIL and CD95L rapidly activated the phosphorylation of IκBα within 15 to 30 minutes in mock transfected cells. Detectable phosphorylation as well as ubiquitination persisted up to 2h after stimulation with subsequent decline, paralleled by the loss of IκBα (see Figure 4.1A). Notably, all cFLIP variants were significantly impaired in NF-κB activation in response only to TRAIL and CD95L, but not to TNF. Thus, cleavage of $cFLIP_L$ does not compromise its inhibitory effect on NF-κB activation by CD95 and TRAIL (see Figure 4.1B).

A)



Furthermore, we also investigated the NF-κB DNA binding activity in HaCaT keratinocytes as a second parameter for NF-κB activation. We noticed that TRAIL induces NF-κB DNA binding with in 60 min whereas TNF-α induces NF-κB DNA binding as early as 20-30 min in mock infected HaCaT keratinocytes. Interestingly we observed suppression of NF-κB DNA binding in all the cFLIP isoforms/mutants overexpressing cells only upon TRAIL treatment. However TNF-α induced NF-κB activating complexes were unchanged (see Figure 4.1C) indicating that cFLIP modulates only TRAIL induced non-apoptotic signalling pathways in keratinocytes. In line with these findings, reporter assays using NF-κB responsive reporter construct showed a 3-fold induction of NF-κB within 6h, whereas all isoforms or mutants of cFLIP completely blocked NF-κB induction (see Figure 4.1D). Collectively, these data indicate that cFLIP protects against death ligand-mediated NF-κB activation independent of processing of cFLIP cleavage in HaCaT keratinocytes.

B)



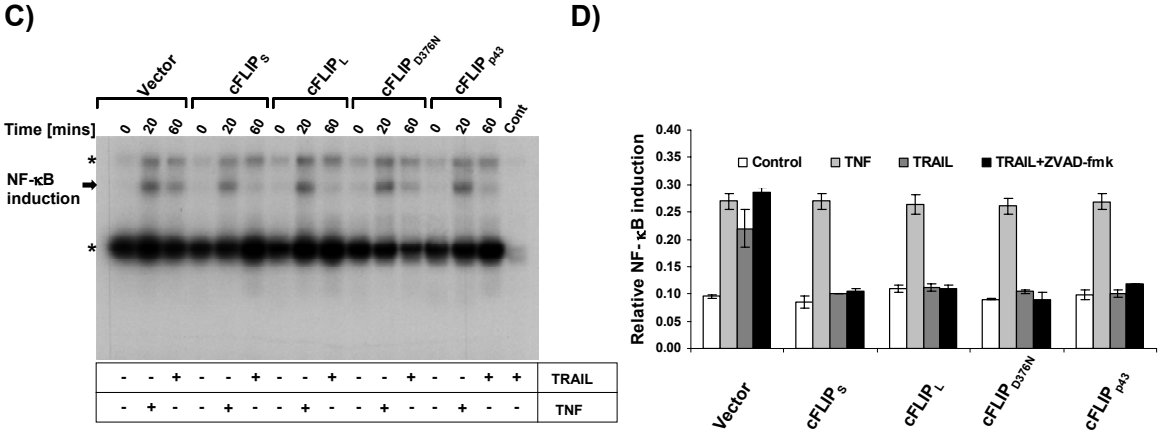
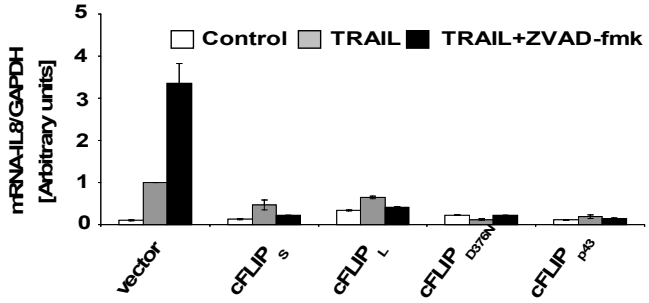


Figure-4.1 DR mediated NF-κB activation is suppressed by cFLIP isoforms or mutants. Control keratinocytes and keratinocytes expressing $cFLIP_L$ (right) or $cFLIP_S$ (left) or all the cFLIP isoforms/mutants (centre) pre incubated in the presence or absence of ZVAD-fmk (20μM) expressing keratinocytes as characterized in Figure 1 were stimulated with TRAIL as shown in **(A)** (0,5μg/ml) or with TNF as in A&B (0,5 μg/ml) and FasL as indicated in **(B)** (0,5μg/ml) for the indicated time points. Cellular lysates were collected and subsequently investigated for IκBα phosphorylation and IκBα degradation using antibodies specific for phosphorylated IκBα (upper panel) or total IκBα (lower panel). β-tubulin served as a loading control. **C)** In parallel experiments, nuclear extracts were analyzed for κB-specific DNA binding by EMSA. The positions of p65/p50 heterodimers or p50 homodimers are indicated. **D)** cFLIP overexpressing HaCaT keratinocytes were seeded in 96 well plate and transfected with 3x κB Firefly (NF-κB promoter elements) along with the internal control Renilla ubiquitin. 24 h after transfection, cells were stimulated with HF-TRAIL (0,5μg/ml) or HF-TNF-α (0,5μg/ml) for 6h and assayed for NF-κB activation as described according to manufactures recommendations. Shown are mean +/- standard error of mean (SD) of representative experiment of total two independent experiments.

4.2 cFLIP isoforms are equally effective in protecting against death ligand-mediated target gene induction

Death ligands such as TNF, TRAIL or CD95L induce the proinflammatory cytokine IL-8 in an NF-κB dependent manner in HaCaT keratinocytes [5;62;63]. Having shown that cFLIP isoforms/mutants block TRAIL-induced NF-κB activation, we therefore next asked how different cFLIP isoforms or mutants impact the induction of chemokine IL-8/CXCL8 [4].

A)



B)

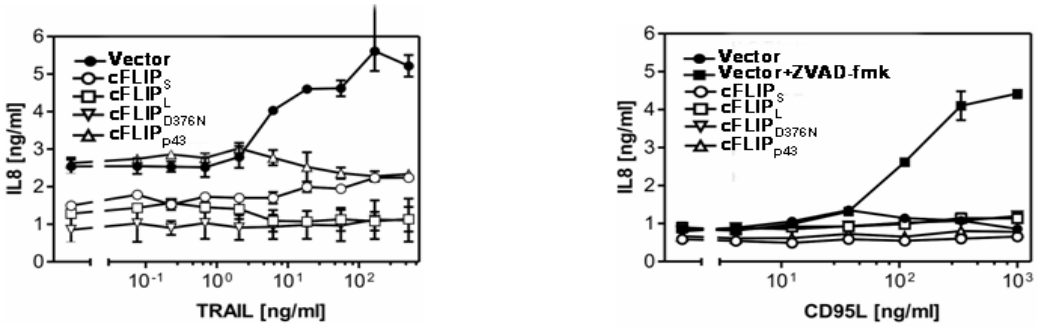


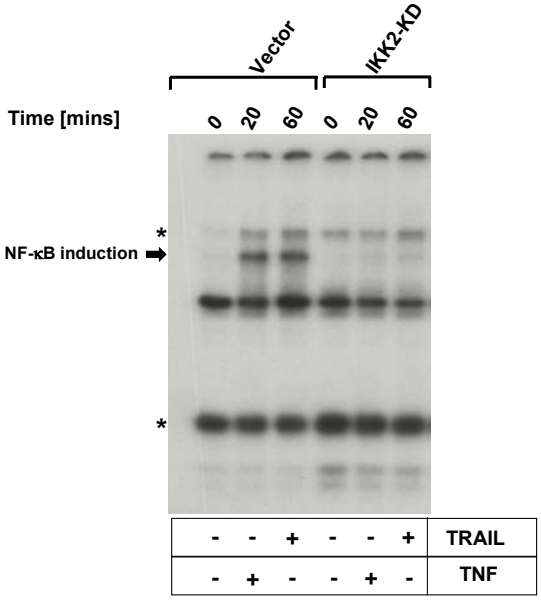
Figure-4.2 Death ligand-mediated IL-8 induction is blocked by cFLIP isoforms as well as cFLIP$_L$ mutants. A) TRAIL induced IL-8 induction is inhibited by cFLIP isoforms and its respective mutants. Following preincubation with either diluent alone or 20μM of ZVAD-fmk for 1h, control or cFLIP isoforms/mutants overexpressing HaCaT keratinocytes were treated with 0.5μg/ml TRAIL for 3h and total RNA was isolated and after synthesis of cDNA, Quantitative PCR (qPCR) performed. IL-8 mRNA expression was normalized with housekeeping gene G3PDH. Shown here is mean +/ - standard error of mean (S.D) of a representative total of three independent experiments. **B)** Upon TRAIL and CD95L treatment in the respective cFLIP mutants, TRAIL and CD95L induced IL-8 secretion is inhibited by different cFLIP isoforms as well as their mutants. Following preincubation with either diluent alone or 20 μM of ZVAD-fmk for 1h, control keratinocytes or the different cFLIP-overexpressing keratinocytes were treated with TRAIL or CD95L, respectively for 24h. Supernatants were assayed for IL-8 secretion by ELISA. Shown are mean +/- standard error of mean (SD) of a total of three independent experiments (ELISA results kindly provided by Dr.Daniela siegmund)

We thus explored if the various cFLIP isoforms/mutants might differentially affect target gene induction. mRNA analysis using qPCR and RPA (Figure 4.2A,B) demonstrated that all different cFLIP mutants or isoforms completely protected the cells against TRAIL-mediated IL-8 mRNA induction. These results are in line with our previous data which suggest that $cFLIP_L$ repress DR-induced IL-8 mRNA induction (see Figure 4.2A) [4], Protein secretion of CXCL8 was induced in a dose-dependent manner by CD95L or TRAIL, in mock transfected cells, provided apoptosis was blocked by ZVAD-fmk. In contrast, all different isoforms of cFLIP or the respective mutants repressed death ligand-mediated target gene IL-8 production (see Figure 4.2C). Taken together, our data support a concept that cFLIP is able to block death receptor-mediated target gene induction by interference with NF-κB activation.

4.3 Inhibition of the NF-κB pathway induces TNF, TRAIL mediated sustained JNK activation in HaCaT keratinocytes

TNF family members includes TRAIL or TNF-α known to induce variety of apoptotic and non apoptotic signalling cascades and also shown to interconnect these contrasting cascades by some unknown mechanisms. We have shown from our previous studies that inhibition of NF-κB pathway sensitizes for TNF-induced apoptosis however TRAIL-induced apoptosis is unchanged in HaCaT keratinocytes [15;34].

A)



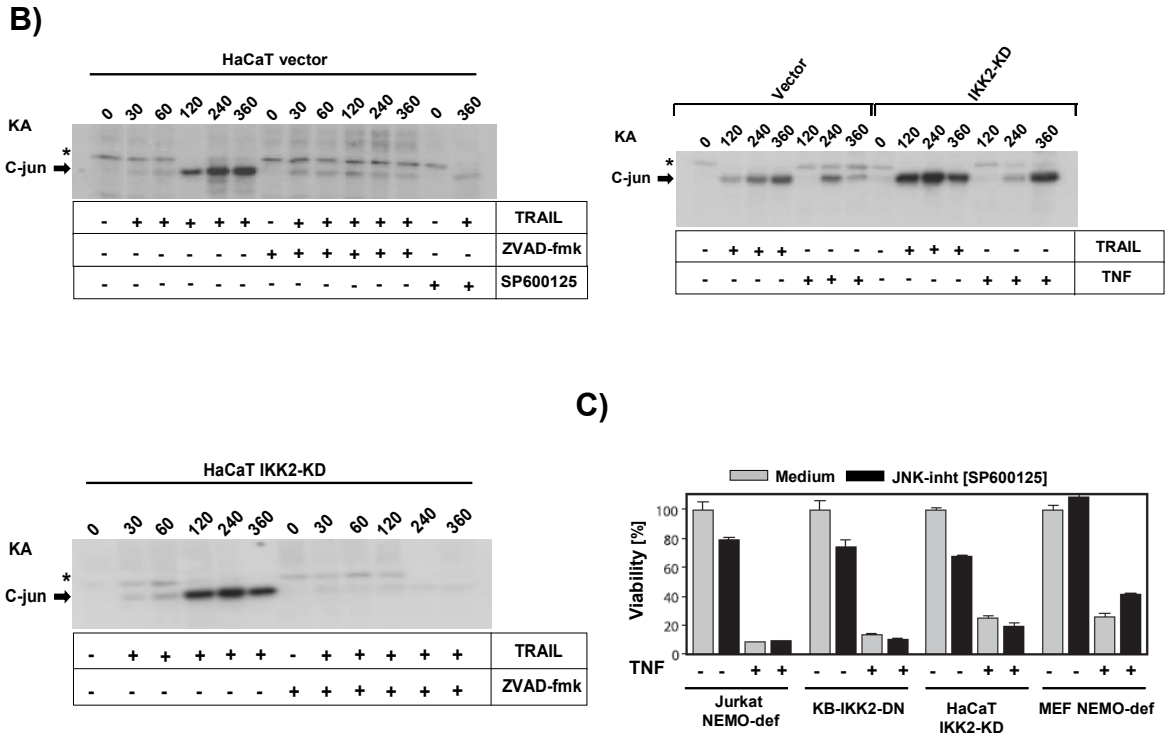


Figure-4.3 TNF family members TNF and TRAIL induces sustained JNK activation in NF-κB inhibited HaCaT cells and sustained JNK activation is dispensable for TNF-induced cell death. A) Infected mock transfected or IKK2-KD HaCaT keratinocytes were stimulated with TNF and TRAIL (0,5µg/ml) and nuclear extracts were prepared and subjected for elctro mobility shift assay as previously indicated and analyzed for NF-κB specific complexes. **B)** Control and IKK2-KD HaCaT keratinocytes were pre incubated with caspase inhibitor (ZVAD-fmk) or JNK inhibitor (SP600125) as In (A left) for 60mins and stimulated with TNF or TRAIL or both (B right) for indicated time points and assayed for C-jun activation by in-vitro kinase assay as indicated previously. **C)** Different NF-κB deleted cell types stimulated with TNF and analyzed for TNF-induced cell death as indicated previously (Viability assay data kindly provided by Dr.Wicovsky.A [34]).

We want to investigate if sustained JNK activation by TNF is essential for JNK mediated cell death in HaCaT keratinocytes. To address this question, we first characterized the phenotype of NF-κB inhibition in HaCaT keratinocytes by electro mobility shift assay. We performed this assay as previously described. From our current findings we noticed that TRAIL and TNF induces NF-κB DNA binding in mock transfected cells and in NF-κB deleted HaCaT cell, we observed

complete blockade of NF-κB DNA binding complexes (see Figure 4.3A). We further analyzed JNK activation in control and IKK2-KD HaCaT keratinocytes by In vitro kinase assay as described previously. Interestingly, we observed c-jun phosphorylation in mock transfected cells however in NF-κB deleted HaCaT cells (IKK2-KD) we noticed sustained JNK activation with both TNF and TRAIL ligands (see Figure 4.3B). In addition to that caspase inhibition results inhibition of TNF or TRAIL induced JNK activation indicating that its caspase dependent. Furthermore to elucidate the functional consequence of prolonged TNF-induced JNK activation in various cellular models including HaCaT cells investigated for this study, we blocked JNK activity with pharmacological JNK inhibitor SP600125 and analyzed TNF mediated cell death. Although TNF-induced c-jun phosphorylation is completely blocked upon JNK inhibition but cell death induction by TNF is unchanged (see Figure 4.3C). Collectively these results suggest that sustained activation of JNKs by TNF is dispensible for cell death induction [34].

5. cFLIP modulates DR-induced DISC composition in HaCaT keratinocytes

5.1 cFLIP isoforms differentially affect posttranslational modification of DISC-associated proteins

Death ligands including TRAIL or CD95L are known to recruit various effector molecules to the death inducing signaling complex (DISC) thereby modulating downstream apoptotic and also non apoptotic signalling pathways in many cellular systems. To investigate the possible differential recruitment and post translational modification of DISC-associated proteins in cFLIP variants overexpressed HaCaT keratinocytes, we characterized the recruitment of various known molecules such as FADD, caspase-8, RIP1 and cFLIP in the CD95 and TRAIL DISC complex. HaCaT-cFLIP$_L$, HaCaT-cFLIP$_S$, HaCaT-cFLIP$_{p43}$, or HaCaT-cFLIP$_{D376N}$ as well as the respective control cells were processed for DISC analysis using ligand affinity precipitation for TRAIL and CD95L (Figure 5.1 A,B).

A)

B)

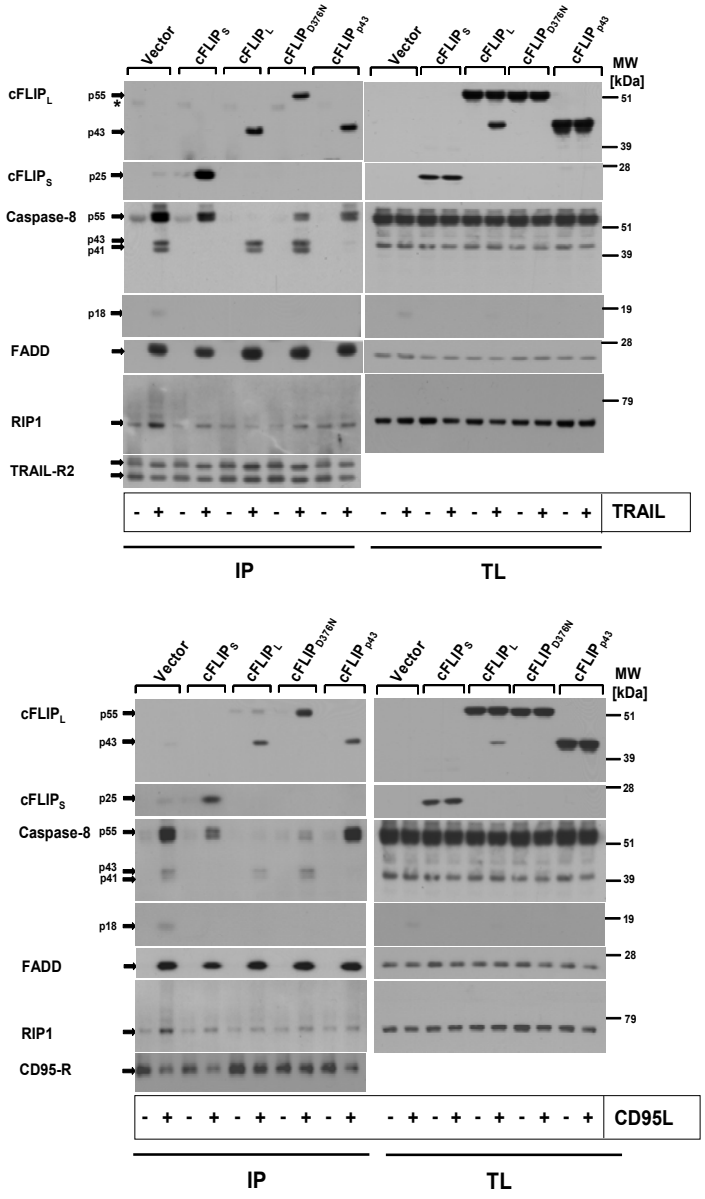


Figure-5.1 Different cFLIP isoforms modulate the composition of the TRAIL or CD95 DISC. A) Differential composition of the TRAIL DISC (A) or CD95 DISC **(B)** in keratinocytes expressing different isoforms or mutants of cFLIP$_L$-expression. DISC analysis (IP) was performed from a total of $5x10^6$ cells. Precipitates of non-stimulated cells served as specificity controls for ligand affinity precipitates and were assayed for comparable immune precipitation of TRAIL-R2 or CD95 as a control. Cells were characterized for cFLIP, caspase-8, FADD, RIP1 recruitment by western blotting. Total cellular lysates (Lysates) were analyzed in parallel from all samples. One of three independent experiments is shown here representatively.

These experiments were performed as indicated in materials and methods, briefly stimulations were performed with TRAIL or CD95L between 30-90mins. Following lysis, immuno precipitation (IP) lysates were subjected to western blotting and analyzed for the DISC recruited molecules. FADD recruitment to both the TRAIL and CD95L DISC did not significantly differ between parental, control and the different cFLIP protein expressing keratinocytes (see Figure 5.1A&B). In control cells, $cFLIP_L$ was only detected as the p43 cleavage fragment in the DISC, indicative of caspase-mediated cleavage in the DISC. A marked increased association of cFLIP p43 was observed in HaCaT-$cFLIP_L$, these findings which are in line with our previous report[4]. The cleavage site-defective $cFLIP_L$ was detected only as a only full length p55 protein in the DISC, indicative of the effective inhibition of its DISC-associated cleavage. Ectopically expressed $cFLIP_{p43}$ was detected at a similar efficiency in the DISC of HaCaT-$cFLIP_{p43}$. We also observed $cFLIP_S$ recruitment in HaCaT-$cFLIP_S$ keratinocytes and which found to be comparably effective as recruitment of other various $FLIP_L$-related FLIP variants. Interestingly we detected RIP1 recruitment and its ubiquitination pattern in control infected HaCaT keratinocytes upon TRAIL or CD95L treatment. Furthermore, we observed suppression of RIP1 recruitment and its characteristic ubiquitination pattern in all the cFLIP variants overexpressing HaCaT keratinocytes. These results are in line with our previous observations where $cFLIP_L$ suppresses TRAIL mediated RIP1 recruitment in HaCaT keratinocytes [4]. Remarkably, caspase-8 pattern detected in the DISC were markedly different: In control cells, the proform of caspase-8 (p55/53) as well as the cleavage product p43/41 were found. In marked contrast, the DISC of HaCaT-$cFLIP_L$ largely contained p43/41 fragments of caspase-8. Expression of $cFLIP_S$ completely inhibited caspase-8 cleavage, but did not interfere with procaspase-8 recruitment to the DISC indicating that $cFLIP_S$ blocked procaspase-8 processing (see Figure 5.1A&B). Similarly, expression of $cFLIP_{p43}$ yielded an increased level of full length caspase-8 within the DISC, suggesting that uncleaved $cFLIP_L$ is required for procaspase-8 cleavage within the DISC. Lastly, HaCaT-$cFLIP_{D376N}$ showed an

increased amount of procaspase-8 p55/53 in the DISC when compared to HaCaT-cFLIP$_L$, indicating that uncleaved cFLIP$_L$ is able to facilitate procaspase-8 recruitment to the DISC when compared to cFLIP$_L$. These data suggest that different steps of procaspase-8 activation are blocked by cFLIP$_{D376N}$ or cFLIP$_{p43}$. In summary, our data demonstrate that different cFLIP isoforms block TRAIL- and CD95L-induced apoptosis of keratinocytes by interference with different steps of caspase-8 processing at the DISC and that full length cFLIP is required for efficient procaspase-8 recruitment and the initial cleavage step to caspase-8 p43/41.

5.2 cFLIP does not interfere with DISC-associated caspase-8 activity and its inhibition stabilizes death receptors induced DISC complex

Recent reports have identified that the functions of cFLIP proteins at the DISC might be more complex then initially thought of and cFLIP isoforms may play a crucial role in caspase-8 activation [15;16;23]. Many other reports suggest that apoptosis induction interferes with proinflammatory gene expression elicited by death receptors because addition of caspase inhibitors such as ZVAD-fmk either leads to increased induction of target genes or is needed for death receptors dependent transcription [4]. In order to investigate if caspase inhibition has any impact on death receptors induced recruitment of DISC associated molecules and their posttranslational modifications HaCaT keratinocytes, we analyzed recruitment of various effector molecules to the DISC upon caspase treatment. These experiments were performed as indicated previously. Briefly, cells were preincubated with caspase inhibitor (ZVAD-fmk) for 60mins and stimulations were performed with CD95L for 90mins. Post lysis and immuno precipitation, IP lysates were subjected to western blotting and analyzed for DISC recruited molecules. In mock transfected cells, cFLIP$_L$ was only detected as the p43 cleavage fragment in the DISC and upon caspase inhibition. We did not observe any significant change in the recruitment of endogenous p43 cleavage fragment in the DISC.

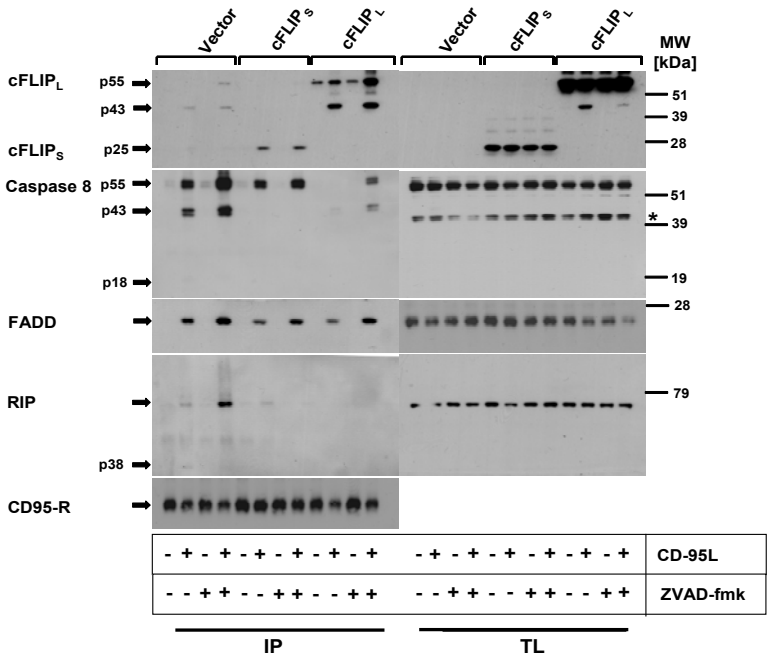


Figure-5.2 Caspase inhibition stabilizes DR-induced DISC composition in HaCaT keratinocytes. A) Differential composition of the CD-95 DISC in keratinocytes expressing different isoforms of cFLIP, $cFLIP_S$, $cFLIP_L$ were preincubated with caspase inhibitor (ZVAD-fmk) and stimulated with CD-95L for 90min. DISC analysis (IP) was performed from a total of $5x10^6$ cells. Precipitates of non-stimulated cells served as specificity controls for ligand affinity precipitates and were assayed for comparable immune precipitation of CD95 as a control. Cells were characterized for cFLIP, caspase-8, FADD, RIP1 recruitment by western blotting. Total cellular lysates were analyzed in parallel from all samples. One of two independent experiments is shown representatively.

As shown previously, marked increase in association of $cFLIP_{p43}$ was observed in HaCaT-$cFLIP_L$. However treatment with ZVAD-fmk we observed marginal enrichment of cleavage product $cFLIP_{p43}$ upon DL-association. Furthermore marginal enrichment of caspase-8 proform p55 and also active p43/p41 fragment were seen but its final cleavage product p18 is completely blocked in mock transfected keratinocytes upon DL-treatment. FADD recruitment to the CD95L DISC complex did not significantly differ between caspase inhibitor (ZVAD-fmk) untreated and treated control keratinocytes upon CD-95 stimulation. Interestingly, we observed DR-induced enrichment of RIP1 recruitment and ubiquitination pattern upon treatment with ZVAD-fmk after stimulation with DL in control

keratinocytes. In line with our previous observations, cFLIP isoforms suppress DR-induced RIP1 recruitment and ubiquitination. Notably caspase inhibition did not show any change in the repression of RIP1 recruitment in our cellular system (see Figure 5.2). Collectively these results suggest that caspase modulation not only alters the caspase activity in the DISC but also it modulates crucial apical modulators (RIP1) recruitment which might be essential in DR-induced non apoptotic signalling pathways.

5.3 TRAF2 not a crucial modulator in DR-induced NF-κB activation in HaCaT keratinocytes

TRAF2, yet another crucial NF-κB inducer which is shown to recruit to DR-induced DISC. In addition to that, it also shown to be crucial for TNF-induced NF-κB activation in many cell types.

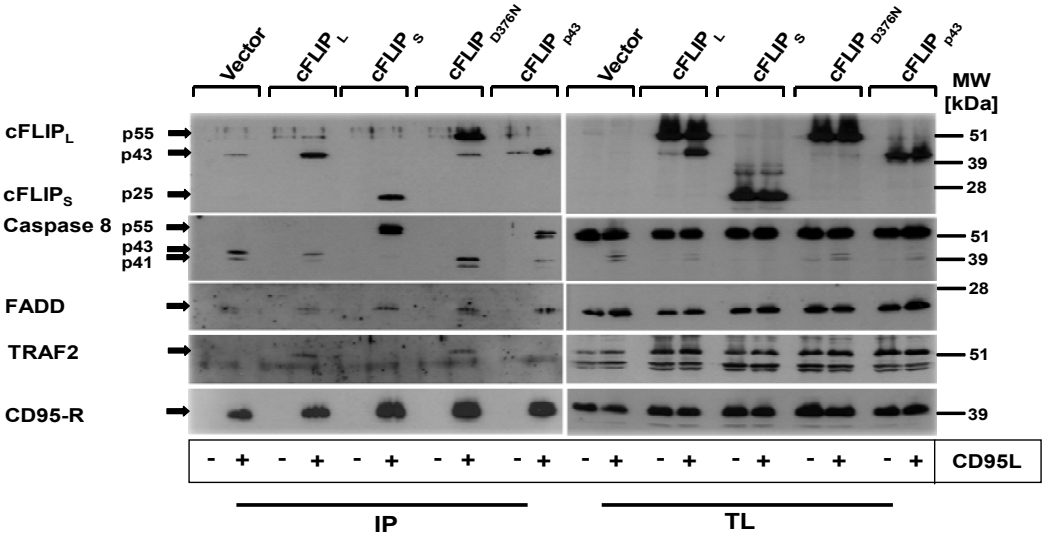


Figure-5.3 Does cFLIP variants modulates DR-induced TRAF2 recruitment in HaCaT keratinocytes A) Different polyclonal cFLIP overexpressing keratinocytes were stimulated with CD-95L and subsequently DISC analysis (IP) was performed from a total of $5x10^6$ cells. Precipitates of non-stimulated cells served as specificity controls for ligand affinity precipitates and were assayed for comparable immune precipitation of TRAIL-R2 or CD95 as a control and further characterized for cFLIP, caspase-8, FADD and TRAF2 recruitment by western blotting. One of two independent experiments is shown representatively.

In addition to that, it also shown to be crucial for TNF-induced NF-κB activation in many cell types. Furthermore TRAF2 is shown to interact specifically with endogenous $cFLIP_L$ and induces NF-κB activation in 293T cells [64]. To investigate if cFLIP modulates recruitment of TRAF2 upon DL treatment in cFLIP isoforms/mutants overexpressing keratinocytes, we stimulated with CD95L for 90mins. Post lysis and immuno precipitation, IP lysates were subjected to western blotting and analyzed for DISC recruited molecules. We observed recruitment of several known effector molecules such as cFLIP, caspase-8, FADD to CD95-R DISC in mock transfected keratinocytes as previously shown. Interestingly we observed recruitment of TRAF2 only in $cFLIP_L$, $cFLIP_{D376N}$ overexpressing HaCaT keratinocytes where we observed similar caspase-8 cleavage pattern upon DR-activation (see Figure 5.3). Surprisingly, TRAF2 is being known activator NF-κB shown to recruit in $cFLIP_L$, and its mutant $cFLIP_{D376N}$ HaCaT cell lines where we observed suppression of DR-induced NF-κB activation. In summary TRAF2 is not one of the crucial modulator in DR-induced NF-κB activation in our cellular system HaCaT keratinocytes.

6. Downregulation of cFLIP sensitizes primary human keratinocytes to TRAIL-induced apoptosis and NF-κB irrespective of apoptosis induction

Primary human keratinocytes are highly resistant to TRAIL-induced cell death. The substantial resistance to DL-induced apoptosis might correlate with the observed high expression of cFLIP protein. Therefore we want to study if cFLIP modulates DR-induced NF-κB activation under endogenous conditions in primary keratinocytes (PK) as our model system. PK are known to express high levels of cFLIP *in vitro* and are also shown to be more resistant to TRAIL then transformed HaCaT keratinocytes [11]. We thus performed knockdown experiments using shRNA against cFLIP as recently described [15;23]. Efficient downregulation of cFLIP isoforms dramatically sensitized primary keratinocytes to TRAIL-mediated cell death (see Figure 6A&B).

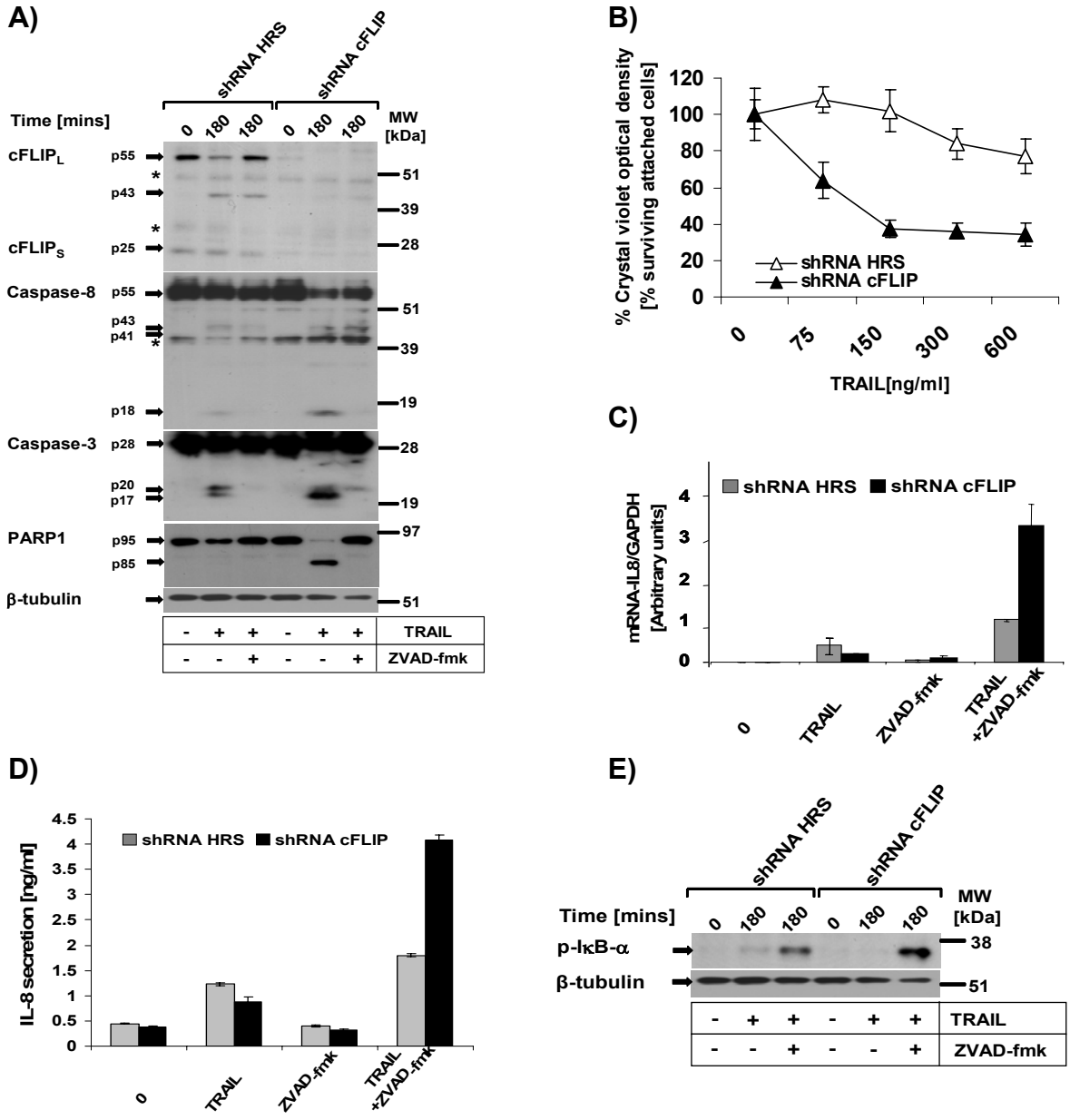


Figure-6 Knockdown of cFLIP proteins leads to increased sensitivity to TRAIL-mediated apoptosis and NF-κB activation in primary human keratinocytes. (PK) A) PK were infected with cFLIP-specific siRNA using recombinant retroviruses and transduced cells were selected with puromycin (1 µg/ml) for 3 days. Hyper random sequence (HRS)-infected cells Vector served as control for cFLIP shRNA-transduced PK. Cells were subsequently stimulated with 150ng of recombinant TRAIL for 3h and analyzed for cFLIP, caspase-8, and caspase-3 expression and cleavage. Arrows indicate molecular weights of protein cleavage fragments. Western blotting of the caspase substrate PARP-1 and detection of the 85kDa fragment served as marker for caspase activity under those conditions. Analysis of β-tubulin protein expression served as loading

control. Molecular weights are indicated on the right side. **B)** PK generated as described in A) were treated with the indicated concentrations of TRAIL for 24 hours. Viability was subsequently examined by crystal violet assay. Shown are mean ± SD of three independent experiments. **C)** cFLIP knock down PK and HRS expressing PK which served as a internal control were stimulated with 150ng of TRAIL for 6h and performed qPCR as mentioned before. Shown are mean +/- standard error of mean (SEM) of a total of three independent experiments. **D)** cFLIP knock down and HRS transduced primary keratinocytes were stimulated with 150ng of TRAIL for 24h and supernatants are assayed for ELISA. Shown are mean +/- standard error of mean (SEM) of a total of three independent experiments. **E)** Cells as indicated in A) were stimulated with 150ng of TRAIL for 3h and subsequently analysed for IκBα phosphorylation by western blotting with antibodies recognizing the phosphorylated forms of IκBα̃. Equal amounts of protein (6µg) were separated by SDS-PAGE gel electrophoresis, and subsequent analysis of β-tubulin protein expression served as loading control. Show is one representative experiment of three independent experiments.

In addition, increased cleavage of initiator caspase-8 and caspase-3 was detectable. Indirect evidence of increased caspase activity in PK with repressed cFLIP levels was obtained by analysis of the caspase substrate PARP1 (see Figure 6A). To investigate if loss of cFLIP may modulate TRAIL-mediated NF-κB activation, we next analyzed TRAIL-mediated phosphorylation and degradation of IκBα in these cells. Repression of cFLIP levels resulted in a rapid phosphorylation of IκBα when compared to control-infected cells (see Figure 6E). Also the loss of cFLIP led to increased expression of the NF-κB target gene IL-8 at the mRNA level (see Figure 6C) and protein level (see Figure 6D) in primary keratinocytes. Taken together these results indicate a physiological role of cFLIP not only for apoptosis protection, but also for inhibition of DL-induced NF-κB activation and target gene induction.

7. Death receptors (TRAIL-R1/R2 and CD95) surface expression in cFLIP overexpressed keratinocytes

HaCaT keratinocytes undergoes apoptosis following treatment with death ligands (TRAIL/CD95L) via surface expressed death receptors which include TRAIL-R1/R2 and CD95-R. Interestingly, in addition to apoptosis it has also been shown

that Death receptors also trigger non-apoptotic signalling pathways such as NF-κB and MAPK p38, JNK. To investigate the death receptors (TRAIL-R1/R2, CD95-R) surface expression in different retro virally generated polyclonal cell lines like HaCaT-cFLIP$_L$, HaCaT-cFLIP$_S$, HaCaT-cFLIP$_{p43}$, or HaCaT-cFLIP$_{D376N}$ as well as the respective control cells were analyzed for the cell surface expression by FACS scan for green fluorescent protein-positive/propidium iodide-negative cells. The cell surface expression of death receptors was not altered between control cells and all the different cFLIP isoforms/mutants overexpressed HaCaT keratinocytes (see Figure 7). This finding suggests that differences of TRAIL sensitivity (see Figure 1B) are not due to the differential surface expression of death receptors (TRAIL/CD95).

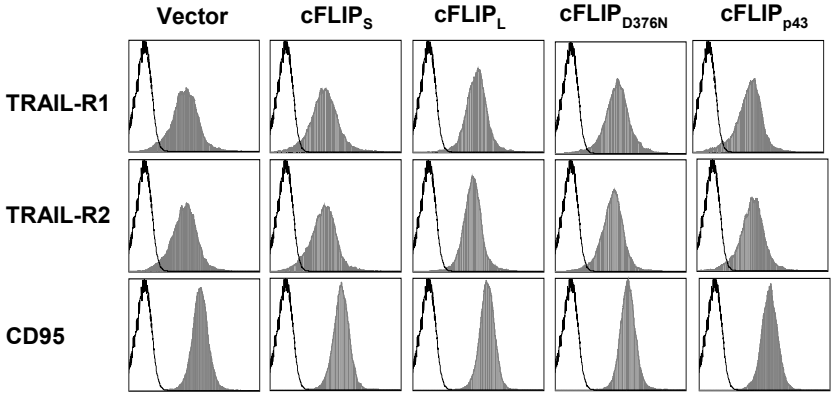


Figure-7 TRAIL-R and CD95-R surface expression of different cFLIP mutants expressing HaCaT keratinocytes. A) TRAIL-R1 -TRAIL-R4 and CD95 cell surface expression of cFLIP isoforms/mutants overexpressed HaCaT keratinocytes were determined by FACS analysis. Cultured cells were stained with TRAIL-R1 (HS101), TRAIL-R2 (HS201), TRAIL-R3 (HS301), TRAIL-R4 (HS402), and CD95 (APO-1 IgG1) primary Abs as well as isotype-matched control Abs. Filled curves indicate receptor specific staining as compared to isotype-matched control staining (open curves).

IV. DISCUSSION

1. cFLIP is a crucial modulator of death receptor mediated signalling pathways

Death receptors belong to the tumor necrosis factor receptor (TNF) super family and can induce apoptosis through activation of procaspase-8. The cellular FLICE-inhibitory protein (cFLIP) is able to modulate the activation of procaspase-8 and thereby prevents the induction of apoptosis mediated by death receptors. As an important physiological modulator of caspase-8, cFLIP regulates life and death in various types of normal cells and tissues, such as lymphoid cells, and renders resistance to death receptor-mediated apoptosis in many types of cancer cells [18;20]. In addition to being an apoptosis modulator, cFLIP has also been shown to exert other physiological functions related to cell proliferation and tumorigenesis [59]. Dysregulation of cFLIP expression has been linked to various diseases, such as cancer and autoimmune diseases, and hence cFLIP might be a critical target for therapeutic intervention [6]. At the onset of this work, the role of death ligand induced cFLIP cleavage in non-apoptotic signalling pathways was controversially discussed. This could be Primarily, due to the transient transfections of cFLIP mutants and also possibly because of the direct effect of cFLIP overexpression in earlier studies [60;64]. We sought to elucidate the role of cleavage of cFLIP, which in turn, could differentially influence non-apoptotic DR signals. Insights into the differential regulation of cFLIP isoforms/mutants could advance our understanding of the physiological and pathophysiological relevance of this molecule in tumor progression and development.

2. cFLIP inhibits DR-mediated apoptosis independent of its cleavage product in HaCaT keratinocytes

Previous studies suggest that, cFLIP is a major anti-apoptotic protein that renders normal and transformed cells resistant to death receptor-mediated

apoptosis [4;18;20;32], even though some reports suggest that cFLIP is a pro-apoptotic protein in death receptors mediated apoptosis. These findings explain the non-physiologic aggregation of large amounts of cFLIP and caspase-8, leading to processing and activation of caspase-8. However, at lower or nearer to physiologic expression levels, cFLIP protects against death receptor–induced apoptosis [65-67]. The anti-apoptotic properties of cFLIP *in vivo* were also supported by the finding that embryonic fibroblasts derived from cFLIP knockout mice exhibit increased susceptibility to death receptor–induced cell death, whereas they show normal responses to drugs or compounds like etoposide, sorbitol, staurosporine and anisomycin [24]. In contrast, recent studies have also suggested that even at low or near-physiologic concentrations, cFLIP can act both as an inhibitor and enhancer of death receptor–mediated apoptosis, indicating that a rheostat effect between the levels of caspase-8 and cFLIP might determine the ultimate sensitivity of cells to apoptosis induced by death receptors [68]. Thus, subtle changes in intracellular cFLIP levels could determine the outcome of death receptor–mediated signalling resulting either in cell death or cell survival. If this scenario proves true, strict control of cFLIP synthesis and degradation would be of utmost importance for the regulation of cell death and survival. Our previous reports suggest an anti-apoptotic role of cFLIP isoforms when overexpressed very close to the physiological level in HaCaT keratinocytes [4;16].

From our current study, the biochemical characterization of cFLIP isoforms/mutants revealed that the overexpression of different cFLIP isoforms or mutants was shown to be comparable in the different polyclonal cell lines (see Figure 1A). When cells were treated with the death ligands TRAIL and CD95L, biochemical analysis showed that parental HaCaT as well as control-infected cells readily cleaved large amounts of the initiator caspase-8 within 3h of stimulation. In contrast, cFLIP isoforms and mutants ($cFLIP_L$, $cFLIP_S$, $cFLIP_{D376N}$, as well as $cFLIP_{p43}$) block caspase-8 cleavage differentially and the p18 fragment was subsequently not detected (see Figure 1A). In line with previous reports,

$cFLIP_L$ containing a cleavage site mutation ($cFLIP_{D376N}$) was fully protected against TRAIL- or CD95L-mediated $cFLIP_L$ cleavage (see Figure 1A, left and right panel) [20]. Furthermore, different isoforms and mutants of $cFLIP_L$ were equally effective in protecting HaCaT cells from TRAIL-or CD95L-mediated cell death, as indicated by crystal violet assay (Figure 1B left-TRAIL or right-CD95L). More interestingly, our results suggest that cFLIP is able to protect HaCaT keratinocytes against CD95L or TRAIL-mediated apoptosis independent of $cFLIP_L$ cleavage. All these observations are in agreement with our own and several previous reports, that cFLIP is an anti-apoptotic molecule that provides protection against death receptors mediated apoptosis [4;16;18;20;23;28].

3. cFLIP and its mutants substantially modulate recruitment and post translational modifications of DISC-associated proteins in HaCaT keratinocytes

The mechanism in which death receptors activate apoptosis is quite clear, namely through the recruitment of caspase-8 into the DISC. Forced dimerization using different systems then suggested activation of caspase-8 through 'induced proximity' involving two active enzymes. Recently, it was demonstrated that procaspase-8 in the DISC gains enzymatic activity prior to its processing suggesting that dimerization induces a conformational change in the zymogen that results in the activation of the active sites [4;16;18;20;23;28].

To date the role of $cFLIP_L$ in apoptosis remains controversially discussed. In most of the reports, $cFLIP_L$ has been described as an anti-apoptotic molecule, largely because of its ability to inhibit apoptosis at high levels of ectopic expression in cell lines in which the level of endogenous $cFLIP_L$ has been approximately 1% of that of endogenous procaspase-8. Since this ratio is so disproportionate it is unclear what precise role $cFLIP_L$ plays in these cells. However, subsequent studies of mice deficient in cFLIP (lacking both $cFLIP_L$ and $cFLIP_S$) revealed the role of cFLIP in death-receptor induced apoptosis. The embryonic fibroblasts (MEFs) derived from these mice (through an *in vitro*

selection process for cell growth) were shown to be more sensitive to CD95-induced apoptosis than the wild-type MEFs. This observation has been widely accepted as a validation of the inhibitory role of $cFLIP_L$ in apoptosis by many other groups. Strikingly, $cFLIP^{-/-}$ mice showed developmental defects that resembled those of caspase-$8^{-/-}$ or $FADD^{-/-}$ mice. These mice died *in utero* between E10.5 and E11.5 with a failure in heart formation accompanied by hemorrhage attributing a function to $cFLIP_L$ that is similar to that of caspase-8 and FADD [21;24].

Previous reports suggest the significant role of $cFLIP_L$ in having an inhibitory effect on the DISC by reducing the generation of active caspase-8. A second cellular splice variant of cFLIP, $cFLIP_S$, also has anti-apoptotic effects. Both cFLIP proteins contain two DEDs and are recruited into the DISC and thereby block the death receptor-mediated apoptosis [4;28]. From our current studies, we provided important insights into the molecular mechanisms of cFLIP-mediated apoptotic inhibition using the HaCaT keratinocytes as our cellular model of this study. cFLIP is expressed very low endogenously in HaCaT keratinocytes. Ectopically expressed cFLIP isoforms, either $cFLIP_L$ or $cFLIP_S$ or the mutants, unprocessed $cFLIP_{D376N}$ and cleavage product $cFLIP_{p43}$, are protected against death receptor-mediated apoptosis in HaCaT keratinocytes. The presence of $cFLIP_S$ or cleavage product $cFLIP_{p43}$ prevents the initial cleavage step of procaspase-8, and therefore its full-length form can be detected at the DISC. In contrast, $cFLIP_L$ or mutant $cFLIP_{D376N}$ allows the initial cleavage step but blocks further processing and the generation of the p18 subunit. The difference in the processing of procaspase-8 in the presence of the two cFLIP isoforms is also reflected in total cell lysates and these results are in line with previous reports thereby shedding new light on caspase-8 activation at the DISC [20].

From our and other previous observations suggested the following model for cFLIP proteins mediated inhibition of death receptor initiated apoptosis has been suggested. In the presence of low concentrations of cFLIP proteins, procaspase-8 represents the major tandem DED-containing protein at the DISC and is

activated by trans-and autocatalytic cleavage due to the close proximity of several procaspase-8 molecules. $cFLIP_L$ that is recruited into the DISC is cleaved by caspase-8. High amounts of $cFLIP_L$ in the DISC abolish their close proximity to procaspase-8 molecules, instead of leading to proximity of $cFLIP_L$ and procaspase-8, thereby resulting in the first but not the second step of procaspase-8 processing. In this conformation, only the p10 subunit of caspase-8 and the p12 subunit of $cFLIP_L$ are generated. Since $cFLIP_L$ itself has no intrinsic catalytic activity, the generation of the p10 subunit of caspase-8 proceeds autocatalytically, whereas the generation of the p18 subunit would require trans-catalytical activity as suggested in BJAB cells [20].

High amounts of $cFLIP_S$ in the DISC totally prevent procaspase-8 cleavage. This indicates that $cFLIP_L$, in contrast to $cFLIP_S$, still induces a conformation change of the DISC that leads to autocatalytic activity of procaspase-8 and the first cleavage step. This hypothesis is supported by the cleavage pattern of procaspase-8 in the presence of $cFLIP_L$ mutants. The uncleavable $cFLIP_L$ mutant ($cFLIP_{D376N}$), like the wild-type $cFLIP_L$, allows the generation of the p10 subunit of caspase-8. Therefore, the full-length protein of $cFLIP_L$, but not its cleavage, facilitates the first cleavage step of procaspase-8. These results from us and others are further supported by the observation that the deletion mutant of $cFLIP_L$ ($cFLIP_{p43}$), which does not contain the p12 subunit, prevents the first cleavage step of procaspase-8, similar to $cFLIP_S$. Both, the p43 cleavage product of $cFLIP_L$ ($cFLIP_{p43}$) and the uncleaved $cFLIP_L$ form ($cFLIP_{D376N}$) form block caspase-8 activation and therefore, inhibit apoptosis.

Given that procaspase-8 interacts with cFLIP proteins in the DISC in a dimeric manner, our results suggest that the generation of the p10 subunit of caspase-8 occurs autocatalytically as suggested previously [20]. In contrast, the second cleavage step leading to the release of the p18 subunit requires trans-catalytic activity. High expression of $cFLIP_S$ also prevents cleavage of $cFLIP_L$ at the DISC.

This phenomenon was also detected in re-stimulated primary T cells [20]. This might be attributed to the spatial interference of $cFLIP_S$ with the interaction of procaspase-8 and $cFLIP_L$. Another possibility is that initial cleavage of procaspase-8, blocked by $cFLIP_S$, is required for its trans-catalytic activity and, thus, $cFLIP_L$ is not cleaved at the DISC upon high expression of $cFLIP_S$.

Thus, so far the biological function of different procaspase-8 cleavage patterns by cFLIP isoforms has not been addressed. One might speculate that the generation of these cleavage products is blocked because they are necessary for the recruitment of yet to be identified molecules into the DISC or, alternatively, they prevent the association of unidentified molecules.

Recent findings further described an *in vitro* DISC reconstitution model, where they clearly demonstrated a two-step activation of procaspase-8 that includes firstly, dimerization and secondly the proteolytic cleavage which is obligatory for the death receptor-induced apoptosis. Furthermore, cellular expression of non-cleavable procaspase-8 mutants, which undergo DISC-mediated oligomerization but not cleavage fails to initiate CD95-induced apoptosis and was shown to undergo CD95-induced survival. These findings represent an important paradigm shift in the current understanding of DISC-mediated procaspase-8 activation, as the reconstituted CD95 DISC model has revealed a key regulatory mechanism whereby activated death receptor complexes can signal for death or survival [69].

Previously, it was reported that cellular FLIP proteins regulate activation of the non-apoptotic signalling pathways such as NF-κB and AP-1 upon stimulation of death receptors, possibly via recruitment and posttranslational modifications of DISC associated proteins RIP1, TRAF1, and TRAF2, A20. However, there are also conflicting reports about the exact role of cFLIP proteins which includes enhanced or suppressive effects in death receptor mediated NF-κB activation [4;28;33;64]. Therefore, the role of cFLIP and its different cleavage products in death receptor induced NF-κB activation, with possible differential recruitment of NF-κB signal inducing proteins remains controversial till date.

In the current study we reported that cFLIP modulated the DL-mediated recruitment and also posttranslational modifications of DISC-associated proteins. In addition, our data confirm that an important function of cFLIP isoforms/mutant is to prevent caspase-8 activation at different levels of procaspase-8 processing at the DISC. In line with previous results, $cFLIP_L$, $cFLIP_{D376N}$ induce a conformational change of procaspase-8 that allows partial but not complete proteolytical processing, whereas in contrast $cFLIP_S$, $cFLIP_{p43}$ even prevent partial procaspase-8 activation at the DISC. However, the adapter protein FADD recruitment remains unchanged. Notably, all the cFLIP isoforms/mutants suppress the recruitment and ubiquitination of receptor interacting protein-1 (RIP1) suggesting that cleavage of cFLIP is irrelevant for the repression of RIP1 in the receptor complex (Figure 5.1-A&B).

A large body of evidence suggests that RIP1 recruitment to the receptor complex (TNF-R, CD95-R, TRAIL-R) and posttranslational modifications are the two crucial events for the activation of transcriptional factor NF-κB [70;71]. Here we show that not only does $cFLIP_L$ suppress the RIP1 modification and recruitment as shown previously [4], but also other isoforms/mutants ($cFLIP_S$, $cFLIP_{p43}$, $cFLIP_{D376N}$) showed repression of RIP1 recruitment and ubiquitination to the receptor complex which results in the suppression of death ligand mediated NF-κB activation (see Figure 9). It is likely that ubiquitinated RIP1 interacts with NEMO (subunit of IKK complex), thereby activating NF-κB activation as previously suggested [71]. Since all the cFLIP isoforms/mutants suppress RIP1 recruitment by an unknown mechanism, which leads to the inhibition of NEMO (one of component of IKK complex). This sort of an interaction finally leads to the suppression of death receptor mediated NF-κB activation. The above results are also in line with earlier reports, suggesting that, RIP1 knockdown substantially inhibited Apo2L/TRAIL induced phosphorylation and degradation of IκB-α in HT1080 cells indicating the importance of RIP1 function in NF-κB signalling [72].

Several other reports suggest that the involvement of TRAF2, yet another effector molecule which is recruited to TNF-R and CD95-R/TRAIL-R receptor complex and influences NF-κB, JNK and MAPK p38 activation [73]. A number of reports indicate that TRAF2 is recruited to DR complexes and thereby influences NF-κB, JNK, and MAPK p38 activation [74]. Moreover, endogenous TRAF2 was shown to interact with $cFLIP_{p43}$ and promote formation of a $cFLIP_{p43}$-caspase-8-TRAF2 tertiary complex. This complex was suggested to be a prerequisite for NF-κB activation in lymphocytes. Our study now investigated the differential outcomes of DR triggered NF-κB activation. The CD95 DISC of HaCaT overexpressing $cFLIP_L$ or the $cFLIP_{D376N}$ mutant contained TRAF2 in a stimulation-dependent manner, in line with another report in pancreatic tumor cells [75]. However, we noticed that CD95-mediated suppression of NF-κB activation was not altered by the different cFLIP isoforms/mutants regardless of the recruitment of TRAF2. This indicates that TRAF2 recruitment to the CD95 DISC is not critical for DR-induced suppression of NF-κB activation (see Figure 5.3). In line, recent evidence for TNF has demonstrated that NF-κB activation in TRAF2 KO murine embryonic fibroblasts (MEFs) is unaltered [76]. Thus, TRAF2 may either require other cFLIP-dependent secondary proteins or binding of TRAF2 to the DISC may be indirect. Taken together these observations indicate that cleavage of $cFLIP_L$ or caspase-8 in the DISC is neither associated with increased NF-κB signalling nor necessary for the inhibitory function of cFLIP isoforms on DR-induced NF-κB signalling.

Interestingly, there is growing evidence that a number of cytosolic complexes formed upon death receptor stimulation are essential for life/death decisions. The formation of complex II comprising of procaspase-8, cFLIP, FADD, RIP, TRADD and TRAF2 was described in detail for TNF signalling. Notably, Larvik et al, reported that upon CD95 stimulation in several T and B cell lines a novel signalling complex is formed, which they termed as complex II [77]. Complex II is composed of the DED (Death Effector Domain) proteins: procaspase-8a/b, three isoforms of cFLIP ($cFLIP_L$, $cFLIP_S$,$cFLIP_R$) and FADD. However, the complex II

does not contain CD95. Based on these findings they suggested that CD95 signalling comprises of two steps. The first step involves the formation of the DISC at the cell membrane. The is followed secondly by the formation of the cytosolic DED protein containing complex that may play an important role in the amplification of caspase activation [77].

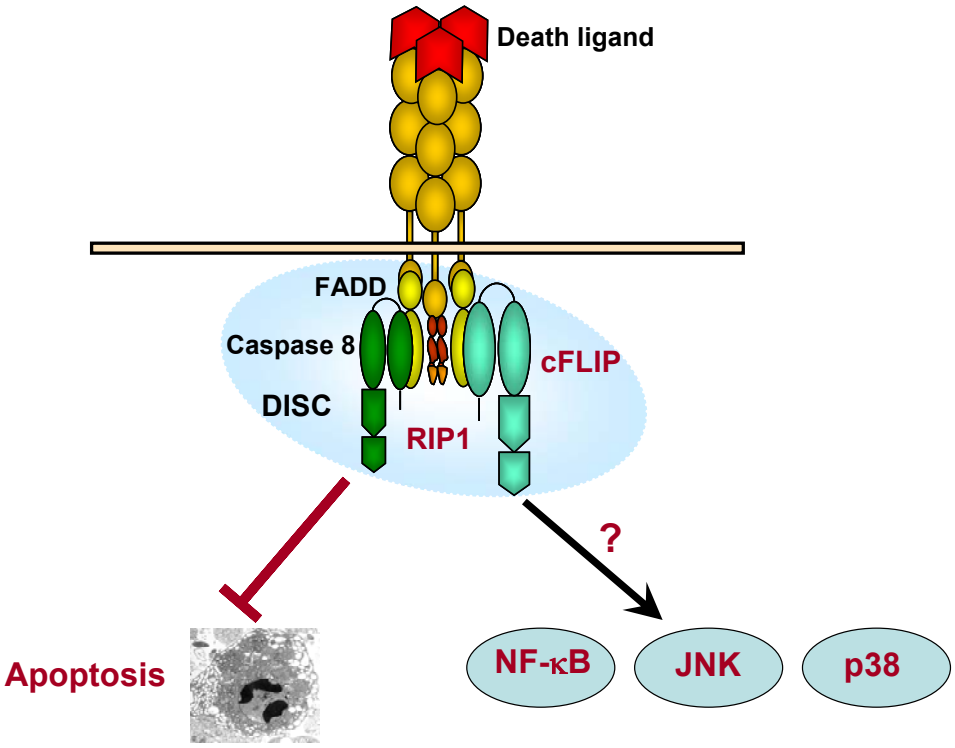


Figure-9 cFLIP proteins suppress DR-induced RIP1 recruitment and its posttranslational modifications irrespective of cFLIP cleavage in HaCaT keratinocytes.

Furthermore, emerging evidence suggests that Apo2L/TRAIL activates kinase pathways by promoting the association of secondary signalling complex subsequent to death-inducing complex (DISC). This secondary complex retains DISC components which are involved in Apo2L/TRAIL-induced kinase pathway such as RIP1, TRAF2, NEMO [72]. On an interesting note, other reports recently demonstrated that upon TRAIL stimulation, the same molecules (FADD, RIP, TRADD and TRAF2, Caspase-8) are involved in the formation of a secondary

complex and this complex is essential for the transduction of both life and death signals [72]. The understanding of the role of complex II in life and death pathways is a matter of future studies.

4. cFLIP proteins modulate DR-mediated non-apoptotic signalling pathways

A large body of evidence till date suggests that three main isoforms of cFLIP-cFLIPS, cFLIPL and $cFLIP_R$, have been reported to block DR mediated apoptosis [4;17;28]. However differential signalling capabilities of various cFLIP isoforms have not yet been studied so far. We reasoned that the cleavage of cFLIP, in turn, could differentially influence non-apoptotic DR signals such as NF-κB and mitogen-activated protein kinases (MAPK). The functions of these signalling pathways may have important and distinct stage-specific pathophysiological consequences during skin tumorigenesis. Targeting these crucial signalling pathways might prove to be a novel strategy for cancer prevention and therapy and would be required prior to systematic cancer treatment of patients with TRAIL or TRAIL-R-agonists [78]. Death receptor mediated non-apoptotic signalling pathways have been a topic of interest scientific debate over the years, in particular due to the fact that TRAIL or TRAIL-R agonists are currently being used in early clinical studies [78]. Although cFLIP isoforms are known for their apoptosis resistance, their non-apoptotic functions have aroused immense interest especially in cellular events that lead to potential detrimental effects whenever TRAIL stimulates tumor cells that carry death receptors at the surface, but do not undergo apoptosis.

4.1 cFLIP represses DR-mediated NF-κB activation independent of cFLIP or caspase-8 processing

The precise role of cFLIP isoforms in death receptors mediated transcriptional factor NF-κB activation is of intense debate over several years. It is also a widely

accepted notion that death receptors not only induce apoptosis but can also trigger other signalling pathways that lead to proliferation. There are also conflicting reports about the molecular mechanisms that switch from death receptor induced apoptosis to proliferation and/or differentiation. There are contradictory reports which suggest that cFLIP, which is upstream to DISC-associated protein, may be pivotal in turning signals for cell death into those for cell survival.

Some reports suggest that transient ectopic expression of cFLIP promotes activation of proliferative signals such as those of transcriptional factor NF-κB and ERK signalling pathways and further indicated that increased levels of cFLIP can lead to the activation of ERK and NF-κB signalling pathways. Furthermore, another additional report suggested that NF-κB-inducing protein TRAF2 specifically interacts with the cleavage product $cFLIP_{p43}$ and enhances NF-κB activation. However, these observations were made from studies of the direct effect of overexpressed $cFLIP_L$ with transient overexpression in 293T cells [60;64]. Therefore, cFLIP might have a role in the regulation of NF-κB dependent gene expression, which could affect cellular proliferation in response to stimulation of death receptors. Consistent with its role in the NF-κB pathway, cFLIP can interact with other NF-κB–related signalling proteins, such as TRAF1, TRAF2, and IKK2 [79]. Contradicting to the earlier reports, retroviral stable overexpression studies revealed that cFLIP isoforms $cFLIP_S$ and $cFLIP_L$ are inhibitory to death receptor induced activation of NF-κB. Our previous studies and other reports confirm that the NF-κB signalling protein RIP1 recruitment and its modification in the death inducing signalling complex (DISC) is crucial for the activation of NF-κB. The ectopic expression of $cFLIP_L$ shown previously in HaCaT cellular system previously is likely to remain at physiologically relevant levels, since we could overcome TRAIL resistance by increasing concentrations of TRAIL. Notably, HaCaT keratinocytes show very low endogenous expression of cFLIP, therefore it is an ideal system for the overexpression studies [4]. It is speculated that the

proteolytic processing of cFLIP isoforms by partially activated procaspase-8 at DISC, might play an important role to induce distinct non-apoptotic signalling events with possible variable recruitment of DISC associated proteins. Many previous studies indicated the significance of cFLIP cleavage products in death receptors mediated non-apoptotic signalling pathways.

In the current study, we investigated the impact of cFLIP isoforms and the role of their cleavage fragments in DR mediated NF-κB activation. We now show that only TRAIL-induced and not TNF-induced NF-κB activation is affected by cFLIP isoforms ($cFLIP_S$, $cFLIP_L$ and its cleavage fragment ($cFLIP_{p43}$), non-processed fragment ($cFLIP_{D376N}$) (see Figure 4,1 A-D). This difference is also reflected by distinctive ligand-mediated IκBα degradation, thereby placing cFLIP isoforms/mutants mediated inhibition upstream of or at the signalosome. What may be the reason for the difference between TNF and TRAIL-mediated NF-κB activation? Since we detected NF-κB activation and IκBα degradation within 15–30 min after stimulation with soluble TNF, the activation of NF-κB via TNF-R2 by endogenous membrane bound TNF was unlikely. Therefore, previous results imply a direct activation of NF-κB via TNF-R1 rather than induction of endogenous TNF. Thus, non-apoptotic death receptor signalling by TNF-R1 is not abrogated by $cFLIP_L$ but rather specifically inhibited for TRAIL, whereas $cFLIP_L$ efficiently blocks pro-apoptotic signals of both TNF and TRAIL [24;33]. These data confirm that pro-apoptotic and gene-inductive signalling pathways utilized by TNF and TRAIL are not identical, proving to be similar to yet another report for CD95L [28].

Previous studies by several groups have shown that apoptosis induction interferes with proinflammatory gene expression elicited by Fas/TRAIL, because the addition of caspase inhibitors such as ZVAD-fmk either leads to increased induction of target genes or is needed for TRAIL-dependent transcription [5;28;80]. Using the physiological caspase-8 inhibitors $cFLIP_L$, $cFLIP_S$ and its mutants $cFLIP_{D376N}$, $cFLIP_{p43}$, we show that cFLIP isoforms/mutants potently inhibit

proinflammatory DR-induced target genes such as IL-8 independent of cFLIP or caspase-8 (see Figure 4,2 A&C) cleavage and thereby demonstrate that the effect exerted by the caspase inhibitor ZVAD-fmk is clearly different from the ability of cFLIP to interfere with TRAIL receptor signalling. Understanding this difference is of great importance, since the clinical use of caspase inhibitors may result in potentially deleterious proinflammatory signals exerted by death receptors when apoptosis induction is blocked by ZVAD-fmk *in vivo*.

Furthermore, we observed that caspase inhibitor ZVAD-fmk stabilizes death-ligand induced DISC complex. Since upon pre-treatment with ZVAD-fmk, CD-95/TRAIL enhances the recruitment of DISC-associated proteins such as cFLIP, caspase-8, RIP1, FADD. Interestingly, there is increased recruitment of the cleavage fragment of cFLIP ($cFLIP_{p43}$), in $cFLIP_L$ overexpressing HaCaT keratinocytes upon ZVAD-fmk treatment indicating that caspase inhibitor (ZVAD-fmk) inhibits caspase-8 activation downstream of DISC (see Figure 5,2). In the current study, we investigated the DR-induced non-apoptotic signals and found that TRAIL or CD95L efficiently activated NF-κB in HaCaT keratinocytes. It is currently uncertain as to how cFLIP isoforms/mutants influence the activation of NF-κB [4;28]. In this report, we have studied how cFLIP isoforms and its DISC-associated cleavage fragments, $cFLIP_{D376N}$ and $cFLIP_{p43}$ modulate DR-mediated gene induction in HaCaT keratinocytes. $cFLIP_L$ was described as a constitutive activator of NF-κB exerted by $cFLIP_L$-mediated recruitment of TRAF-2 to the caspase-like domain of $cFLIP_L$. Furthermore, It was suggested that heterodimers of the p43 fragment of $cFLIP_L$ and the p43/41 fragments of caspase-8 are formed at the CD95 DISC and thereby activate NF-κB [60;64].

However, these results were based on direct ectopic expression of the cleavage product $cFLIP_{p43}$ rather then the direct effect of death receptor ligation. In contrast to these reports, our group and others have suggested that death receptor-mediated NF-κB activation was inhibited by $cFLIP_L$ and $cFLIP_S$ [4;28]. In these reports, they did not observe any changes in basal NF-κB DNA binding to

casapase-8 in cells expressing different isoforms of cFLIP ($cFLIP_{S/L}$). The discrepancy with the report in 293T cells might be explained by the direct effect of overexpressed $cFLIP_L$ in this study [64]. Our previous results and those of others are in accordance to the findings by many other groups [4;28] demonstrating an inhibitory role of cFLIP isoforms ($cFLIP_{Short}$ and $cFLIP_{Long}$ for death receptor-induced NF-κB activation. From our current results, we demonstrate that not only the cFLIP isoforms ($cFLIP_L$, $cFLIP_S$) but also its mutants ($cFLIP_{D376N}$, $cFLIP_{p43}$) completely inhibited DL-mediated IκBα phosphorylation and degradation, NF-κB DNA binding and transcription and induction of the target gene IL-8. These observations support previous reports that $cFLIP_L$ knockdown accelerates TRAIL-induced NF-κB activation in HT1080 cells [72]. Our data clearly demonstrate that the cleavage of cFLIP is not necessary for the death ligand mediated NF-κB activation (see Figure 10). These results also confirm that the differential processing of caspase-8 by cFLIP isoforms/mutants at the DISC is neither sufficient for apoptosis induction nor does it predominantly lead to gene induction.

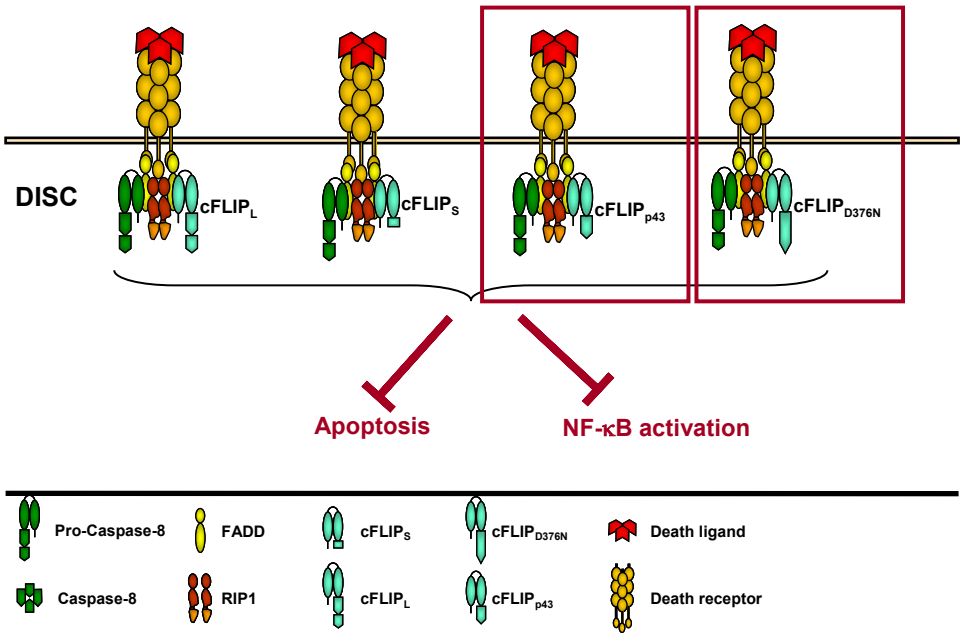


Figure-10 Cleavage of cFLIP is NOT necessary for DR-induced NF-κB activation in HaCaT keratinocytes.

In summary, cFLIP isoforms are not only potent inhibitors of DL-mediated apoptosis, but also block DL-mediated non-apoptotic signalling pathways such as NF-κB. This indicates that cleavage of $cFLIP_L$ or caspase-8 in the DISC is neither associated with increased NF-κB signalling nor necessary for the inhibitory function of cFLIP isoforms on DR-induced NF-κB. Taken together, our data highlight the importance of cFLIP and its isoforms for the inhibition of DR-induced non-apoptotic signals that might be of crucial importance during tumorigenesis of keratinocyte skin cancer in order to escape the activation of innate or adaptive immune responses.

4.2 Sustained activation of JNKs by TNF is dispensable for cell death induction

JNK activation can mediate not only pro-apoptotic but also anti-apoptotic effects in death receptor signalling, especially after TNFR1 stimulation [34;79;81;82]. The pleiotropic cellular effects of TNF are in part the consequence of intense and multifold cross talk between the major TNF-induced intracellular signalling pathways especially those leading to apoptosis or activation of NF-κB and JNK. In the recent years, many reports revealed that JNK pathway can tip the balance between cell death induction and survival signalling in TNF-stimulated cells in both directions. The cell death promoting effect of JNK in TNF signalling however has been attributed to a second sustained phase of JNK activation that follows on the early phase of JNK activation especially in cells sensitized to apoptosis by cycloheximide (CHX) treatment or inhibition of NF-κB (for review [83]). Several mechanisms by which TNF-induced cell death have been identified include caspase independent cleavages of BID, proteasomal degradation of $cFLIP_L$ and ROS production [81;82]. Whether TNF-induced cell death signalling is also involved in the modulation of JNK signalling is yet largely unknown, more so, in particular the relationship of TNF-induced apoptotic caspases and prolonged JNK signalling is unclear. It seems possible that there is interplay between JNK and

caspases in other cell types, where apoptosis induction by TNF is more prominent. We therefore chose the HaCaT keratinocytes as our model of study with respect to the relationship of cell death induction, caspase activation and JNK and NF-κB stimulation in the context of TNF signalling. The prolonged JNK activation by TNF has been ascribed to the relief from the inhibitory actions of the NF-κB pathway. From our previous observations we demonstrated that inhibition of NF-κB dramatically sensitizes human keratinocytes to TNF- but not to TRAIL-induced apoptosis and that this sensitization to TNF was largely independent of cIAP2.[34]. In the current study, we report that TNF family members, TNF and TRAIL induce sustained JNK activation in NF-κB inhibited HaCaT keratinocytes (see Figure 4.3 B). Furthermore, the pharmacological JNK inhibitor SP600125, efficiently blocked JNK activity but had no effect on TNF induced cell death in many cellular systems we used in our present study, including in the HaCaT keratinocytes (see Figure 4.3 C). Therefore, TNF-induced prolonged JNK activation was cell type dependent and mediated by caspases and had no essential role in cell death induction by TNF in the cellular models investigated in this study.

4.3 cFLIP isoforms inhibit death ligand mediated JNK, MAPK p38 activation in HaCaT keratinocytes

Several tumor types have been reported to have inappropriately elevated levels of cFLIP, e.g. melanoma, colon carcinoma, and Hodgkin lymphoma. High expression levels of cFLIP in tumor cells could lead to resistance to death ligand induced apoptosis induction. Interestingly, in immunocompetent mouse models, cFLIP overexpressing B lymphoma tumor cells developed into more aggressive tumors than the respective control cells, both in syngenic and semi-allogeneic tumor-host systems, indicating that cFLIP expression was associated with a selective advantage for tumor growth. Furthermore, cFLIP overexpression in tumor cells was capable of preventing tumor rejection by perforin-deficient NK cells. These results provided compelling evidence that, deregulated cFLIP

expression in tumor cells could act as a tumor-promoting factor and assist the cancer cells to evade the milieu of immune surveillance mechanisms [84]. Various lines of evidence suggest that death-inducing proteins have an additional role in regulating innate immunity. Subsequent reports generated conditional deletion of cFLIP using CD19-Cre to analyze the function of cFLIP in B cells. The resulting B cell-specific cFLIP-deficient mice were found to have reduced numbers of peripheral B cells that were hypersensitive to Fas-induced apoptosis and impaired proliferation induced by Toll like receptors (TLRs) and the B-cell receptor (BCR). Furthermore, there was aberrant expression of co-stimulatory proteins and activation markers in cFLIP deficient B cells. Whereas LPS-induced activation of NF-κB and Erk1/2 appears to be unaffected, p38 and JNK were spontaneously activated and hyperinduced in cFLIP-deficient B cells. Therefore, these data revealed novel functions of cFLIP in B cells [84].

In the current study we observed that death ligands (TRAIL/CD95L) induce JNK phosphorylation while cFLIP isoforms ($cFLIP_S$, $cFLIP_L$) inhibit death ligand induced JNK phosphorylation (see Figure 2.1A). Surprisingly, we also observed that the cFLIP mutants ($cFLIP_{D376N}$, $cFLIP_{p43}$) suppress death ligand mediated JNK suppression, indicating that the cleavage of cFLIP is not necessary for the JNK activation (see Figure 2.1B). We also observed that caspase activation is essential for the death ligand mediated JNK phosphorylation, since the caspase inhibitor (ZVAD-fmk) completely blocked the TRAIL-induced JNK activation in HaCaT keratinocytes. We went on to investigate TRAIL mediated AP-1 activation which is the downstream target of JNK. Also identical with the JNK results, we also observed that cFLIP isoforms/mutants suppress TRAIL mediated AP-1 activation (see Figure 2.1C). To sum up, cFLIP inhibits TRAIL mediated JNK phosphorylation and its downstream target transcriptional factor AP-1 activation indicating that cFLIP or caspase-8 cleavage is not necessary for the death ligand mediated activation of JNK and transcriptional factor AP-1 in HaCaT keratinocytes. Our studies are consistent with the previous reports suggesting

that cFLIP plays a crucial role in modulating the activation of both caspases and stress MAPKs in immune cells [84].

Death ligands also activate MAPK p38 activation in many cellular systems. Our studies indicate that TRAIL activates MAPK p38 activation in a biphasic manner. TRAIL activates the first peak as early as 15-30mins and lasts for 2h (second peak) and finally the activation of p38 goes down at later time points. Interestingly, all the cFLIP isoforms/mutants suppress the TRAIL mediated second peak MAPK p38 phosphorylation. Furthermore, isoforms/mutants of cFLIP did not modulate TRAIL mediated first peak of MAPK p38 activation. Strikingly, TRAIL mediated MAPK p38 activation is inhibited upon treatment with caspase inhibitor ZVAD-fmk. ZVAD-fmk only inhibited the second peak but not the first peak (see Figure 3,1A). These results clearly demonstrated that cFLIP and its cleavage products suppress TRAIL mediated MAPK p38 activation independent of cFLIP cleavage (see Figure 11). These results are comparable to many other reports suggesting that $cFLIP_L$ exerts its anti-apoptotic activity partially by inhibiting MAPK p38 activation [85].

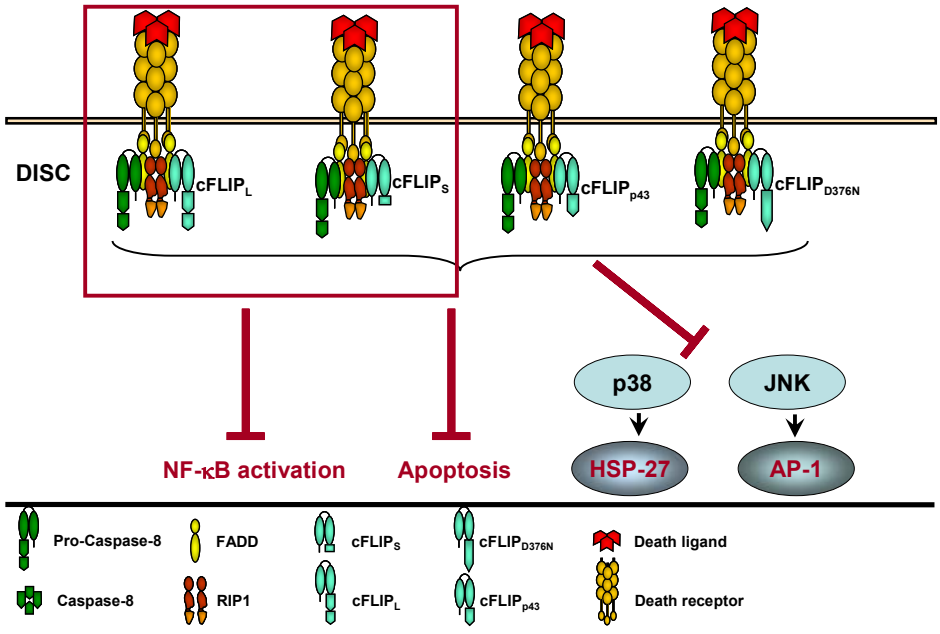


Figure-11 cFLIP proteins inhibit DR-induced p38 and JNK activation In HaCaT keratinocytes.

Alike to these findings, all the cFLIP isoforms/mutants suppress RIP1 recruitment at CD95/TRAIL DISC, which in turn results in the suppression of TRAIL-induced MAPK p38 and JNK activation. These results confirm many other observations, indicating that RIP null ($RIP^{-/-}$) mouse fibroblast cells show decreased activation of TNF-induced JNK and MAPK p38 activation. Also the reconstitution of wild type RIP in $RIP^{-/-}$ cells partially restored the activation of MAPK p38 and JNK in response to TNF-α. RIP1 kinase domain is only essential for the activation of ERK but not p38, JNK. This indicates that RIP1 is likely to interact with different downstream targets to activate different MAPKs.[74]. Our results are in concordance with the findings by Wajant et al and many others, demonstrating an inhibitory role of $cFLIP_L$ for death receptor-induced non-apoptotic signalling pathways [30;84-86].

5. cFLIP knockdown enhances TRAIL-induced apoptosis and NF-κB activation

Tumor cells have developed different strategies to escape apoptosis induced by death receptors, triggering effects such as down-regulation of death receptors (TRAIL-R/R2, CD-95R), loss or mutation. The other known mechanisms elaborated by tumor cells to develop cell death resistance include aberrant expression of anti-apoptotic molecules such as cFLIP, Bcl_2, Bcl-x_L, survivin and IAP family proteins (cIAP1/2, XIAP, livin). The current understanding is that perturbations in apoptotic death regulation constitute a vital step in cancer evolution. There is increasing evidence, which suggests that the anti-apoptotic function of cFLIP can be considered as a tumor progression factor. At present, the role of cFLIP, as an anti-apoptotic protein involved in the regulation of the DR extrinsic apoptotic pathway, remains unclear. However, the identification of the precise role of cFLIP in cancer cells is an essential step to further target and restore deregulated death pathways [6;25;87].

Primary human keratinocytes are highly resistant to TRAIL-induced cell death. The substantial resistance to DL-induced apoptosis might correlate with the

observed high expression of cFLIP proteins. Therefore, we want to study if cFLIP modulates DR-induced NF-κB activation under endogenous conditions using the primary keratinocytes (PK) as our model system. PK are known to express high levels of cFLIP *in vitro* and are also shown to be more resistant to TRAIL than transformed HaCaT keratinocytes [11].

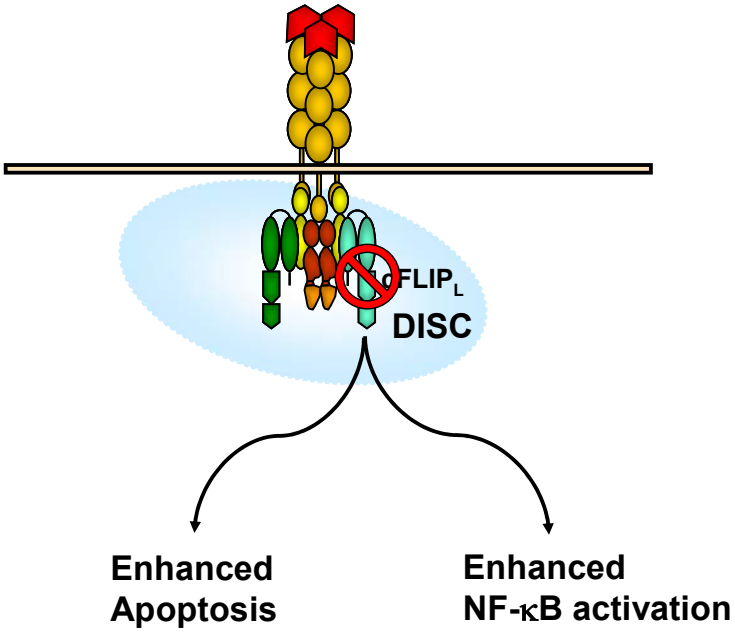


Figure-12 cFLIP knockdown not only enhances TRAIL-induced apoptosis but also enhances NF-κB activation

Throughout our study, we corroborated the inhibitory role of cFLIP isoforms to death ligand- mediated NF-κB activation by overexpression studies, as reported previously by others [4;28]. Most of the information known so far about the inhibitory action of the cFLIP molecule has been obtained under non-physiological conditions. In the present study, we have shown the physiological role of cFLIP in death ligand-mediated NF-κB activation by using primary keratinocytes as a model system. Consistent with previous observations, we also found enhanced activation of caspase-8 and caspase-3 and caspase substrate PARP-1 in the cFLIP knock down primary keratinocytes (see Figure 6 A&B). Interestingly, we

also found TRAIL-induced pronounced activation of phosphorylation of IκBα in cFLIP knock down cells (see Figure 6E). Furthermore, we also observed enhanced activation of NF-κB target gene IL-8 in the absence of cFLIP, supporting the hypothesis that cFLIP is required in the skin (see Figure 12) to inhibit proliferative signals through mechanisms such as the NF-κB signalling pathway (see Figure 6 C&D).

In the skin there is a constant need to repress proinflammatory gene induction and unwanted cell death, at least in the basal cell layer of the epidermis [88]. This corresponds well to our present data showing that cFLIP isoforms block inflammatory CD95 and TRAIL-R signalling irrespective of cell death induction. In the absence of caspase activity in cells expressing low levels of cFLIP, we show augmented NF-κB activation and target gene induction. Thus, blockade of NF-κB by cFLIP is not an epiphenomenon of cell death protection or due to overexpression, supporting the notion that cFLIP has an indispensible role in the skin to downregulate inflammatory responses.

V. APPENDIX

1. Abbreviations

HaCaT	**Human adult keratinocytes**
TRAIL	**Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand**
TNF	**Tumor Necrosis Factor**
TRAIL-R	**TRAIL receptor**
CD-95R	**CD-95 receptor**
ZVAD-fmk	**Z-Val-Ala-Asp-fluoromethyl ketone**
NF-κB	**Nuclear factor-kappa B**
MAPK	**Mitogen protein kinase**
JNK	**JUN NH_2-terminal kinase**
ERK	**Extracellular signal related kinase**
MAPK	**p38, Mitogen activated protein kinase p38**
AP-1	**Activated protein kinase-1**
IL-8	**Interleukin-8**
cFLIP	**Cellular FLICE proteins**
$cFLIP_L$	**Cellular FLICE protein long form**
$cFLIP_S$	**Cellular FLICE protein long form**
FADD	**Fas-associated death domain protein**
TRAF	**TNF receptor-associated factor**
DD	**Death domain**
DED	**Death effector domain**
IAP	**Inhibitor of apoptosis protein**
KD	**Kinase dead**
siRNA	**Small interfering RNA**
XIAP	**X-linked inhibitor of apoptosis**
Rpm	**Rotations per minute**
FSC	**Forward scatter**
SSC	**Sideward scatter**

2. References

1. Boukamp,P., R.T.Petrussevska, D.Breitkreutz, J.Hornung, A.Markham, and N.E.Fusenig. 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* 106:761-771.

2. Raj,D., D.E.Brash, and D.Grossman. 2006. Keratinocyte apoptosis in epidermal development and disease. *J. Invest Dermatol.* 126:243-257.

3. Leverkus,M., M.Yaar, and B.A.Gilchrest. 1997. Fas/Fas ligand interaction contributes to UV-induced apoptosis in human keratinocytes. *Exp. Cell Res.* 232:255-262.

4. Wachter,T., M.Sprick, D.Hausmann, A.Kerstan, K.McPherson, G.Stassi, E.B.Brocker, H.Walczak, and M.Leverkus. 2004. cFLIPL inhibits tumor necrosis factor-related apoptosis-inducing ligand-mediated NF-kappaB activation at the death-inducing signaling complex in human keratinocytes. *J. Biol. Chem.* 279:52824-52834.

5. Leverkus,M., M.R.Sprick, T.Wachter, T.Mengling, B.Baumann, E.Serfling, E.B.Brocker, M.Goebeler, M.Neumann, and H.Walczak. 2003. Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation. *Mol. Cell Biol.* 23:777-790.

6. Leverkus,M., P.Diessenbacher, and P.Geserick. 2008. FLIP ing the coin? Death receptor-mediated signals during skin tumorigenesis. *Exp. Dermatol.* 17:614-622.

7. Hanahan,D. and R.A.Weinberg. 2000. The hallmarks of cancer. *Cell* 100:57-70.

8. Roy,S. and D.W.Nicholson. 2000. Cross-talk in cell death signaling. *J. Exp. Med.* 192:F21-F25.

9. Zhang,L. and B.Fang. 2005. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther.* 12:228-237.

10. Walczak,H. and P.H.Krammer. 2000. The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp. Cell Res.* 256:58-66.

11. Leverkus,M., M.Neumann, T.Mengling, C.T.Rauch, E.B.Brocker, P.H.Krammer, and H.Walczak. 2000. Regulation of tumor necrosis factor-related apoptosis-inducing ligand sensitivity in primary and transformed human keratinocytes. *Cancer Res.* 60:553-559.

12. Sheridan,J.P., S.A.Marsters, R.M.Pitti, A.Gurney, M.Skubatch, D.Baldwin, L.Ramakrishnan, C.L.Gray, K.Baker, W.I.Wood, A.D.Goddard, P.Godowski, and A.Ashkenazi. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 277:818-821.

13. van Noesel,M.M., B.S.van, G.S.Salomons, P.A.Voute, R.Pieters, S.B.Baylin, J.G.Herman, and R.Versteeg. 2002. Tumor-specific down-regulation of the tumor necrosis factor-related apoptosis-inducing ligand decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation. *Cancer Res.* 62:2157-2161.

14. Deveraux,Q.L., N.Roy, H.R.Stennicke, A.T.Van, Q.Zhou, S.M.Srinivasula, E.S.Alnemri, G.S.Salvesen, and J.C.Reed. 1998. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J.* 17:2215-2223.

15. Diessenbacher,P., M.Hupe, M.R.Sprick, A.Kerstan, P.Geserick, T.L.Haas, T.Wachter, M.Neumann, H.Walczak, J.Silke, and M.Leverkus. 2008. NF-kappaB inhibition reveals differential mechanisms of TNF versus TRAIL-induced apoptosis upstream or at the level of caspase-8 activation independent of cIAP2. *J. Invest Dermatol.* 128:1134-1147.

16. Geserick,P., M.Hupe, M.Moulin, W.W.Wong, M.Feoktistova, B.Kellert, H.Gollnick, J.Silke, and M.Leverkus. 2009. Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment. *J. Cell Biol.* 187:1037-1054.

17. Golks,A., D.Brenner, C.Fritsch, P.H.Krammer, and I.N.Lavrik. 2005. c-FLIPR, a new regulator of death receptor-induced apoptosis. *J. Biol. Chem.* 280:14507-14513.

18. Krueger,A., S.Baumann, P.H.Krammer, and S.Kirchhoff. 2001. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol. Cell Biol.* 21:8247-8254.

19. Roth,W. and J.C.Reed. 2004. FLIP protein and TRAIL-induced apoptosis. *Vitam. Horm.* 67:189-206.

20. Krueger,A., I.Schmitz, S.Baumann, P.H.Krammer, and S.Kirchhoff. 2001. Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J. Biol. Chem.* 276:20633-20640.

21. Peter,M.E. and P.H.Krammer. 2003. The CD95(APO-1/Fas) DISC and beyond. *Cell Death. Differ.* 10:26-35.

22. Chau,H., V.Wong, N.J.Chen, H.L.Huang, W.J.Lin, C.Mirtsos, A.R.Elford, M.Bonnard, A.Wakeham, A.I.You-Ten, B.Lemmers, L.Salmena, M.Pellegrini, R.Hakem, T.W.Mak, P.Ohashi, and W.C.Yeh. 2005. Cellular FLICE-inhibitory protein is required for T cell survival and cycling. *J. Exp. Med.* 202:405-413.

23. Geserick,P., C.Drewniok, M.Hupe, T.L.Haas, P.Diessenbacher, M.R.Sprick, M.P.Schon, F.Henkler, H.Gollnick, H.Walczak, and M.Leverkus. 2008. Suppression of cFLIP is sufficient to sensitize human melanoma cells to T. *Oncogene* 27:3211-3220.

24. Yeh,W.C., A.Itie, A.J.Elia, M.Ng, H.B.Shu, A.Wakeham, C.Mirtsos, N.Suzuki, M.Bonnard, D.V.Goeddel, and T.W.Mak. 2000. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity.* 12:633-642.

25. Reed,J.C. 2006. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. *Nat. Clin. Pract. Oncol.* 3:388-398.

26. Degterev,A., A.Lugovskoy, M.Cardone, B.Mulley, G.Wagner, T.Mitchison, and J.Yuan. 2001. Identification of small-molecule inhibitors of interaction between the BH3 domain and Bcl-xL. *Nat. Cell Biol.* 3:173-182.

27. Bertrand,M.J., S.Milutinovic, K.M.Dickson, W.C.Ho, A.Boudreault, J.Durkin, J.W.Gillard, J.B.Jaquith, S.J.Morris, and P.A.Barker. 2008. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol. Cell* 30:689-700.

28. Kreuz,S., D.Siegmund, J.J.Rumpf, D.Samel, M.Leverkus, O.Janssen, G.Hacker, O.ttrich-Breiholz, M.Kracht, P.Scheurich, and H.Wajant. 2004. NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *J. Cell Biol.* 166:369-380.

29. Choi,C., X.Xu, J.W.Oh, S.J.Lee, G.Y.Gillespie, H.Park, H.Jo, and E.N.Benveniste. 2001. Fas-induced expression of chemokines in human glioma cells: involvement of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase. *Cancer Res.* 61:3084-3091.

30. Wajant,H. 2004. TRAIL and NFkappaB signaling--a complex relationship. *Vitam. Horm.* 67:101-132.

31. Neumann,M. and M.Naumann. 2007. Beyond IkappaBs: alternative regulation of NF-kappaB activity. *FASEB J.* 21:2642-2654.

32. Kreuz,S., D.Siegmund, P.Scheurich, and H.Wajant. 2001. NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. *Mol. Cell Biol.* 21:3964-3973.

33. Micheau,O., S.Lens, O.Gaide, K.Alevizopoulos, and J.Tschopp. 2001. NF-kappaB signals induce the expression of c-FLIP. *Mol. Cell Biol.* 21:5299-5305.

34. Wicovsky,A., N.Muller, N.Daryab, R.Marienfeld, C.Kneitz, S.Kavuri, M.Leverkus, B.Baumann, and H.Wajant. 2007. Sustained JNK activation in response to tumor necrosis factor is mediated by caspases in a cell type-specific manner. *J. Biol. Chem.* 282:2174-2183.

35. Schaeffer,H.J. and M.J.Weber. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* 19:2435-2444.

36. Kyriakis,J.M. and J.Avruch. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev.* 81:807-869.

37. Bardwell,L. and J.Thorner. 1996. A conserved motif at the amino termini of MEKs might mediate high-affinity interaction with the cognate MAPKs. *Trends Biochem. Sci.* 21:373-374.

38. Enslen,H. and R.J.Davis. 2001. Regulation of MAP kinases by docking domains. *Biol. Cell* 93:5-14.

39. Tournier,C., C.Dong, T.K.Turner, S.N.Jones, R.A.Flavell, and R.J.Davis. 2001. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev.* 15:1419-1426.

40. Wada,T., K.Nakagawa, T.Watanabe, G.Nishitai, J.Seo, H.Kishimoto, D.Kitagawa, T.Sasaki, J.M.Penninger, H.Nishina, and T.Katada. 2001. Impaired synergistic activation of stress-activated protein kinase SAPK/JNK in mouse embryonic stem cells lacking SEK1/MKK4: different contribution of SEK2/MKK7 isoforms to the synergistic activation. *J. Biol. Chem.* 276:30892-30897.

41. Derijard,B., M.Hibi, I.H.Wu, T.Barrett, B.Su, T.Deng, M.Karin, and R.J.Davis. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 76:1025-1037.

42. Tournier,C., A.J.Whitmarsh, J.Cavanagh, T.Barrett, and R.J.Davis. 1999. The MKK7 gene encodes a group of c-Jun NH2-terminal kinase kinases. *Mol. Cell Biol.* 19:1569-1581.

43. Kishimoto,H., K.Nakagawa, T.Watanabe, D.Kitagawa, H.Momose, J.Seo, G.Nishitai, N.Shimizu, S.Ohata, S.Tanemura, S.Asaka, T.Goto, H.Fukushi, H.Yoshida, A.Suzuki, T.Sasaki, T.Wada, J.M.Penninger, H.Nishina, and T.Katada. 2003. Different properties of SEK1 and MKK7 in dual phosphorylation of stress-induced activated protein kinase SAPK/JNK in embryonic stem cells. *J. Biol. Chem.* 278:16595-16601.

44. Derijard,B., J.Raingeaud, T.Barrett, I.H.Wu, J.Han, R.J.Ulevitch, and R.J.Davis. 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 267:682-685.

45. Raingeaud,J., A.J.Whitmarsh, T.Barrett, B.Derijard, and R.J.Davis. 1996. *Mol. Cell Biol.* 16:1247-1255.

46. Brancho,D., N.Tanaka, A.Jaeschke, J.J.Ventura, N.Kelkar, Y.Tanaka, M.Kyuuma, T.Takeshita, R.A.Flavell, and R.J.Davis. 2003. Mechanism of p38 MAP kinase activation in vivo. *Genes Dev.* 17:1969-1978.

47. Karin,M. 2005. Inflammation-activated protein kinases as targets for drug development. *Proc. Am. Thorac. Soc.* 2:386-390.

48. Kataoka,T. 2005. The caspase-8 modulator c-FLIP. *Crit Rev. Immunol.* 25:31-58.

49. Thome,M. and J.Tschopp. 2001. Regulation of lymphocyte proliferation and death by FLIP. *Nat. Rev. Immunol.* 1:50-58.

50. Wajant,H., K.Pfizenmaier, and P.Scheurich. 2002. TNF-related apoptosis inducing ligand (TRAIL) and its receptors in tumor surveillance and cancer therapy. *Apoptosis.* 7:449-459.

51. Mitsiades,N., C.S.Mitsiades, V.Poulaki, K.C.Anderson, and S.P.Treon. 2002. Intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human multiple myeloma cells. *Blood* 99:2162-2171.

52. Berg,D., M.Lehne, N.Muller, D.Siegmund, S.Munkel, W.Sebald, K.Pfizenmaier, and H.Wajant. 2007. Enforced covalent trimerization increases the activity of the TNF ligand family members TRAIL and CD95L. *Cell Death. Differ.* 14:2021-2034.

53. Garcia-Calvo,M., E.P.Peterson, B.Leiting, R.Ruel, D.W.Nicholson, and N.A.Thornberry. 1998. Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J. Biol. Chem.* 273:32608-32613.

54. Schmidt,M., M.Goebeler, G.Posern, S.M.Feller, C.S.Seitz, E.B.Brocker, U.R.Rapp, and S.Ludwig. 2000. Ras-independent activation of the Raf/MEK/ERK pathway upon calcium-induced differentiation of keratinocytes. *J. Biol. Chem.* 275:41011-41017.

55. Nicoletti,I., G.Migliorati, M.C.Pagliacci, F.Grignani, and C.Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* 139:271-279.

56. Berberich,I., G.Shu, F.Siebelt, J.R.Woodgett, J.M.Kyriakis, and E.A.Clark. 1996. Cross-linking CD40 on B cells preferentially induces stress-activated protein kinases rather than mitogen-activated protein kinases. *EMBO J.* 15:92-101.

57. Kyriakis,J.M., P.Banerjee, E.Nikolakaki, T.Dai, E.A.Rubie, M.F.Ahmad, J.Avruch, and J.R.Woodgett. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369:156-160.

58. Chang,D.W., Z.Xing, V.L.Capacio, M.E.Peter, and X.Yang. 2003. Interdimer processing mechanism of procaspase-8 activation. *EMBO J.* 22:4132-4142.

59. Golks,A., D.Brenner, P.H.Krammer, and I.N.Lavrik. 2006. The c-FLIP-NH2 terminus (p22-FLIP) induces NF-kappaB activation. *J. Exp. Med.* 203:1295-1305.

60. Kataoka,T., R.C.Budd, N.Holler, M.Thome, F.Martinon, M.Irmler, K.Burns, M.Hahne, N.Kennedy, M.Kovacsovics, and J.Tschopp. 2000. The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways. *Curr. Biol.* 10:640-648.

61. Goebeler,M., R.Gillitzer, K.Kilian, K.Utzel, E.B.Brocker, U.R.Rapp, and S.Ludwig. 2001. Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. *Blood* 97:46-55.

62. Banno,T., A.Gazel, and M.Blumenberg. 2005. Pathway-specific profiling identifies the NF-kappa B-dependent tumor necrosis factor alpha-regulated genes in epidermal keratinocytes. *J. Biol. Chem.* 280:18973-18980.

63. Farley,S.M., A.D.Dotson, D.E.Purdy, A.J.Sundholm, P.Schneider, B.E.Magun, and M.S.Iordanov. 2006. Fas ligand elicits a caspase-independent proinflammatory response in human keratinocytes: implications for dermatitis. *J. Invest Dermatol.* 126:2438-2451.

64. Kataoka,T. and J.Tschopp. 2004. N-terminal fragment of c-FLIP(L) processed by caspase 8 specifically interacts with TRAF2 and induces activation of the NF-kappaB signaling pathway. *Mol. Cell Biol.* 24:2627-2636.

65. Goltsev,Y.V., A.V.Kovalenko, E.Arnold, E.E.Varfolomeev, V.M.Brodianskii, and D.Wallach. 1997. CASH, a novel caspase homologue with death effector domains. *J. Biol. Chem.* 272:19641-19644.

66. Irmler,M., M.Thome, M.Hahne, P.Schneider, K.Hofmann, V.Steiner, J.L.Bodmer, M.Schroter, K.Burns, C.Mattmann, D.Rimoldi, L.E.French, and J.Tschopp. 1997. Inhibition of death receptor signals by cellular FLIP. *Nature* 388:190-195.

67. Srinivasula,S.M., M.Ahmad, S.Ottilie, F.Bullrich, S.Banks, Y.Wang, T.Fernandes-Alnemri, C.M.Croce, G.Litwack, K.J.Tomaselli, R.C.Armstrong, and E.S.Alnemri. 1997. FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR1-induced apoptosis. *J. Biol. Chem.* 272:18542-18545.

68. Micheau,O., M.Thome, P.Schneider, N.Holler, J.Tschopp, D.W.Nicholson, C.Briand, and M.G.Grutter. 2002. The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J. Biol. Chem.* 277:45162-45171.

69. Hughes,M.A., N.Harper, M.Butterworth, K.Cain, G.M.Cohen, and M.MacFarlane. 2009. Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. *Mol. Cell* 35:265-279.

70. Li,H., M.Kobayashi, M.Blonska, Y.You, and X.Lin. 2006. Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. *J. Biol. Chem.* 281:13636-13643.

71. Ea,C.K., L.Deng, Z.P.Xia, G.Pineda, and Z.J.Chen. 2006. Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Mol. Cell* 22:245-257.

72. Varfolomeev,E., H.Maecker, D.Sharp, D.Lawrence, M.Renz, D.Vucic, and A.Ashkenazi. 2005. Molecular determinants of kinase pathway activation by Apo2 ligand/tumor necrosis factor-related apoptosis-inducing ligand. *J. Biol. Chem.* 280:40599-40608.

73. Devin,A., A.Cook, Y.Lin, Y.Rodriguez, M.Kelliher, and Z.Liu. 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity.* 12:419-429.

74. Devin,A., Y.Lin, and Z.G.Liu. 2003. The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. *EMBO Rep.* 4:623-627.

75. Siegmund,D., S.Klose, D.Zhou, B.Baumann, C.Roder, H.Kalthoff, H.Wajant, and A.Trauzold. 2007. Role of caspases in C. *Cell Signal.* 19:1172-1184.

76. Zhang,L., K.Blackwell, G.S.Thomas, S.Sun, W.C.Yeh, and H.Habelhah. 2009. TRAF2 suppresses basal IKK activity in resting cells and TNFalpha can activate IKK in TRAF2 and TRAF5 double knockout cells. *J. Mol. Biol.* 389:495-510.

77. Lavrik,I.N., T.Mock, A.Golks, J.C.Hoffmann, S.Baumann, and P.H.Krammer. 2008. CD95 stimulation results in the formation of a novel death effector domain protein-containing complex. *J. Biol. Chem.* 283:26401-26408.

78. Ashkenazi,A. 2008. Targeting the extrinsic apoptosis pathway in cancer. *Cytokine Growth Factor Rev.* 19:325-331.

79. Chaudhary,P.M., M.T.Eby, A.Jasmin, and L.Hood. 1999. Activation of the c-Jun N-terminal kinase/stress-activated protein kinase pathway by overexpression of caspase-8 and its homologs. *J. Biol. Chem.* 274:19211-19219.

80. Harper,N., S.N.Farrow, A.Kaptein, G.M.Cohen, and M.MacFarlane. 2001. Modulation of tumor necrosis factor apoptosis-inducing ligand- induced NF-kappa B activation by inhibition of apical caspases. *J. Biol. Chem.* 276:34743-34752.

81. Kennedy,N.J. and R.J.Davis. 2003. Role of JNK in tumor development. *Cell Cycle* 2:199-201.

82. Chang,L., H.Kamata, G.Solinas, J.L.Luo, S.Maeda, K.Venuprasad, Y.C.Liu, and M.Karin. 2006. The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* 124:601-613.

83. Liu,J. and A.Lin. 2005. Role of JNK activation in apoptosis: a double-edged sword. *Cell Res.* 15:36-42.

84. Zhang,H., S.Rosenberg, F.J.Coffey, Y.W.He, T.Manser, R.R.Hardy, and J.Zhang. 2009. A role for cFLIP in B cell proliferation and stress MAPK regulation. *J. Immunol.* 182:207-215.

85. Grambihler,A., H.Higuchi, S.F.Bronk, and G.J.Gores. 2003. cFLIP-L inhibits p38 MAPK activation: an additional anti-apoptotic mechanism in bile acid-mediated apoptosis. *J. Biol. Chem.* 278:26831-26837.

86. Lee,T.H., J.Shank, N.Cusson, and M.A.Kelliher. 2004. The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. *J. Biol. Chem.* 279:33185-33191.

87. Yu,J.W. and Y.Shi. 2008. FLIP and the death effector domain family. *Oncogene* 27:6216-6227.

88. Kerstan,A., M.Leverkus, and A.Trautmann. 2009. Effector pathways during eczematous dermatitis: where inflammation meets cell death. *Exp. Dermatol.* 18:893-899.

Publications

1. **Cellular FLICE-inhibitory Protein (cFLIP) Isoforms Block CD95- and TRAIL Death Receptor-induced Gene Induction Irrespective of Processing of Caspase-8 or cFLIP in the Death-inducing Signaling Complex**.; ***Kavuri S***, Geserick P, Berg D, Dimitrova DP, Feoktistova M, Siegmund D, Gollnick H, Neumann M, Wajant H, Leverkus M.;***J Biol Chem*. 2011** May 13;286(19):16631-46. Epub 2011 Mar 22.

2. **Glycogen synthase kinase-3β is a crucial mediator of signal-induced RelB degradation;** Neumann M, Klar S, Wilisch-Neumann A, Hollenbach E, ***Kavuri S***, Leverkus M, Kandolf R, Brunner-Weinzierl MC, Klingel K.*;**Oncogene* ; 2011** May 26;30(21):2485-92. Epub 2011 Jan 10.

3. **The contact allergen nickel sensitizes primary human endothelial cells and keratinocytes to TRAIL-mediated apoptosis**; Schmidt M, Hupe M, Endres N, Raghavan B, ***Kavuri S***, Geserick P, Goebeler M, Leverkus M.;***J Cell Mol Med*. 2010** Jun;14(6B):1760-76. Epub 2009 Jun 16.

4. **Sustained JNK activation in response to tumor necrosis factor is mediated by caspases in a cell type-specific manner;** Wicovsky A, Müller N, Daryab N, Marienfeld R, Kneitz C, ***Kavuri S***, Leverkus M, Baumann B, Wajant H;***J Biol Chem.* 2007** Jan 26;282(4):2174-83. Epub 2006 Nov 22.