Apoptotic and non-apoptotic death receptor signaling pathways and their regulation by cFLIP, cIAPs and RIP1 in the skin

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Summary

The worldwide increasing incidence of skin-derived cancer and their diversity requires development of novel therapeutic strategies. The malignant melanoma, for example, is a highly aggressive cancer with the capacity to metastasize at early stages. Together with resistance to chemotherapeutic agents it results in overall poor prognosis of melanoma patients once metastasis has occurred. Death receptor-induced cell death may represent an innovative therapeutic approach for the specific elimination of skin tumor cells. Activation of death receptors leads to induction of several apoptotic and non-apoptotic signaling pathways, which are regulated in a complex manner by different proteins. In this context cIAP1 and cIAP2 were recently described to play a substantial role in resistance to extrinsic, in particular for TNF-induced, cell death. This study shows that cIAPs are essential to prevent CD95-mediated cell death. Loss of cIAPs caused by a chemical IAP antagonist leads to dramatic sensitization of skin tumor cells to CD95 ligand. The cell death mediated by CD95 and IAP antagonist is neither entirely caspase-dependent nor caspase-independent. Only a combination of caspase and RIP1 kinase inhibition is sufficient to block CD95-mediated cell death in absence of cIAPs. RIP1 is a known target of cIAP activity. Here it is demonstrated that cIAPs inhibit recruitment of RIP1 to the CD95 DISC and suppress formation of the secondary receptor-independent complex (complex II). In addition, loss of RIP1 protects cells from IAP angagonist-mediated sensitization to CD95L. These underline that RIP1 is a key regulator of CD95-initiated cell death pathways which is on its part regulated by cIAPs.

cFLIP is a highly efficient anti-apoptotic protein and therefore also an important regulator of death receptor-mediated cell death. Increased cFLIP expression confers resistance of tumor cells to death ligands and promotes tumor progression. This study shows that cFLIP inhibits death receptor-mediated cell death by limiting activation of the pro-apoptotic molecule Caspase-8 at the DISC. Suppression of cFLIP causes increased Caspase-8 activation at the DISC and is therefore sufficient to sensitize cells to death ligands. Interestingly, it was revealed that cFLIP isoforms differentially contribute to resistance to CD95L in absence of cIAPs. Only $cFLIP_L$ and not $cFLIP_S$ interfere with RIP1 recruitment to the DISC and the subsequent formation of the complex II. This protects $cFLIP_L$, but not $cFLIP_S$, expressing cells from death ligand-induced cell death in absence of cIAPs. This study demonstrates a remarkable and previously unexpected specificity concerning the mechanism of death inhibition by cFLIP isoforms.

These findings provide a new insight in the fundamental roles of cFLIP, cIAPs and RIP1 in regulation of death receptor signaling and may help to develop novel therapeutic approaches to overcome death ligand resistance of skin tumors.

Zusammenfassung

Das weltweit stark ansteigende Auftreten von Hautkrebs jeglicher Art und die ungenügenden therapeutischen Behandlungsmöglichkeiten erfordern die Entwicklung neuer therapeutischer Interventionsstrategien. Das maligne Melanom zum Beispiel ist ein hochgradig aggressiver Tumor mit der Fähigkeit relativ frühen zu metastasieren. Aufgrund der Aggressivität und der zunehmenden Resistenz gegenüber chemotherapeutischen Wirkstoffen ist das malignen Melanoms die am häufigsten tödlich verlaufende Hautkrankheit. Der Todesrezeptor-vermittelte Zelltod könnte ein innovativer Therapieansatz zur spezifischen Eliminierung von Hauttumoren darstellen. Die Aktivierung von Todesrezeptoren führt zur Aktivierung verschiedenster, höchst komplex regulierter apoptotischer und nicht-apoptotischer Signalwege. Im Kontext der Regulierung von Todesrezeptor-induzierten Signalwegen wurden kürzlich cIAP1 und cIAP2 als Resistenzfaktoren des TNF-vermittelten Zelltodes beschrieben. Die vorliegende Studie beschreibt die zentrale Funktion der cIAP Moleküle für die Inhibierung des CD95-vermittelten Zelltodes. Der herbeigeführte Verlust der cIAP's durch einen chemischen IAP Antagonisten, führt zu einer dramatischen Sensitivierung von Hauttumorzellen gegenüber CD95 Ligand. Der durch CD95- und IAP Antagonist-Stimulation herbeigeführte Zelltod der Hauttumorzellen ist weder ausschließlich Caspase abhängig noch Caspase unabhängig. In der Abwesenheit von cIAP's kann ausschließlich die Kombination von Caspase und RIP1 Kinase Inhibitoren die Zellen vor dem CD95-induziertem Zelltod schützen. RIP1 ist ein Todesrezeptor-assoziiertes Molekül und bekanntes Ziel der cIAP Ubiquitinilierungsaktivität. Die Studie zeigt, dass die cIAP Moleküle die Rekrutierung von RIP1 in den CD95 Rezeptorkomplex (DISC) inhibieren und somit die Formierung eines sekundären zytoplasmatischen Komplexes (Komplex II) supprimieren. Darüber hinaus ist gezeigt, dass der Verlust von RIP1 Hauttumorzellen vor der IAP Antagonist-vermittelten Sensitivierung gegenüber CD95L schützt. Dieses unterschreiecht die Schlüsselrolle des RIP1, welches von cIAP's reguliert wird, in der Regulation von CD95-induzierten Signalwegen.

cFLIP ist ein hoch effizientes anti-apoptotisches Protein und somit auch ein wichtiger Regulator des Todesrezeptor-vermitteltem Zelltodes. Erhöhte Level von cFLIP verleihen Tumorzellen Resistenz gegenüber Todesliganden und fördern somit die Tumor Entwicklung. In der Studie ist gezeigt, dass cFLIP den Todesrezeptor-induzierten Zelltod durch die Inhibierung der Aktivierung des pro-apoptotischen Moleküls Caspase-8 im DISC blockiert. Repression von cFLIP im Gegenzug, bewirkt eine verstärkte Caspase-8 Aktivierung im DISC und ist somit hinreichend zur Sensitivierung von Tumorzellen gegenüber Todesliganden. Interessanterweise tragen die cFLIP Isoformen in der Abwesenheit von cIAP's unterschiedlich zur Resistenz von Hauttumorzellen gegenüber CD95L bei. $cFLIP_L$, aber nicht

$cFLIP_S$, interferiert mit der Rekrutierung von RIP1 in den DISC und mit der anschließenden Formierung des Komplexes II. Dieses wiederum schützt $cFLIP_L$-exprimierende Zellen vor dem Todesliganden-induzierten Zelltod in der Abwesenheit von cIAP's, im Gegensatz zu $cFLIP_S$-exprimierende Zellen. Die vorliegende Studie demonstriert eine deutliche und unerwartete Spezifität der cFLIP Isoformen bezüglich des Mechanismus der Inhibition von Zelltod.

Diese Befunde erlauben einen neuen Einblick in die fundamentalen Rollen von cFLIP, cIAPs and RIP1 in die Regulation von Todesrezeptor-vermittelten Signalwegen und helfen möglicherweise bei der Entwicklung neuer therapeutischer Ansätze zur Beseitigung von Resistenzmechanismen gegen Todesliganden-vermittelten Zelltod in Tumoren der Haut.

Contents

Figures and Tables

1 Introduction

1.1 The physiological role of apoptotic and necrotic cell death

The worldwide increasing incidence of keratinocyte, epithelial and melanocyte-derived skin cancer and their diversity requires the development of novel therapeutic strategies (Ghavami et al., 2009). For example, the malignant melanoma is a highly aggressive cancer with a dramatically increasing incidence. Its capacity to metastasize at early stages and the resistance to chemotherapeutic agents results in overall poor prognosis of melanoma patients once metastasis has occurred (Miller and Mihm, 2006). Death receptor-induced cell death may represent an innovative therapeutic approach for the specific elimination of skin tumor cells. Cell death is a necessity for cell homeostasis and essential for the development and maintenance of multi cellular organisms (Trosko and Ruch, 1998). Cell death can occur via several pathways and can be classified according to its appearance, in particular apoptosis or necrosis (Kroemer et al., 2009).

Apoptosis is a physiological and highly regulated form of programmed cell death. It has an essential role in adjustment to changing conditions like removal of unnecessary cells during embryonic development or elimination of virus infected and tumor cells (Strasser et al., 2000). During the course of apoptosis cells round-up and retract their pseudopodes. Reduction of cellular volume, condensation of chromatin and nuclear fragmentation are detectable. Apoptosis is biochemically defined by the activation of Cysteinyl-Aspartate Specific Proteases (caspases) and detection of their cleaved substrates (Roy and Nicholson, 2000). Plasma membrane remains intact, preventing leakage of cellular content into the environment. Membrane blebbing occurs and results in formation of apoptotic bodies that are finally recognized and phagocytozed by macrophages or neighboring cells (Kroemer et al., 2009).

Necrotic cell death can be induced by injury, infection, toxins or inflammation. Necrosis is morphologically characterized by gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents (Kroemer et al., 2009). The release of intracellular content causes the inflammatory phenotype (Festjens et al., 2006). In the past, necrosis has been considered as an accidental uncontrolled form of cell death. More recent findings suggest a fine tuned mechanism controlled by a set of signal transduction and metabolic pathways (Golstein and Kroemer, 2007). For instance, death receptors can induce Receptor-Interacting Protein 1 (RIP1)-dependent necrosis (Holler et al., 2000).

1.2 Death receptors mediated signaling

Cell death can be initiated by several internal (intrinsic) and external (extrinsic) stimuli. The intrinsic or mitochondrial pathway is activated by extra- and intracellular stresses, like oxidative stress, serum starvation or cytotoxic drugs (Ghavami et al., 2008; Ghavami et al., 2004). It is defined by release of mitochondrial factors such as Cytochrome C, Apoptosis-Inducing Factor (AIF) or Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI (SMAC/DIABLO) from the mitochondrial inter-membranous space into the cytosol (Du et al., 2000; Kim, 2005; Lorenzo et al., 1999). The mitochondria-dependent pathway is mediated and controlled by members of the B-cell lymphoma 2 (Bcl-2) family (Youle and Strasser, 2008). In contrast, the extrinsic pathway is induced by activation of death receptors.

Tumor Necrosis Factor (TNF), TNF Related Apoptosis Inducing Ligand (TRAIL) and FS7-associated cell surface antigen Ligand (FasL, also named CD95L) are death ligands and essential cytokines with pleiotropic effects on various cells (Ashkenazi and Dixit, 1998; Locksley et al., 2001). These ligands bind to their specific cognate death receptors on the cell surface. These receptors are members of the TNF-superfamily. Six death receptors have been identified in humans to date: CD95 (or Fas), TNF-receptor 1 (R1), TRAIL-R1, TRAIL-R2 (2a and 2b), TRAIL-R3 and TRAIL-R4 (Fig. 1). With the exception of TRAIL-R3, these receptors are characterized by presence of a cytosolic domain called Death Domain (DD). DDs mediate homotypic interactions and can appear in four death-fold motifs: DD itself, Death Effector Domain (DED), Caspase Activation and Recruitment Domain (CARD) and Pyrin domain (Fesik, 2000). These folds allow regulation of death receptor signaling in a highly regulated but complex manner.

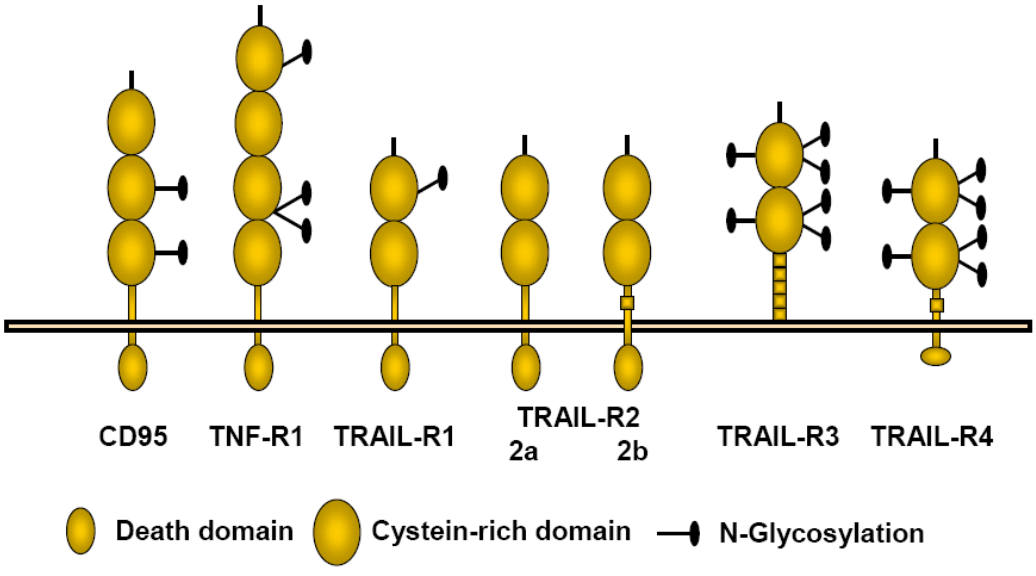


Figure 1: Structure of death receptors of the TNF receptor family. Distribution of domain composition is indicated.

Stimulation of death receptors leads to recruitment of one of two DD-containing molecules: Fas-Associated Death Domain protein (FADD) for CD95 and TRAIL receptors or TNF Receptor Associated Death Domain (TRADD) for TNF-R1. These adaptors bind to the intracellular DD of death receptors and act as anchors for further receptor associated proteins (Fig. 2). FADD contains a DED domain that enables recruitment of cellular FLICE-Inhibitory Protein (cFLIP) and the initiator caspase Procaspase-8 via their DED domains (Medema et al., 1997; Neumann et al., 2010; Scaffidi et al., 1999). These associated proteins are the core of the intracellular membrane-associated Death Inducing Signaling Complex (DISC) (Peter and Krammer, 2003). RIP1 can be recruited via FADD and TRADD or bind directly to the DD of all death receptors (Festjens et al., 2007). It is known that RIP1 is involved in recruitment of TNF Receptor Associated Factor-2 (TRAF2) and cellular Inhibitor of Apoptosis Protein-1 (cIAP1) and cIAP2 to the membrane-bound TNF-R complex I (Mace et al., 2010; Rothe et al., 1995; Rothe et al., 1994; Wertz and Dixit, 2010).

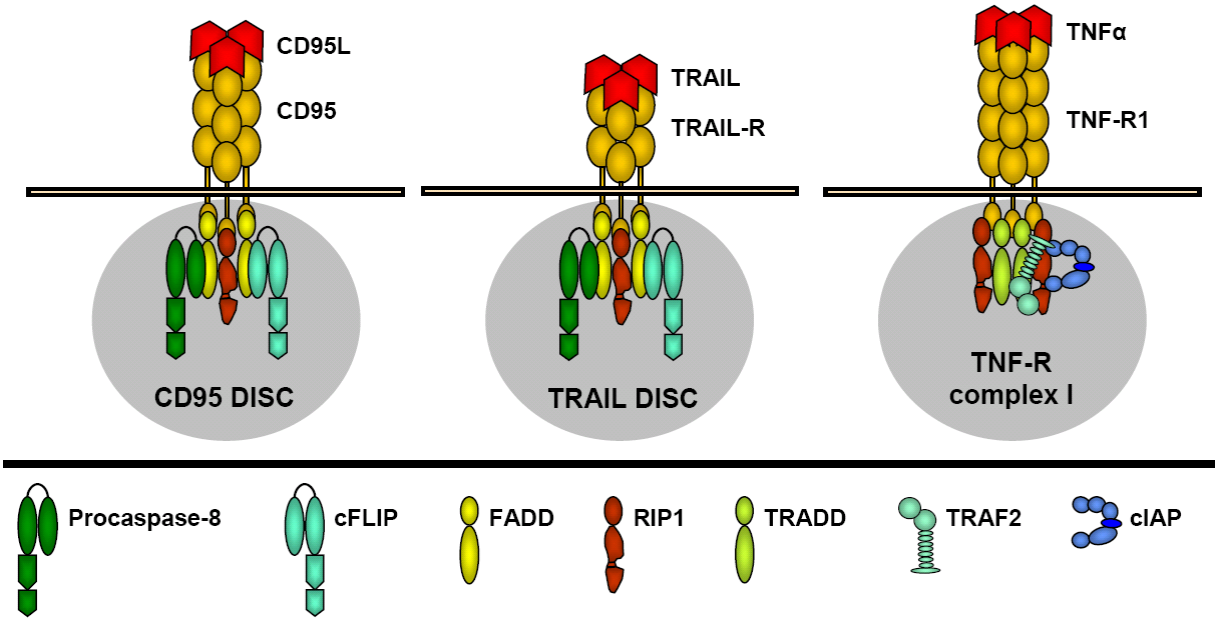


Figure 2: Formation of membrane bound death receptor complexes. Activation of death receptors triggers recruitment of different receptor associated molecules to the receptor. Formation of a membrane bound receptor complex occurs.

Formation of death receptor complex I can induce cell death, which in turn can be either apoptotic (via activation of caspases) or non-apoptotic (e.g. necrosis). Additionally, complex I formation can induce other non-apoptotic signals like differentiation, proliferation and inflammation (Fig. 3). These pathways are activated via Mitogen-Activated Protein Kinase (MAPK) or transcription factors, as Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) (Kroemer et al., 2009; Wajant et al., 2003a; Wertz and Dixit, 2010). These pathways may antagonize cell death pathways, or facilitate cell death induction depending on the cellular context.

In addition to formation of the membrane-associated complex I, death receptors can also induce formation of cytoplasmic signaling platforms. Formation of these distinct secondary complexes was first discovered for TNF (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004) and later also shown for TRAIL (Varfolomeev et al., 2005) and CD95L (Lavrik et al., 2008) pathways. Mechanisms leading to formation of these secondary complexes and their regulation are still poorly understood and further complicate understanding of death receptor signaling.

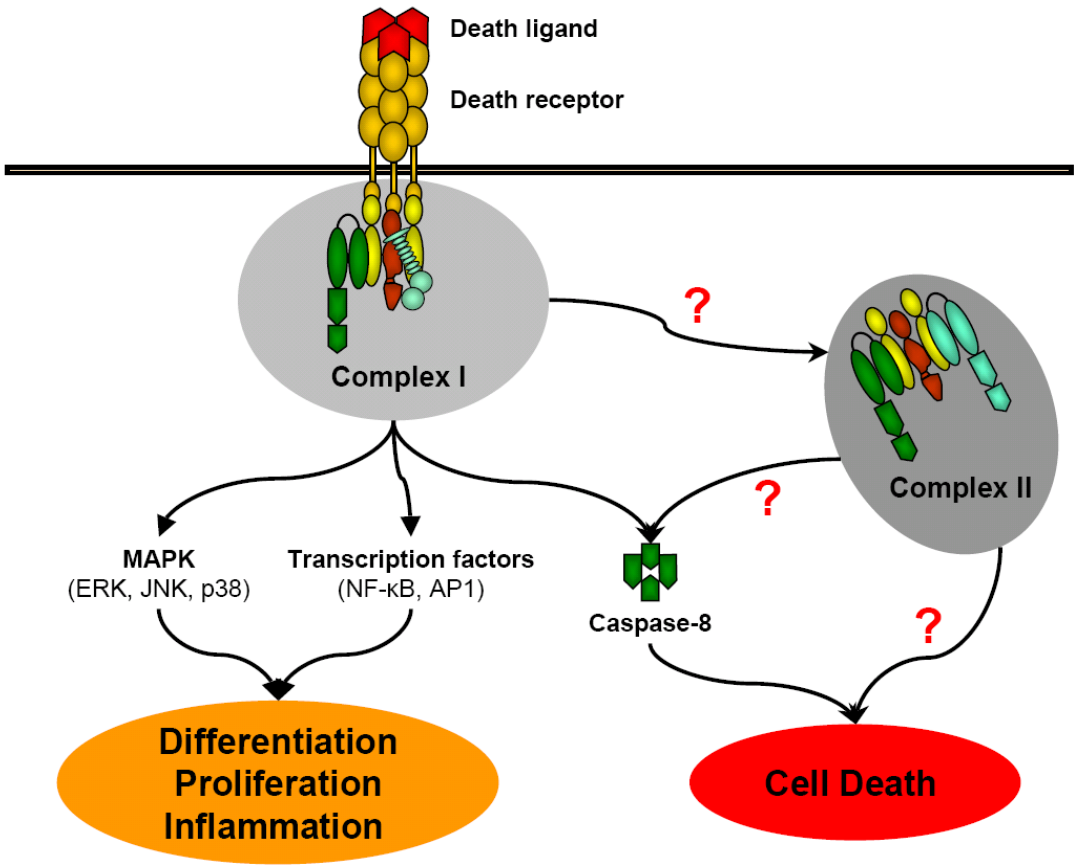


Figure 3: Mechanism of death receptor-mediated signal transduction. After activation of the death receptor recruitment of distinct receptor associated molecules occur in a membrane bound complex I. On one hand, complex I can induce cell survival by activation of specific MAPK (ERK, JNK, p38) or transcription factors (NF-κB, AP1). On the other hand, complex I can induce cell death via cleavage and activation of Caspase-8. The intracellular complex II is believed to increase efficacy of cell death induction but mechanisms leading to formation of complex II are still unclear.

Death receptors can induce activation of NF-κB signaling pathways. NF-κB molecules are dimeric transcription factors found in all cell types. They are involved in cellular responses to diverse stimuli like stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens. Dysregulation of NF-κB has been observed in cancer, inflammatory and autoimmune diseases, viral infection and improper immune development (Liang et al., 2006).

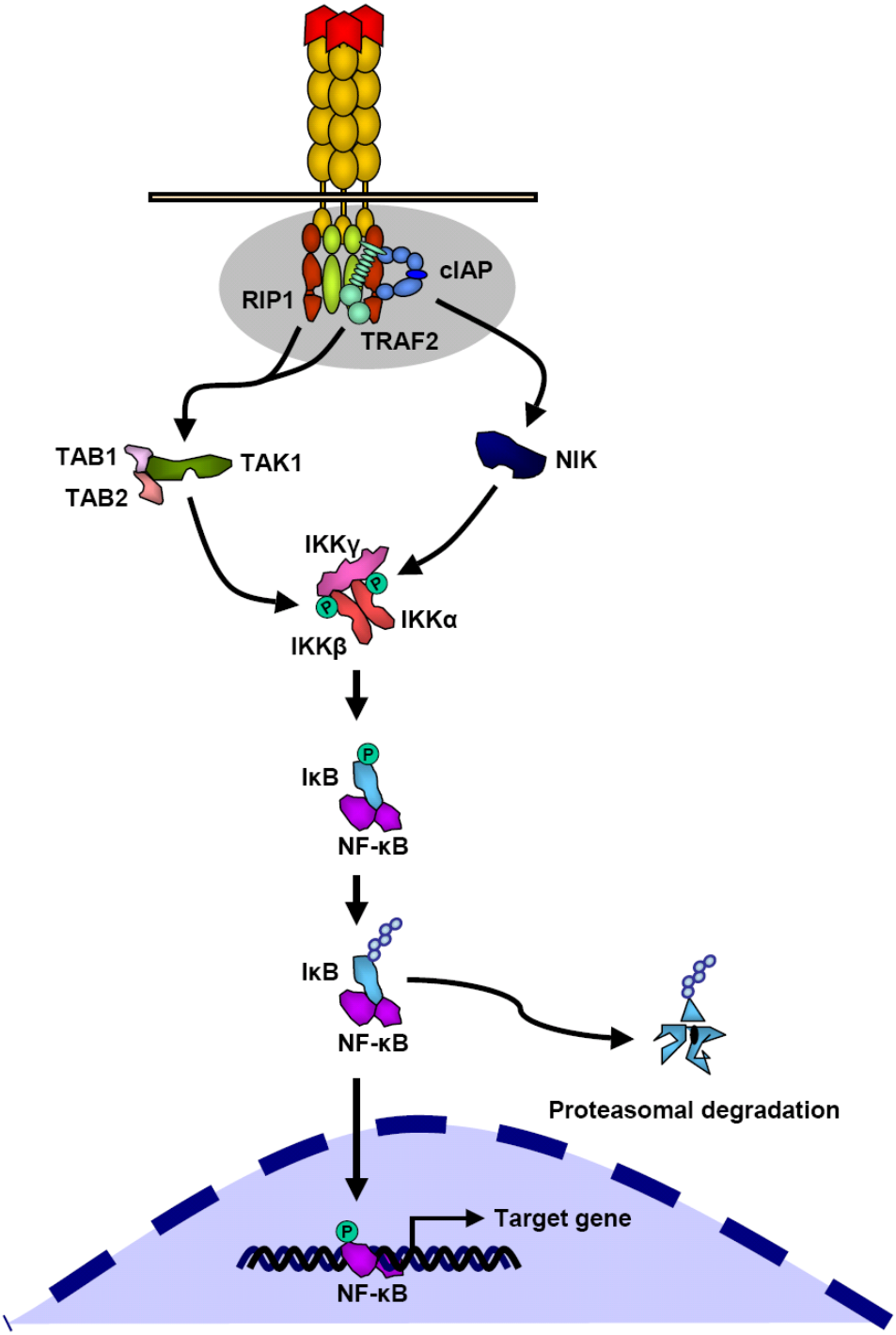


Figure 4: NF-κB Signaling Pathways. Different stimuli trigger canonical (TAK1-dependent) or noncanonical (NIK-dependent) IKK phoshorylation and activation. The activated IKK complex induces phosphorylation and ubiquitination of IκB proteins. Proteasomal degradation of IκB releases NF-κB dimmers which are further activated through various posttranslational modifications. Translocated NF-κB dimers bind to specific DNA sequences in the nucleus and promote transcription of NF-κB target genes.

Downstream of death receptor complex I several kinases have been implicated in activation of the Inhibitor of κB Kinase (IKK) complex (Fig. 4). This IKK complex consists two catalytic subunits (IKKα and IKKβ) and a regulatory component (IKKγ) also called NF-κB Essential Modulator (NEMO) (Karin and Greten, 2005). In case of the canonical NF-κB

pathway Transforming Growth Factor-β (TGF-β)-Activated Kinase 1 (TAK1), TAK Binding protein 1 (TAB1) and TAB2 are recruited and activated at the receptor complex (Ishitani et al., 2003; Shibuya et al., 1996; Takaesu et al., 2000). Subsequently the TAK/TAB complex causes the phosphorylation and activation of the IKK complex in a RIP1-dependent manner (Ea et al., 2006; Kanayama et al., 2004; Li et al., 2006; Wu et al., 2006). In the so called noncanonical pathway, NF-κB-Inducing Kinase (NIK) is required for phosphorylation and activation of IKKα in the IKK complex (Senftleben et al., 2001; Xiao et al., 2004; Xiao et al., 2001). The activated IKK complexes lead to degradation of Inhibitor of NF-κB (IκB), which sequesters NF-κB, or processing of a NF-kB precursor protein into mature subunits. NF-κB dimers can translocate to the nucleus and regulate transcription of target gens (Hayden and Ghosh, 2008).

1.2.1 cFLIP critically regulates death receptor-mediated apoptosis

Activation of the extrinsic cell death pathways is an important mechanism for elimination of unwanted or transformed cells. These pathways plays an important role in many physiological processes, especially development and maintaining of proper functioning of immune system (Gerl and Vaux, 2005; Krammer, 2000). Death receptor signaling pathways can be regulated at multiple levels and in particular by inhibitors such as cFLIP, IAPs, or Bcl-2 family proteins such as Bcl-2, Bcl-X_L (Meier and Vousden, 2007). Expression of these anti-apoptotic molecules has been suggested to correlate with tumor progression (Valente et al., 2006). Therefore inhibition of anti-apoptotic molecules, e.g. cFLIP, may serve as a potent therapeutic tool to overcome apoptosis resistance in tumor therapies (Day et al., 2009).

cFLIP proteins are extensively studied and described for their physiological relevance and inhibitory role in apoptosis signaling. cFLIP knockout mice die prenatal (E10.5–11.5) showing impaired heart development (Yeh et al., 2000). The cFLIP gene can give rise to 11 distinct isoforms by alternative splicing but in most cells a long (cFLIP$_L$) and a short (cFLIP$_S$) isoforms are the only detectable ones (Budd et al., 2006; Kataoka, 2005). cFLIP$_L$ is highly homologous to Procaspase-8, possessing two DEDs at its N-terminus and a catalytically inactive protease domain at its C-terminus (Fig. 5). cFLIP$_S$ contains two N-terminal DEDs followed by a short C-terminal stretch and is structurally related to viral FLIP (Golks et al., 2005; Krueger et al., 2001; Thurau et al., 2006). cFLIP isoforms interact with FADD and Caspase-8 and are recruited to the DISC after TRAIL or CD95L stimulation. cFLIP$_S$ interferes with caspase activation within this signaling platform and thereby block cell death induction (Condorelli et al., 1999). In contrast, the function of cFLIP$_L$ was initially reported to be either pro-apoptotic (Goltsev et al., 1997; Han et al., 1997; Inohara et al., 1997; Shu et al., 1997) or

anti-apoptotic (Hu et al., 1997; Irmler et al., 1997; Rasper et al., 1998; Srinivasula et al., 1997). Further studies suggested that $cFLIP_L$ can either promote or inhibit apoptosis depending on its expression level. Highly expressed $cFLIP_L$ compete with Procaspase-8 for recruitment into the DISC thereby preventing caspase activation. However, at physiological expression levels (~1% of Procaspase-8 expression levels) (Scaffidi et al., 1999), $cFLIP_L$ can still be targeted into the DISC, where it promotes Procaspase-8 activation and enhances apoptotic signaling (Yu and Shi, 2008). It is postulated that this Procaspase-8 activation occurs via heterodimerization with the catalytically inactive protease domain of $cFLIP_L$ (Boatright et al., 2004; Chang et al., 2002; Micheau et al., 2002).

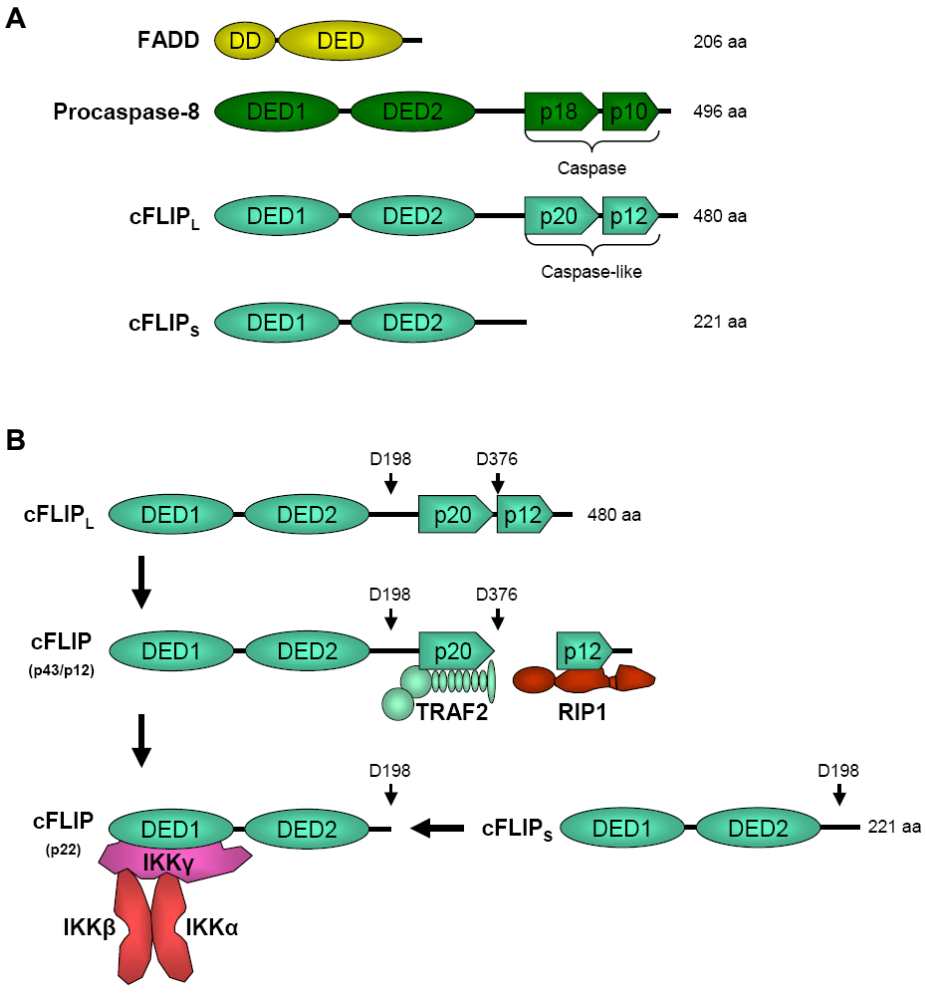


Figure 5: Structures of $cFLIP_L$ and $cFLIP_S$. A) Diagram of domain composition of cFLIP isoforms and interacting proteins. Both cFLIP isoforms processes two DEDs at the N-terminus, required for interaction with FADD. $cFLIP_L$ contains in addition a caspase-like domain which is highly homologous to the caspase domain of Procaspase-8.Number of amino acids (aa) indicate the lengths of proteins. **B**) Suggested cFLIP-mediated NF-κB activation from (Yu and Shi, 2008). Procaspase-8 and possibly mature Caspase-8 cleave cFLIP at D376 and D198 to generate products as shown. Cleaved cFLIP is supposed to interact with key signaling proteins to activate NF-κB. Specific regions of cFLIP that bind TRAF2 or RIP1 are still not defined. cFLIP p22 is suggested to interact with IKKγ, the regulatory subunit of the IKK complex (Golks et al., 2006).

Functions of different isoforms of cFLIP in activation of non-apoptotic signaling pathways are still not well defined (Falschlehner et al., 2007; Lavrik et al., 2005). The role of cFLIP in NF-κB activation is under further discussion. On one side, high levels of cFLIP seem to repress activation of NF-κB (Wachter et al., 2004). On the other side, it is suggested that moderate expression levels of $cFLIP_L$ can activate Procaspase-8, but restricts its full activation. In presence of $cFLIP_L$ activated Procaspase-8 is only partially processed and thereby retained in the DISC. Thus, non-released Caspase-8 can cleave only substrates located in close proximity to the DISC, such as $cFLIP_L$. Cleaved $cFLIP_L$ however, is supposed to recruit specific additional proteins and may initiate non-apoptotic signaling pathways like NF-κB activation (Yu and Shi, 2008).

Diverse cellular responses to death receptor signaling are elicited by the dynamic regulation of Procaspase-8 activation. This regulation is achieved by modulation of cFLIP expression levels (Krueger et al., 2001). Based on the discussed role of cFLIP in NF-κB activation it will be interesting to further analyze the distinct role of cFLIP isoforms.

1.2.2 Role of cIAPs in apoptosis resistance

IAPs were first identified in baculovirus (Crook et al., 1993). All IAP family members are characterized by presents of 1-3 zinc-binding Baculoviral IAP Repeat (BIR) motifs (Hinds et al., 1999; Wei et al., 2008). The BIR domains consist of approximately 70 amino acids that contain a characteristic sequence. To date, three subtypes of BIR domains (BIR1, BIR2, and BIR3) are classified. BIR motifs of some IAPs are able to bind and inhibit caspases (Huang et al., 2000; Liston et al., 2003; Salvesen and Duckett, 2002), the reason for the name of the protein family as inhibitors of apoptosis. On the other side, this nomenclature can be misleading since other BIR-domain-containing proteins (e.g. mammalian Survivin) are not believed to inhibit cell death (Vaux and Silke, 2005b).

cIAP1, cIAP2 and X-linked IAP (XIAP) are regulators of cell death which can interact with a variety of inducers and effectors of apoptotic cell death (Deveraux and Reed, 1999; Salvesen and Abrams, 2004; Vucic, 2008). They contain three BIR domains and a C-terminal Really Interesting New Gene (RING) domain (Fig. 6). When compared to XIAP, cIAP1 and cIAP2 additionally possess a CARD domain (Hofmann et al., 1997; Wei et al., 2008). The RING motif functions as ubiquitin ligase for modification of IAP interacting proteins (Salvesen and Duckett, 2002; Vaux and Silke, 2005b). cIAPs are critical regulators of TNF-R1 signaling, and it has been shown that they can inhibit CD95 and TRAIL-R induced apoptosis (McEleny et al., 2004; Wang et al., 2005). IAPs can inhibit several caspases by promoting their degradation or by binding and sequestering of these caspases (Tenev et al., 2005). For cIAP1 and cIAP2 it was demonstrated that they do not act as direct caspase inhibitors

because they are weak inhibitors of caspase activity (Eckelman and Salvesen, 2006). Given that cIAPs can inhibit induction of cell death, it was interesting to investigate their regulatory mechanisms in death receptor-mediated signaling pathways.

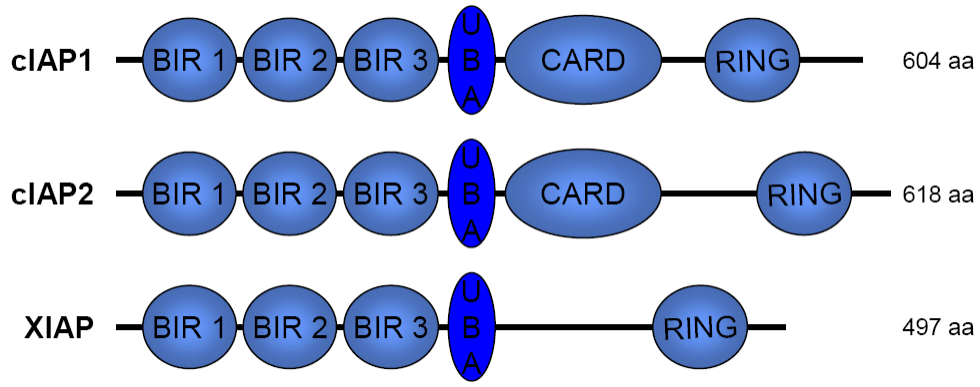


Figure 6: Schematic structures of cIAP1, cIAP2 and XIAP. All IAPs contain three BIR and one RING motif. cIAP1 and cIAP2 also contain a CARD domain. Recently identified UBA domain is located behind the third BIR domain. Number of amino acids (aa) indicate lengths of proteins.

cIAP1 and cIAP2 were originally identified due to their ability to interact directly with TRAF2 (Rothe et al., 1995; Rothe et al., 1994). They regulate TNF-mediated signaling (Shu et al., 1996; Wang et al., 1998). cIAPs are essential regulators (Varfolomeev and Vucic, 2008), but also targets (Karin and Lin, 2002) of NF-κB signaling pathways. They are required for RIP1 ubiquitination and NF-κB activation in the canonical NF-κB pathway (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008). In the non-canonical NF-κB pathway cIAPs ubiquitinate NIK. This, in turn causes proteasomal degradation of NIK and abrogation of NF-κB signaling (Varfolomeev et al., 2007).

Protein ubiquitination has emerged as an important secondary protein modification that regulates various biological processes (Fang and Weissman, 2004; Wertz and Dixit, 2010). The process of ubiquitination involves three sequential reactions catalyzed by ubiquitin activating (E1), ubiquitin conjugating (E2) and ubiquitin ligase (E3) enzymes of the ubiquitin system (Hershko and Ciechanover, 1998). RING domains of IAPs are required for tow functions: dimerization and E3 ligase activity (Hu and Yang, 2003; Silke et al., 2005). The RING motif acts as a molecular scaffold bringing together the E2 enzyme and a target protein. This causes a direct transfer of ubiquitin from E2 to the target. RING domain sequences are variable but all have a conserved structure with two zinc ions coordinated by invariant cysteine and histidine residues (Borden and Freemont, 1996). However, mechanisms by which RING domains interact with E2 ligases and promote ubiquitin transfer remain elusive. Some RING domain containing proteins, including IAPs, are able to regulate their own abundance by autoubiquitylation. It is suggested that IAP autoubiquitylation depends on their dimerization (Mace et al., 2008). The regulatory mechanism of cIAP-

dependent auto- and target-ubiquitinilation is still unclear (Wei et al., 2008). Recently two groups have described a novel ubiquitin-binding domain of IAP proteins (Blankenship et al., 2009; Gyrd-Hansen et al., 2008). The Ubiquitin-Associated (UBA) domain is located between the BIR motifs and the CARD or the RING domain (Fig. 6). The UBA domain of cIAP1 binds monoubiquitin as well as lysine-48 (K-48) and K-63-linked polyubiquitin chains with low micro molar affinities. Authors of the original papers suggested that ubiquitin-binding may be an important mechanism for rapid turnover of autoubiquitinated cIAP1 and cIAP2 (Blankenship et al., 2009; Gyrd-Hansen et al., 2008).

Tumor cells must be resistant to transformation-induced apoptosis in order to progress to clinically relevant invasive or metastatic disease (Hanahan and Weinberg, 2000). Recently developed tumor therapies try to overcome apoptosis resistance and death receptor agonists are currently in clinical trials (Ashkenazi, 2008). Presumably due to their genetic instability, resistant tumor cells are frequently selected during treatment. This resistance is the cause of subsequent therapeutic failure. Therefore there is substantial interest in developing novel treatments that can further sensitize tumors to death receptor-mediated apoptosis in order to allow efficient combination therapies. Different approaches were developed to sensitize cancer cells by interference with critical cell death pathways. Initial attempts, using antisense RNA and more recently small molecules antagonizing Bcl-2, are currently in early clinical trials (Cotter, 2009). In parallel to these promising concepts using small antagonists to apoptosis-related molecules, a new approach was the development of IAP antagonists (SMAC mimetics) for sensitization of tumor cells. IAP antagonists are synthetic compounds that were modeled on the N-terminal IAP-binding motif of the mitochondrial protein SMAC/DIABLO (Wright and Duckett, 2005). The SMAC-mimetic compounds were developed to inhibit XIAP, because interference with XIAP function is crucial for therapeutic efficiency of TRAIL in xenograft tumor models (Vogler et al., 2008). XIAP is also a critical regulator of CD95-sensitivity in type II cells such as murine hepatocytes (Jost et al., 2009). Recently it was shown that compounds, originally designed to target XIAP, also target cIAPs. They induce rapid autoubiquitylation and proteasomal degradation of cIAP1 and cIAP2 and sensitize tumor cells to TNF-mediated cell death (Bertrand et al., 2008; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). Less is known about possible synergies of SMAC and/or SMAC mimetics with TRAIL (Fulda et al., 2002) or CD95L to induce apoptosis. Given that cIAPs regulate RIP1 in TNF-R1 and RIP1 also plays a role in other death receptor signaling pathways (e.g. CD95), it was interesting to investigate mechanisms of death receptor-mediated cell death in the context of cIAP inhibition. Understanding of these functions of cIAP proteins might be useful to overcome apoptosis resistance in tumor therapy, and may also be relevant during virus infection or tumor immunity (Lotze et al., 2007).

1.2.3 RIP1 is a key mediator of non-apoptotic signaling pathways

A further complication hindering complete understanding of the extrinsic cell death pathway is the ability of death receptors to activate non-apoptotic caspase-independent cell death or cause non-apoptotic responses such as inflammatory gene induction (Kroemer et al., 2009; Wajant et al., 2003a). Physiological significance of these pathways is still debated since mechanisms underlying these cellular responses are not fully understood. Novel studies indicate that these non-apoptotic pathways depend on RIP1. RIP1 is constitutively expressed in many tissues, e.g. skin. This protein is an essential sensor of cellular stress and a crucial regulator of cell survival and death (Meylan and Tschopp, 2005). Evidences for an important role of RIP1 in death receptor-associated signaling pathways come from studies on RIP1 knock-out mice which die days after birth, as a result of an increased sensitivity to TNF (Cusson et al., 2002; Kelliher et al., 1998). RIP1 belongs to the RIP family of serine-threonine kinases which shares homologous kinase domains (Festjens et al., 2007). Additionally to the N-terminal kinase domain RIP1 contain an intermediate domain, a RIP Homotypic Interaction Motif (RHIM) and a C-terminal DD (Fig. 7). The DD of RIP1 was originally identified as an interaction partner for the DD of CD95 (Stanger et al., 1995). Upon death ligand stimulation RIP1 is able to directly bind to the DD of death receptors or is indirectly recruited by the DD-containing adaptor proteins TRADD and FADD.

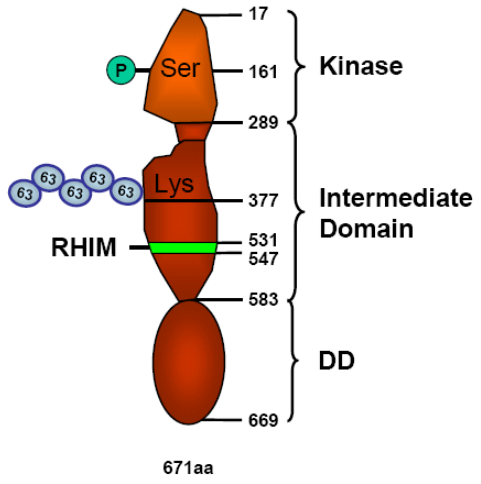


Figure 7: Schematic representation of the structure of RIP1. Number of aa that corresponding to each domain and suggested modifications important for NF-κB activation (K-63-linked polyubiquitin chains on K-377) or necrosis (phosphorylation on Ser-161) are indicated.

RIP1 is recruited to the TNF-R1 (Hsu et al., 1996) and can interact with TRAFs and cIAPs (Hayden and Ghosh, 2008; Takeuchi et al., 1996; Vandenabeele et al., 2010). Activated death receptors trigger polyubiquitination of RIP1 which is regulated by cIAPs (Mahoney et al., 2008; Varfolomeev et al., 2008). Depending on the cellular context the ubiquitin modification of RIP1 can determine activation of prosurvival signaling pathways that counteract cell death (Zheng et al., 2006). cIAPs mediate presumably K-63 linkage of RIP1

(Bertrand et al., 2008; Park et al., 2004; Varfolomeev et al., 2008) which subsequently functions as a scaffold for the binding of IKKγ (Devin et al., 2000; Ea et al., 2006; Hur et al., 2003). Recruitment of the IKK complex to the death receptor signaling platform can promote downstream activation of MAPKs and transcription factors like NF-κB. K-63 ubiquitination of RIP1 can be removed by deubiquitinases like A20 (Wilson et al., 2009a). A20 was shown to exert two opposing activities: sequential K-63 deubiquitination followed by K-48 ubiquitination of RIP1. This K-48 linkage leads to proteasomal degradation of RIP1 (Heyninck and Beyaert, 2005) and repression of NF-κB signaling (Wertz et al., 2004). When ubiquitination of RIP1 and death receptor-mediated NF-κB activation are reduced, RIP1 switches its function to that of a promoter of cell death (Wang et al., 2008). RIP1 is supposed to be critical for death receptor-mediated necrosis (Degterev et al., 2005; Holler et al., 2000). It was hypothesized that the DD of RIP1 is important for this necrotic activity (Holler et al., 2000). Recent development of specific RIP1 kinase inhibitors has facilitated experiments examining the functional role of RIP1 kinase in necrosis (Degterev et al., 2008). Precise role or potential targets of the kinase activity of RIP1 are still poorly understood (Hitomi et al., 2008). However novel studies revealed that the interaction between RIP1 and RIP3 via their RIHM domains seems to be crucial for activation of necrotic cell death (Cho et al., 2009; Zhang et al., 2009).

A recent report suggested that RIP1 interaction with Caspase-8 depends on the type of a ligand (membrane-bound vs. soluble) that activates death receptors, further complicating the possible outcomes of death receptor signaling (Morgan et al., 2009). Therefore it will be crucial to reveal the correlation between important death receptor associated proteins as RIP1, cIAPs and cFLIP to understand antagonizing effects of death receptor-mediated apoptotic and non-apoptotic signaling pathways.

1.3 Aims

Skin-derived cancer has a worldwide increasing incidence. Therefore, understanding of the tumor biology is necessary for the development of novel and potent therapeutic strategies. Tumor progression requires several alterations like resistance to death ligand-induced cell death. Expression of anti-apoptotic molecules, like cFLIP or cIAP, has been suggested to correlate with death ligand resistance of tumor cells and consequently with tumor progression. Given these, it was the aim of this study to identify the role of the anti-apoptotic molecules cFLIP and cIAPs, as well as the cIAP target RIP1, in modulation of death receptor-mediated signaling pathways in different primary and skin tumor cells. Activation of death receptors leads to formation of distinct receptor and intracellular signaling platforms and all three molecules can be recruited to these complexes. To this end the precipitation of receptor and intracellular complexes was established and modified to analyze

cFLIP, cIAP and RIP1-dependent complex formations under various conditions. Based on this technique and applying further technologies established in the laboratory it was the goal of this project to answer several open questions in death receptor signaling of primary and transformed skin cells.

1. How does cFLIP regulate death ligand-induced cell death in skin and primary endothelia cells? Death ligand-induced apoptosis is tightly regulated by Caspase-8 activation in the membrane-associated DISC and cFLIP is a critical regulator of the caspase-dependent apoptosis. Given this and to answer the initial question, endogenous expression levels of cFLIP were manipulated either by knockdown or by stable retroviral overexpression. Ligand affinity precipitation of the DISC allowed to analyze cFLIP-dependent complex formation and Caspase-8 activation.
2. How do cIAP1 and cIAP2 regulate CD95-mediated cell death in skin tumor cells? To investigate roles of cIAPs in CD95 signaling endogenous expression levels of cIAPs were manipulated using either an IAP antagonist or inducible lentiviral overexpression of cIAP1 or cIAP2. Sensitivity to CD95L of stimulated skin tumor cells were analyzed using cytotoxicity assays.
3. How do cIAPs regulate CD95-mediated formation of signaling complexes? cIAPs are involved in formation of the TNF-induced signaling platform. Therefore, it was of particular interest to monitor the CD95-induced DISC and the complex II formation in a cIAP-dependent manner. Ligand affinity precipitation of the CD95 DISC and Caspase-8 immunoprecipitation allowed to analysis cIAP-related DISC and complex II formation.
4. How does RIP1 regulate CD95-mediated cell death and complex formation in human keratinocytes? RIP1 is regulated by cIAPs and involved in the formation of TNF-induced signaling platform. To answer the initial question, RIP1 expression levels were down regulated by specific knockdown in human keratinocytes and sensitivity to CD95L as well as CD95L-dependent DISC and complex II formation of these cells were analyzed.
5. Does cFLIP influences cIAP-dependent CD95 signaling pathways in the skin? Both, cFLIP as well as cIAPs, were described for their anti-apoptotic function. Therefore, to answer the question whether cFLIP can antagonize IAP antagonist-mediated sensitization of skin tumor cells to CD95L the sensitivity of stable cFLIP overexpressing skin tumor cells to CD95L in presence and absence of cIAPs were analyzed. In addition, interdependency of cFLIP and cIAP in recruitment and function of signaling complexes were investigated by precipitation of CD95-induced DISC and complex II of human keratinocytes.

2 Material and Methods

2.1 Material

The kits and chemicals that were used in this work were purchased from the described companies. The quality of the reagents was of analytical grade. If special reagents were used for experiments, it is described at the beginning of each section.

2.1.1 Enzymes and molecular biology reagents

Item	Company
Alkaline Phosphatase from calf intestine (CIAP)	Promega
Deoxynucleoside Triphosphate Set (dNTPs)	Promega, Fermentas
Endonucleases (Restriction enzymes)	Promega, Fermentas
NuPAGE® Novex 4-12% Bis-Tris Gels	Invitrogen
Oligonucleotides (Primer)	MWG-operon
Polyvinylidene fluoride (PVDF) membrane	GE Healthcare
Protein G beads	Roche
T4 DNA ligase	Promega
Taq DNA polymerase	Promega, Fermentas

Table 1: Molecular biology reagents

2.1.2 Kits

Item	Company
DC Protein Assay Reagents Package	Bio-Rad
ECL Detection Reagents	GE Healthcare
ECL Plus™ Western Blotting Detection Reagents	GE Healthcare
EndoFree Plasmid Maxi Kit	Qiagen
Wizard Plus SV Miniprep DNA Purification System	Promega
Wizard® SV Gel and PCR Clean-Up System	Promega

Table 2: Kits used in biochemical and molecular biological assays

2.1.3 Phoshpatase and protease inhibitors

Inhibitor	Company (Catalog number)
AEBSF Hydroclorid	AppliChem (A1421)
Aprotinin	Roth (A162.3)
Benzamidine	Fluka (12072)
Complete (Protease Inhibitor Cocktail Tablets)	Roche (11836145001)
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich (G6376)
Leupeptin hemisulfate salt	Sigma-Aldrich (L2884)
Sodium orthovanadate	Sigma-Aldrich (S6508)
Sodium pyrophosphate tetrabasic decahydrate	Sigma-Aldrich (S6422)

Table 3: Phoshpatase and protease inhibitors

2.1.4 Pharmacological stimulating substances

Substance	Company (Catalog number)
Compound A (CompA)	provided by TetraLogic Corp. (Vince et al., 2007)
Necrostatin-1	Sigma-Aldrich (N9037)
Nickel standard ($NiCl_2$ in H_2O)	Merck (109989)
4-Hydroxytamoxifen (4-HT)	Sigma-Aldrich (H7904)
z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (ZVAD-fmk)	Bachem (N-1560)

Table 4: Pharmacological stimulating substances

2.1.5 Stimulating Cytokine

Cytokine	Source
His-Flag-TNFα (HF-TNF)	P. Diessenbacher (Diessenbacher et al., 2008)
TNF-R2-Fc	M. Feoktistova (Geserick et al., 2009)
Flag-TRAIL (F-TRAIL)	M. Schmidt (Schmidt et al., 2009)
His-Flag-TRAIL (HF-TRAIL)	P. Diessenbacher (Diessenbacher et al., 2008)
CD95L-Fc	M. Feoktistova (Geserick et al., 2009)

Table 5: Stimulating Cytokine

2.1.6 Molecular weight markers

DNA molecular weight markers	**Company**
GeneRuler™ 1 kb DNA Ladder, ready-to-use	Fermentas
GeneRuler™ 100 bp Plus DNA Ladder, ready-to-use	Fermentas
Protein molecular weight markers	**Company**
SeeBlue® Pre-Stained Standard	Invitrogen

Table 6: Molecular weight markers for DNA and proteins

2.1.7 Buffers

Buffer	**Composition**
1 x PBS	2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4, pH 7.4
1 x T-PBS	1xPBS, 0.1% Tween 20
5 x Laemmli sample buffer	60 mM Tris-HCl (pH 6.8), 2% SDS, 10%l Glycerol, 5% β-Mercaptoethanol, 0.01% Bromophenol blue
AB-buffer	3% nonfat dry milk, 1.5% BSA in 1 x T-PBS
Blocking buffer	5% nonfat dry milk, 3% BSA in 1 x T-PBS
Crystal violet staining solution	0.5% crystal violet, 20% methanol
DNA sample buffer	30% (v/v) Glycerine, 50 mM EDTA, 0.25% Bromophenol-blue, 0.25% Xylene Cyanol
Lysis buffer	30 mM TRIS-HCL (pH 7.5), 120 mM NaCl, 10% Glycerol, 1% Triton X, 2 tablets Complete (Protease Inhibitor) per 100 ml
Lysis-buffer (for IP)	Lysis buffer, 1mM Sodium orthovanadate, 50 mM β-Glycerophosphate, 20 mM Sodium pyrophosphate, 1 mM AEBSF, 5 mg/ml Aprotinin, 5 mg/ml Leupeptin, 5 mM Benzamidine
SDS Running Buffer (MES)	50 mM MES, 50 mM Tris-base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.3
SDS Running Buffer (MOPS)	50 mM MOPS, 50 mM Tris-base, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.7
TAE buffer	40 mM Tris Base, 20 mM Acetic acid, 10 mM EDTA
Transfection buffer A	0.5 M $CaCl_2$
Transfection buffer B	140 mM NaCl, 50 mM Hepes, 1.5 mM Na_2PO_4, pH 7.05
Transfer buffer	25 mM Tris, 192 mM glycine, 10% methanol

Table 7: Buffers used in biochemical and molecular biological assays

2.1.8 Primary antibodies for western blot analysis

Antibody (clone)	Company (Catalog number)
β-actin (A2103)	Sigma (A2103)
Caspase-8 (C15)	provided by P.H. Krammer, (DKFZ, Heidelberg, Germany)
Caspase-8 (C20)	Santa Cruz (sc-6136)
CD95 (C20)	Santa Cruz (sc-715)
CD95 (Apo-1 IgG3)	provided by P.H. Krammer, (DKFZ, Heidelberg, Germany)
FADD (1)	BD Transduction Laboratories (F36620)
Flag (M2)	Sigma (F3165)
cFLIP (NF-6)	Enzo Life Sciences, Inc
GFP-HRP	Clonetech (8369-1)
cIAP-1 (1E1)	provided by J. Silke (Silke et al., 2005)
cIAP-2 (207)	provided by J. Silke (Vince et al., 2009)
IKKβ	Cell signaling (2684)
RIP1 (38)	BD Transduction Laboratories (R41220)
TNF-R1 (H-5)	Santa Cruz (sc-8436)
TRADD (37)	BD Transduction Laboratories (T50320)
TRAF2 (C20)	Santa Cruz (sc-876)
TRAIL-R1	Abcam (ab8414)
TRAIL-R2	Abcam (ab8416)
TRAIL-R4 (C20)	Santa Cruz (sc-7550)
ß-Tubulin	Sigma (T4026)
XIAP (48)	BD Transduction Lab (H62120)

Table 8: Primary antibodies for western blot analysis

2.1.9 HRP-coupled secondary antibodies

Antibody (clone)	Company (Catalog number)
Mouse IgG1	Southern biotech (1070-05)
Mouse IgG2a	Southern biotech (1080-05)
Mouse IgG2b	Southern biotech (1090-05)
Rabbit IgG	Southern biotech (4030-05)
Rat IgG	Southern biotech (3050-05)

Table 9: HRP-coupled secondary antibodies for western blot analysis

2.1.10 Vectors

Vector	Reference
Expression vector: PCR-3	Invitrogen
Expression vector: pQE32	Qiagen
Lentiviral packaging vector: pMD2.G	(Rubinson et al., 2003)
Lentiviral packaging vector: pMDlg/pRRE	(Rubinson et al., 2003)
Lentiviral packaging vector: pRSV-Rev	(Rubinson et al., 2003)
Lentiviral vector: pFGEV16-Super-PGKHygro	(Vince et al., 2007)
Lentiviral vector: pF5xUAS-W-SV40-Puro	(Vince et al., 2007)
Retroviral vector: pCF65-IEGZ	(Denk et al., 2001)
Retroviral vector for siRNA: pRS-MIG	(Diessenbacher et al., 2008)

Table 10: Vectors used for expression

2.1.11 siRNA-sequences for knock down

Target	Sequence
cIAP2	64-mer oligomers containing cIAP2-targeting sequence nucleotide start position +316
cIAP2	64-mer oligomers containing cIAP2-targeting sequence nucleotide start position +466
cFLIP	64-mer oligomers containing cFLIP-targeting sequence nucleotide start position +911
cFLIP	targeting sequence: GGAGCAGGGACAAGTTACA
RIP1	64-mer oligomers containing RIP1-targeting sequence nucleotide start position +193

Table 11: siRNA-sequences for knock down

2.1.12 Bacteria cells

Bacterial Cells	Company
E.coli DH5α	Clontech
E.coli M15 (pREP4)	Qiagen
E.coli TOP10F	Invitrogen
E.coli XL10 Gold	Stratagene

Table 12: Bacteria cells

2.1.13 Culture media and additives for bacterial cells

Item	Company (Catalog number)
Ampicillin sodium salt (100µg/ml)	Roth (K029.2)
Kanamycin sulfate (30µg/ml)	Roth (T832.1)
LB-Agar (Lennox)	Roth (X965.2)
LB-medium (Lennox)	Roth (X964.2)
SOC Medium	Sigma-Aldrich (S1797)

Table 13: Media and Reagents for bacterial cell culture. All media were autoclavated at 121°C for 15 min. The additives were filtered with a 0.2 µm filter-unit and stored at -20°C.

2.1.14 Human cells

Human cells	Source
A5RT3 parental (SCC cell line)	Mueller et al., 2001: provided by P. Boukamp (DKFZ, Heidelberg, Germany)
HaCaT parental (transformed keratinocytes)	Boukamp et al., 1988: were provided by P. Boukamp (DKFZ, Heidelberg, Germany)
HaCaT pCF65	P. Diessenbacher (Diessenbacher et al., 2008)
HaCaT $cFLIP_L$	P. Diessenbacher (Diessenbacher et al., 2008)
HaCaT $cFLIP_S$	P. Diessenbacher (Diessenbacher et al., 2008)
HaCaT IKKβ-EE	P. Diessenbacher (Diessenbacher et al., 2008)
HaCaT IKKβ-KD	P. Diessenbacher (Diessenbacher et al., 2008)
HaCaT pCF65 GEV16 cIAP2	M.Hupe (Diessenbacher et al., 2008)
HaCaT IKKβ-KD GEV16 cIAP2	M.Hupe (Diessenbacher et al., 2008)
HaCaT pCF65 GEV16 GFP	M.Hupe (Geserick et al., 2009)
HaCaT pCF65 GEV16 cIAP1-Flag	M.Hupe (Geserick et al., 2009)
HaCaT pRS-MIG	M. Feoktistova (Geserick et al., 2009)
HaCaT pRS-RIP1	M. Feoktistova (Geserick et al., 2009)
HEK 293T (Human Embryonic Kidney 293 that stably express the large T-antigen of SV40)	American Type Culture Collection (ATCC)
HUVEC (Human Umbilical Vein Endothelial Cells)	Cambrex (Schmidt et al., 2009)
HUVEC pRS-MIG	M. Schmidt (Schmidt et al., 2009)
HUVEC pRS-cFLIP	M. Schmidt (Schmidt et al., 2009)
IGR parental (melanoma cell line)	Odh et al., 1994
IGR pRS-MIG	P. Geserick (Geserick et al., 2009)

IGR pRS-cFLIP	P. Geserick (Geserick et al., 2009)
MET1 parental (SCC cell line)	Poppet al., 2000: provided by I. Leigh (Skin Tumor Laboratory, London, UK)
MET1 pCF65	P. Geserick (Geserick et al., 2009)
MET1 $cFLIP_L$	P. Geserick (Geserick et al., 2009)
MET1 $cFLIP_S$	P. Geserick (Geserick et al., 2009)
ΦNX (amphotropic producer cell line derived from HEK 293T)	American Type Culture Collection (ATCC)
RPM-EP parental (melanoma cell line)	Kikuchi et al., 1996
RPM-EP pCF65	P. Geserick (Geserick et al., 2008)
RPM-EP $cFLIP_L$	P. Geserick (Geserick et al., 2008)
RPM-EP $cFLIP_S$	P. Geserick (Geserick et al., 2008)

Table 14: Human cells

2.1.15 Cell culture media and reagents for human cells

Item	**Company (Catalog number)**
Amphotericin B solution	Sigma-Aldrich (A2942)
Dulbecco´s Modified Eagle Medium (D-MEM)	Invitrogen (41965-062)
EGM Bulletkit	LONZA (CC-3124)
Fetal Bovine Serum “Gold” (FBS)	PAA (A15-151)
Gentamicin solution	Sigma-Aldrich (G1397)
HEPES	Sigma-Aldrich (H3375)
Hexadimethrine bromide	Sigma-Aldrich (H9268)
Hygromycin B	MERK (400050)
Liquemin® N25000 (Heparin-natrium)	Roche
Medium 199	PAA (E15-834)
MEM Amino Acids	PAA (M11-003)
MEM NEAA	
MEM Vitamins	PAA (N11-002)
Dulbecco´s Phosphate-Buffered Salines (D-PBS)	Invitrogen (14190)
Puromycin dihydrochloride	Sigma-Aldrich (P9620)
Sodium Pyruvate	PAA (S11-003)
Trypsin EDTA	PAA (L11-004)
Zeocin™	Invitrogen (R250-01)

Table 15: Media and reagents for human cell culture

2.2 Methods

2.2.1 Molecular biological methods

All molecular biological work was carried out corresponding to standard protocols. All methods are described in the literature in detail: Current Protocols in Molecular Biology (Ausubel et al., 1990) and Molecular Cloning (Sambrook et al., 1989). Therefore, only a brief description will be given and the modifications will be described in more detail if this applies.

2.2.2 Polymerase chain reaction (PCR)

PCR was performed using 0.5 µg DNA mixed with PCR reagents in a volume of 25 µl. The final concentrations of the PCR reagents were: 0.5 1 pM forward primer, 1 pM reverse primer, 2.5 mM $MgCl_2$, 0.1 units/µl Taq DNA polymerase, 0.2 mM dNTPs and 1 x PCR buffer.

Process	**Time and temperature**	**Cycles**
Initial denaturation	1 min at 95°C	1
Denaturation Annealing Extension	30 sec at 95°C 30 sec at 50-70°C 60 sec at 72°C	30-40
Final extension	1 min at 72°C	1

Table 16: PCR programmes

2.2.3 DNA restriction enzyme digestion

For analytical digestions 1 µg of DNA, harboring 1 restriction site, was incubated with 1 U of enzyme for 1 h (alternatively, 0.5 U for 2 h). For preparative digestions 5 to 10 fold overdigestion was made. Reaction mixture was incubated at 37°C, unless another temperature was recommended by manufacturer.

2.2.4 DNA agarose gel electrophoresis

DNA fragments were separated according to their size by one-dimensional agarose gel electrophoresis. To visualize the DNA under UV light, 5-10 µl Ethidium bromide solution (10 mg/ml in H_2O) was added before gel polymerization. The DNA samples were prepared in DNA sample buffer and were loaded onto the gel. Gels were run at 80 V in TAE buffer. The DNA fragments were visualized under UV-light and photographed.

2.2.5 Cloning

DNA fragments of interest were purified subjected to enzymatic digestion and ligated with T4 DNA ligase to the pre-digested vector. The ligations were performed at 16-20°C for 2-8 h. The used DNA fragment/vector ratio was 3:1. To select for positive clones, the ligated fragment-vectors were transformed into competent bacterial cells for subsequent DNA isolation.

2.2.6 Heat shock transformation

The DNA ligation mixture was incubated together with 100 µl of heat shock competent bacteria cells for 30 min on ice. Heat shocking for 30 sec at 42°C was followed by incubation on ice for 2 min. Then 1 ml of pre-warmed SOC Medium was added and the tube incubated at 37°C for 1 h shaking at low speed. Bacterial cells were spined down at 1000 g for 1 min and the supernatant was decanted. The pellet was resuspented by vortexing in the remaining drops of liquid. The entire suspension was plated on LB-Agar plates containing the respective antibiotics. Plates were incubated over night at 37°C.

2.2.7 Plasmid isolation

DNA plasmids were purified using Plasmid Kits and following their instructions. The DNA concentration was determined by spectrophotometrical quantification at 260 nm by A_{260} * 50 = x µg/µl.

2.2.8 Cell culture techniques

Cells were cultured in their respective media in 5% CO_2 at 37°C and a humidity of 95%. All media were pre-incubated at 37°C before addition to cells.

Human cells	**Medium**
A5RT3	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 10% heat inactivated FBS
HEK 293T	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 10% heat inactivated FBS
HaCaT	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 10% heat inactivated FBS
HUVEC	HUVEC Mix: 2 parts Medium 199 complete and 1 part EGM Medium 199 complete: 500ml Medium 199, 50ml FBS, 300µl Gentamicin (50mg/ml), 30µl Amphothericin B (250µg/ml), 10µl Liquemin (5000 I.E./0,5ml) EGM: 500ml EBM, Bulletkit (FBS, GA-1000, BBE, hEGF, Hydrocortison)

IGR	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 10% heat inactivated FBS
MET1	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 1% MEM NEAA, 1%MEM Vitamins, 10% heat inactivated FBS
ΦNX	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 1% MEM Amino Acids, 10% heat inactivated FBS,
RPM-EP	D-MEM, 1% HEPES, 1% Sodium Pyruvate, 10% heat inactivated FBS

Table 17: Media and references for cell culture

2.2.9 Transfection with Ca phosphate method

Cells were grown in 75 cm^2 flasks in there respective medium. 0.5 ml of Transfection buffer A was mixed with 25 µg DNA (for double transfection; 12.5 µg of each DNA). Then, 0.5 ml of Transfection buffer B, were added and, after 1 min, applied to cells in culture. The cells were incubated for 4-8 h at 37°C in 5% CO_2 atmosphere before exchange of growth media. Cells were grown for 24 h before applied for further analyses.

2.2.10 Generation von stimulating cytokines

For expression of HF-TRAIL and HF-TNF, the complementary DNAs encoding the extracellular domain of TRAIL (aa 95-281) or TNF (aa 78-233) were N-terminally fused to a Flag tag (DYKDDDDK) by PCR cloning and ligated into the BamHI- and NotI-restricted pQE32 vector. The proteins were expressed in E.coli M15 (pREP4) at 18°C overnight and purified using a standard Ni-NTA column (Qiagen).

The CD95L-Fc expression construct was cloned in the PCR-3 vector and encodes the hemagglutinin signal peptide, the Fc portion of human immunoglobulin G (IgG) (aa 108 to 338 of accession number AAC82527, excluding the stop codon), a linker sequence (RSPQPQPKPQPKPEPEGSLQ), and CD95L (aa 139 to 281). Proteins were expressed transiently in HEK 293T cells. Supernatants were harvested, filtered (0.2 µm, Millipore) and stored at -20°C.

2.2.11 Retroviral infection

The amphotropic producer cell line ΦNX was transfected with 10 µg of the retroviral vectors pCFG5-IEGZ and pCF65 containing cDNA of interest. The supernatants were harvested 24 h post-transfection and filtered (0.45, Millipore). The virus containing supernatants were added to target cells with 5 µg/ml Hexadimethrine bromide. Target cells were spin-infected (3 h at 30°C). Stable cell lines were selected in Zeocin for 10-14 days. Western

blot analyses were performed on polyclonal cells to confirm ectopic expression of the respective molecules.

2.2.12 Stable siRNA expression

RIP1 siRNA as well as a random sequence not matched by any gene in the National Center for Biotechnology Information database (Vogler et al., 2007) were used. The random sequence construct was provided by S. Fulda (Ulm University, Ulm, Germany). For generation of the constructs, cDNA 64-mer oligomers containing RIP1 targeting sequence (nucleotide start position +193) were cloned into the pRS-MIG retroviral vector using HindIII and BglII restriction sites. For generation of the cIAP2 and cFLIP constructs, complementary DNA 64-mer oligomers containing either cIAP2 (nucleotide start position +316 or +466) or cFLIP (nucleotide start position +911) targeting sequences were cloned into the pRS-MIG retroviral vector using HindIII and BglII restriction sites. The resulting vectors were transfected into the amphotropic producer cell line ΦNX. The virus containing supernatants were added to target cells with 5 µg/ml Hexadimethrine bromide. Target cells were spin-infected (3 h at 30°C). Stable cell lines were selected in Puromycin (1 µg/ml) for 3 days. Western blot analyses were performed on polyclonal cells to analyze expression of the down regulated proteins.

2.2.13 Lentiviral infection

To generate lentiviral supernatants, HEK 293T cells were transfected with pMD2.G, pMDlg/pRRE, and pRSV-Rev of the lentiviral packaging vectors together with the pFGEV16-Super-PGKHygro and molecule of interest containing pF5xUAS-W-SV40-Puro. The supernatants were harvested 24 h post-transfection and filtered (0.45 µm, Millipore). The virus containing supernatants were added to target cells with 5 µg/ml Hexadimethrine bromide. Target cells were spin-infected (3 h at 30°C). Stable cell lines were selected in hygromycin (100-150 mg/ml), puromycin (1 mg/ml), or both. Cells were subsequently tested for expression of the respective proteins after 24 h of induction with 10 to 100 nM 4-HT.

2.2.14 Cytotoxicity assay

Crystal violet staining of attached, living cells was performed 18-24 h after stimulation with the indicated concentrations of death ligands in 96-well plates. Plates were washed two times with 1 x PBS. Subsequently, 50 µl of Crystal violet staining solution were added per well. After incubation for 20 min at room temperature, plates were washed four times with

water. Plates were air dried, and 200 µl methanol was added per well and incubated for 30 min. The optical density of the wells was subsequently measured by a plate reader (Victor3; PerkinElmer). The optical density of control cultures was normalized to 100% and compared with stimulated cells. For statistical analysis, the Standard Error of Mean (SEM) was determined for at least three independent experiments of each cell line and stimulatory condition.

2.2.15 Western blot analysis

Cells were washed twice with ice-cold 1 x PBS and lysed for 30 min on ice by the addition of Lysis buffer. Cellular debris was removed by centrifugation at 20,000 g for 10 min. 5 µg of total cellular proteins was supplemented with 5 x Laemmli sample buffer and boiled at 95°C. Proteins were separated by SDS-PAGE on 4-12% gradient gels using SDS Running Buffer. Proteins were transfer to PVDF membrane using Transfer buffer. Membranes were incubated for 1 h in Blocking buffer, washed with 1 x T-PBS, and incubated AB-buffer and primary antibodies overnight. After washing in 1 x T-PBS, blots were incubated with HRP-conjugated isotype-specific secondary antibody in 1 x T-PBS. After washing of the blots with 1 x T-PBS, bands were visualized with ECL detection kits.

2.2.16 Ligand affinity precipitation of receptor complexes

For precipitation of the death ligand-induced receptor complex, 5×10^6 cells were used for each condition. Cells were washed once with medium at 37°C and subsequently pre-stimulated with indicated conditions. Flag containing death ligands were pre-complexed with 5 mg/ml anti-Flag M2 antibody for 30 minutes. Cells were incubated with pre-complexed or Fc containing death ligands for indicated times. Receptor complex formation was stopped by washing the monolayer four times with ice-cold 1 x PBS. Cells were lysed on ice by the addition of 2 ml of Lysis-buffer (for IP) for 30 min. The lysates were centrifuged two times at 20,000 g for 5 min and 30 min, respectively, to remove cellular debris. A minor fraction of these clear lysates was used to control for the input of the respective proteins. For the precipitation of the CD95 receptor and stimulation-dependent recruited proteins, CD95 (Apo-1 IgG3) antibody was added to the lysates prepared from non-stimulated as well as stimulated cells to precipitate the CD95-interacting proteins. Receptor complexes were precipitated from the lysates using 40 µl Protein G beads for 16-24 h on an end over end shaker at 4°C. Ligand affinity precipitates were washed four times Lysis-buffer (for IP) before the protein complexes were eluted from dried beads by the addition of 5 x Laemmli sample

buffer and boiling at 95°C. Receptor complex components were analyzed by Western blot analysis.

2.2.17 Caspase-8 immunoprecipitation of complex II

After precipitation of the membrane bound receptor comlex, remaining lysates were centrifuged two times at 20,000 g for 5 min. Subsequently, 1 µg Caspase-8 (C20) antibody was added to all lysates. The Caspase-8 containing complexes were precipitated from the lysates by co-incubation with 40 µl of Protein G beads for 16-24 h on an end over end shaker at 4°C. Precipitates were washed four times Lysis-buffer (for IP) before the protein complexes were eluted from dried beads by the addition of 5 x Laemmli sample buffer and boiling at 95°C. Caspase-8 interacting components were analyzed by Western blot analysis.

3 Results

3.1 cFLIP dynamically regulates Caspase-8 activation

Death ligand-induced apoptosis is tightly regulated by Caspase-8 activation in the membrane-associated DISC. cFLIP is a well known inhibitor of Caspase-8. Given these, the question arose: how is cFLIP mechanistically regulating death receptor-mediated Caspase-8 activation in the DISC and consequently cell death in melanoma and primary endothelia cells?

3.1.1 $cFLIP_L$ and $cFLIP_S$ block death ligand-induced activation of Caspase-8 in the DISC in RPM-EP melanoma cells

Preliminary findings indicated that RPM-EP melanoma cells are sensitive to TRAIL- and CD95L-induced cell death, while overexpression of $cFLIP_L$ or $cFLIP_S$ provides resistance to death ligands in these cells (Geserick et al., 2008). To analyze the function of $cFLIP_L$ and $cFLIP_S$ for caspase-8 modulation in the DISC, ligand affinity precipitations of TRAIL and CD95 receptor complexes were preformed of parental, control vector, RPM-EP-$cFLIP_L$ and RPM-EP-$cFLIP_S$ cells (Fig. 8).

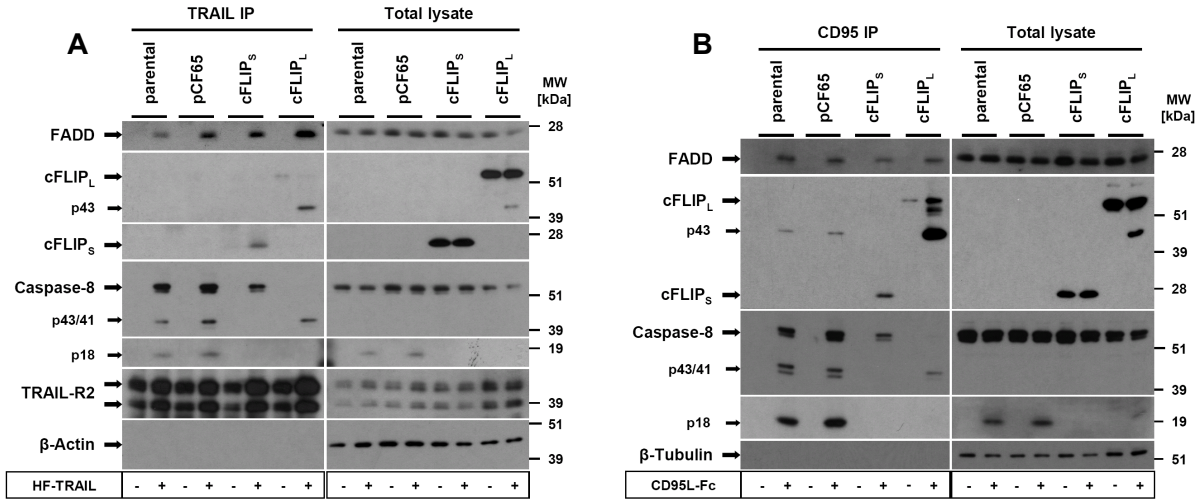


Figure 8: $cFLIP_L$ and $cFLIP_S$ block TRAIL- and CD95L-induced activation of Caspase-8 in the DISC in RPM-EP melanoma cells. Parental and transduced RPM-EP cells were either stimulated with (**A**) 2.5 mg/ml HF-TRAIL or (**B**) 250 U/ml CD95L-Fc for 30 min. TRAIL or CD95L DISC were precipitated by ligand affinity precipitation as described in Materials and Methods. Equal amounts of the DISC (IP) or total cellular lysates (Total lysate) were subsequently analyzed by Western blotting.

Upon stimulation FADD is equally recruited into the DISC in all examined cells, indicating that cFLIP overexpression did not significantly alter the recruitment of this adapter molecule. In parental and control vector transduced RPM-EP melanoma cells enrichment of cleaved

(p43/41) and activated (p18) fragments of Caspase-8 were observed in both receptor complexes. $cFLIP_L$ was mainly detected as p43 cleavage fragment in the DISC, indicating Caspase-8-mediated cleavage. Significantly increased recruitment of cFLIP p43 was observed in $cFLIP_L$ overexpressing cells. Furthermore increased amounts of partially cleaved Caspase-8 (p43/41), but decreased levels of Procaspase-8 and p18 fragment were found in TRAIL and CD95 DISCs of RPM-EP-$cFLIP_L$ cells. In contrast, $cFLIP_S$ completely inhibited Caspase-8 cleavage, but did not interfere with Procaspase-8 recruitment into the DISC, indicating that $cFLIP_S$ blocked Procaspase-8 processing in the DISC. This data suggested that both cFLIP isoforms block TRAIL- and CD95L-induced apoptosis of RPM-EP melanoma cells by inhibition of fully Caspase-8 procession into p18 in the DISC.

3.1.2 Repression of cFLIP by siRNA increases death ligand-induced Caspase-8 activation in the DISC in IGR melanoma cells

IGR melanoma cells are resistant to TRAIL- and CD95L-induced cell death, while downregulation of cFLIP by siRNA dramatically sensitize IGR cells to these death ligands (Geserick et al., 2008). Concerning the results with RPM-EP cells, it had to be shown whether suppression of cFLIP increase death receptor-mediated Caspase-8 activation in the DISC. Therefore, TRAIL- and CD95-induced complex formation of parental, control and cFLIP-knockdown IGR cells were analyzed (Fig. 9).

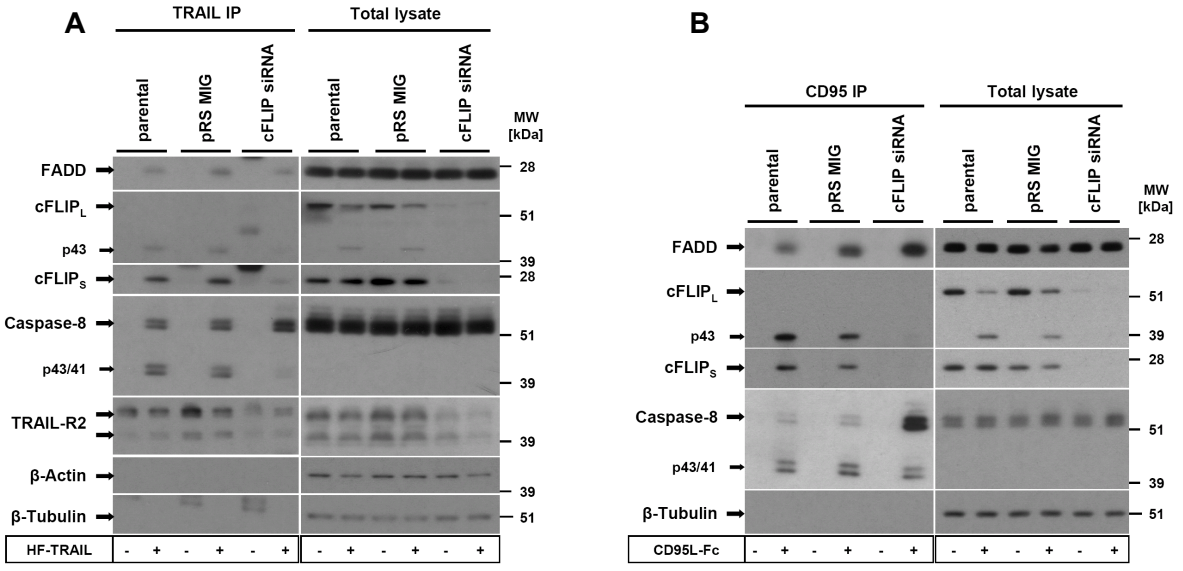


Figure 9: Knockdown of cFLIP leads to increased activation of Caspase-8 in the DISC in IGR melanoma cells. Parental and transduced IGR cells were either stimulated with (**A**) 2.5 mg/ml HF-TRAIL or (**B**) 250 U/ml CD95L-Fc for 30 min. TRAIL or CD95L DISC were precipitated by ligand affinity precipitation as described in Materials and Methods. Equal amounts of the DISC (IP) or total cellular lysates (Total lysate) were subsequently analyzed by Western blotting.

FADD was recruited into the TRAIL and CD95 DISC of all IGR cell lines upon ligand stimulation. In parental and control vector transduced IGR melanoma cells enrichment of cleaved (p43/41) fragment of Caspase-8 was observed in both receptor complexes. $cFLIP_L$ was mainly detected as p43 cleavage fragment in the DISC. Downregulation of cFLIP led to decreased amount of p43 cFLIP, while increased recruitment of uncleaved Procaspase-8 was observed in cFLIP-siRNA expressing cells. In several experiments, the Caspase-8 cleavage fragment p18 was not specifically detectable within the DISC in IGR cells. These data suggest that cleaved Caspase-8 is rapidly released from the receptor complex when cFLIP is absent and thereby promote increased apoptotic cell death.

Taken together these data suggested that the ratio of cFLIP and Caspase-8 is crucial to determine the sensitivity of death ligand-induced cell death in melanoma cells. cFLIP interfered with Caspase-8 activation in the DISC and thereby block apoptotic cell death induction.

3.1.3 cFLIP interferes with TRAIL-induced activation of Caspase-8 in the DISC in human endothelial cells

Primary endothelial cells are fully resistant to TRAIL-mediated apoptosis, while downregulation of cFLIP by siRNA dramatically sensitize these cells to TRAIL (Schmidt et al., 2009). To investigate the molecular mechanism of cFLIP action in endothelial cells, Human Umbilical Vein Endothelial Cells (HUVEC) expressing cFLIP siRNA were analyzed for TRAL-mediated activation of Caspase-8. As shown, both isoforms of cFLIP were efficiently suppressed by the siRNA (Fig. 10 A). TRAIL treatment of control vector-infected cells did not induce substantial Caspase-8 cleavage, while a prominent cleavage of $cFLIP_L$ to its p43 fragment was detected. In contrast, TRAIL treatment of cFLIP siRNA-expressing HUVEC led to substantial cleavage of Caspase-8 to its active fragment p18. These implicated that cFLIP blocks TRAIL-mediated cell death in HUVEC by inhibition of Caspase-8 activation. To investigate at which level cFLIP interferes with TRAIL-induced Caspase-8 activation in HUVEC, composition of the TRAIL DISC was analyzed (Fig. 10 B). FADD and Caspase-8 (pro form as well as the cleavage fragment p43/41) were recruited into the TRAIL DISC of control HUVEC. $cFLIP_L$ was mainly detectable as the p43 fragment at the receptor complex. Expression of cFLIP siRNA led to decreased amount of p43 cFLIP, while increased recruitment of unprocessed Procaspase-8 was observed in the DISC. The receptors TRAIL-R1, TRAIL-R2, and TRAIL-R4 functioned as control for the IP. The ratio of cFLIP and Caspase-8 was also crucial to determine the sensitivity of TRAIL-induced cell death in HUVEC. These findings were consistent with the repression of cFLIP in IGR melanoma cells

(Fig. 9) and supported the hypothesis that cleaved Caspase-8 is rapidly released from the receptor complex when cFLIP is absent.

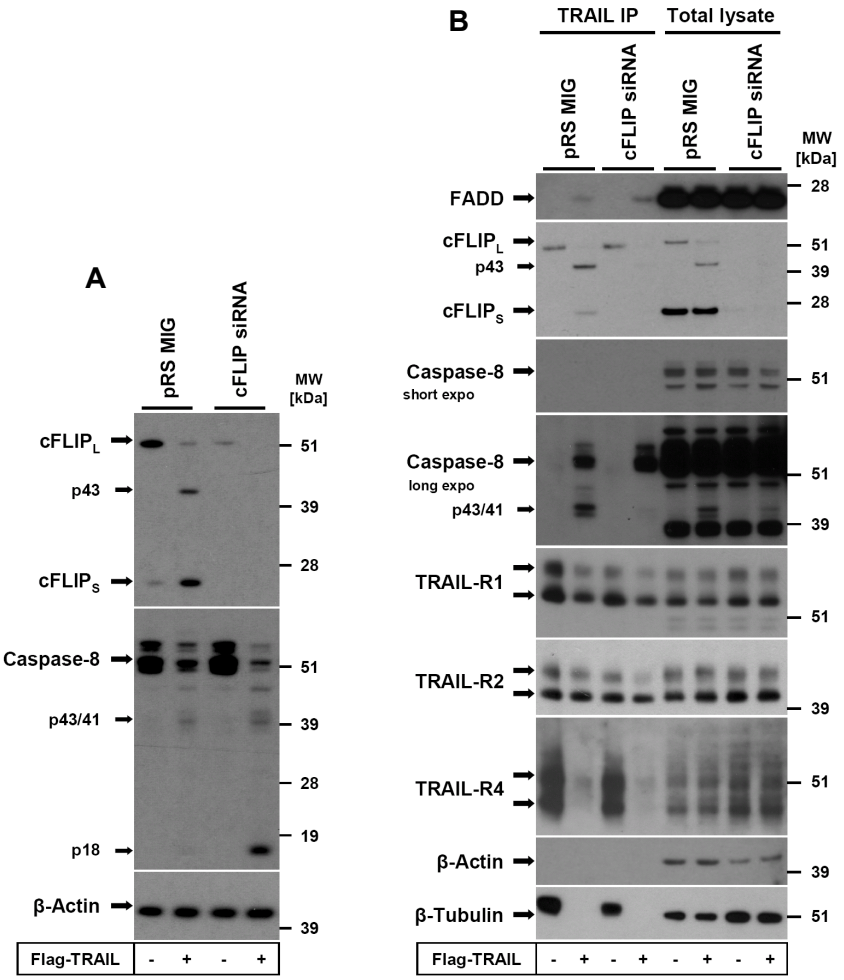


Figure 10: Knockdown of cFLIP by siRNA leads to increased activation of Caspase-8 in the TRAIL DISC and causes intracellular accumulation of Caspase-8 p18 in HUVEC. A) HUVEC infected with control vector or cFLIP siRNA were stimulated with 32 ng/ml of Flag-TRAIL or diluent for 3 h. Equal amounts of total cellular lysates were analyzed by Western blotting for indicated molecules afterwards. **B**) Transduced HUVEC were stimulated with 2.5 mg/ml Flag-TRAIL for 30 min. Receptor complexes (TRAIL IP) were precipitated via FLAG antibody as described in Materials and Methods. Precipitation of TRAIL receptors following lysis (-) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of total cellular lysates (Total lysate) or ligand affinity precipitates (TRAIL IP) were subsequently analyzed by Western blotting. IgG heavy chain of anti-Flag anti body was detected at 55 kDa in the cFLIP and β-Tubulin IP blots.

3.1.4 Prolonged nickel stimulation amplifies TRAIL-induced Caspase-8 activation by repressed cFLIP recruitment in the DISC

To analyze the physiological relevance of cFLIP repression in primary endothelial cells, HUVEC were treated with the proinflammatory and potent contact allergen Ni^{2+}. Previous results demonstrated that nickel stimulation represses cFLIP at mRNA and protein levels in HUVEC and sensitizes these cells to TRAIL-induced cell death in vitro (Schmidt et

al., 2009). To test whether nickel-dependent loss of cFLIP protein correlates with increased TRAIL sensitivity by increased activation of Caspase-8 at the receptor complex, DISC analyses in nickel-treated HUVEC were preformed (Fig. 11).

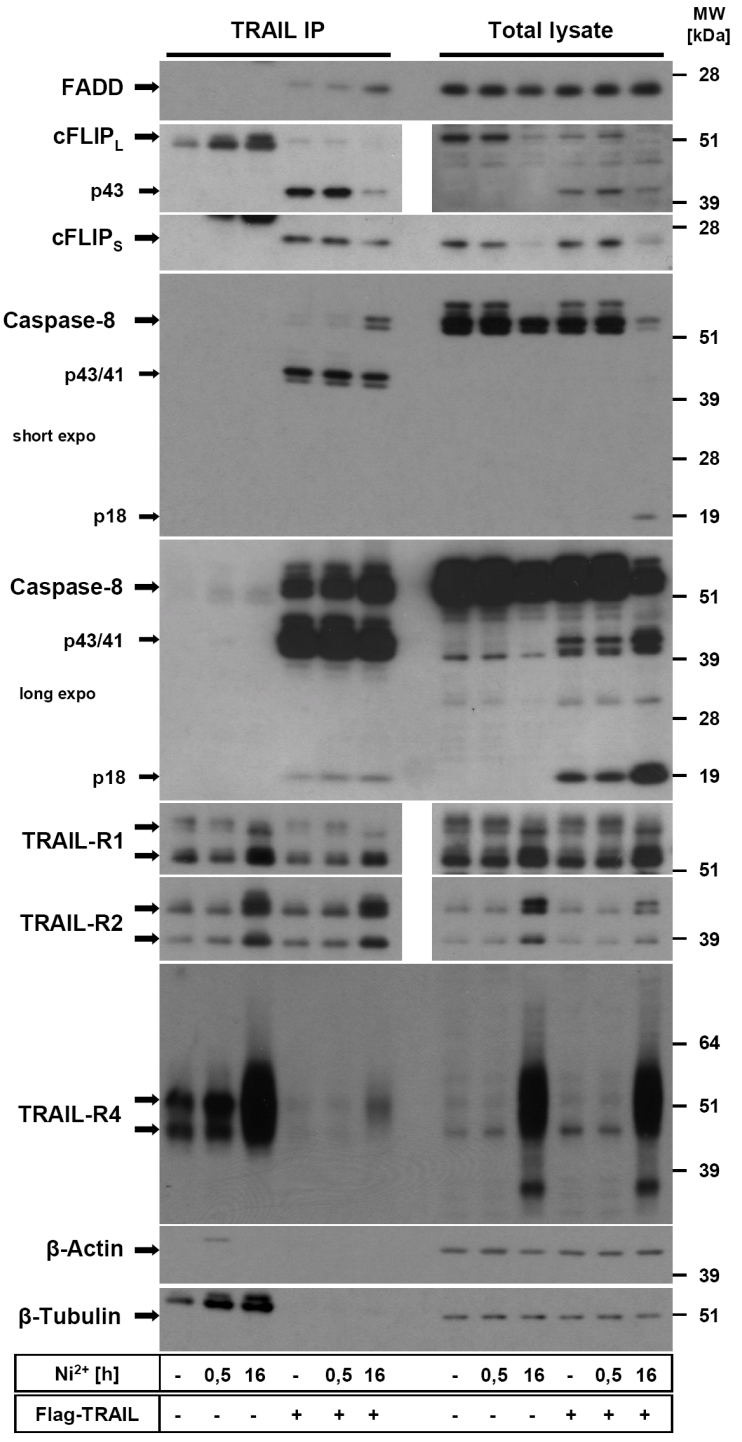


Figure 11: Prolonged Ni^{2+}-treatment triggers TRAIL-dependent activation of Caspase-8 by reduced cFLIP recruitment into the DISC. Parental HUVEC were pre-treated with Ni^{2+} or diluent for indicated time and subsequently with 2.5 mg/ml Flag-TRAIL for 30 min. Receptor complexes (TRAIL IP) were precipitated via FLAG antibody as described in Materials and Methods. Precipitation of TRAIL receptors following lysis (-) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of total cellular lysates (Total lysate) or ligand affinity precipitates (TRAIL IP) were subsequently analyzed by Western blotting. IgG heavy chain of anti-Flag anti body detected at 55 kDa at the cFLIP and β-Tubulin IP blot.

Prolonged nickel pretreatment resulted in a detectable upregulation of TRAIL-R1, TRAIL-R2, and TRAIL-R4 in the IP and total cellular lysates. Proteolytic cleavage of $cFLIP_L$ to p43 was evident under all conditions in the TRAIL DISC, suggesting the presence of functional receptor complexes in all cases. However, compared to both TRAIL-stimulated as well as Ni^{2+}-costimulated cells, clearly reduced amounts of p43 as well as $cFLIP_S$ were observed in the TRAIL-DISC of nickel-prestimulated cells despite enhanced precipitation of all three TRAIL receptors. Furthermore, an elevated amount of uncleaved Procaspase-8 was recruited into the TRAIL DISC of nickel-pretreated cells. Remarkably, the pattern of nickel-prestimulated cells closely resembled the TRAIL-DISC following depletion of cFLIP by siRNA. Thus, enhanced Procapase-8 recruitment most likely reflects an accelerated turnover of Caspase-8 within the DISC and subsequent rapid release of cleaved fragments from the receptor complex (similar to results in IGR melanoma cells; Fig. 9). In line with this hypothesis, fully cleaved Caspase-8 (p18) was strongly enriched while cFLIP was repressed in total cellular lysates in TRAIL-treated samples of Ni^{2+}-preexposed HUVEC. In contrast, neither fully cleaved Caspase-8 nor loss of total cFLIP protein was observed in lysates from nickel-cotreated or solely TRAIL-treated cells. These results suggest a physiological relevance of cFLIP in maintaining natural resistance of primary cells to death ligands. Dysregulation of cFLIP by proinflammatory agents like Ni^{2+} causes misbalance in death ligand sensitivity and thereby may provide allergic reactions.

In summary the data led to the suggestion that death receptor-mediated apoptosis is dynamically regulated by activation of Caspase-8 at DISC. This regulation is achieved by modulation of cFLIP expression. High expression of cFLIP isoforms increases, while repression of cFLIP decreases resistance of tumor cells to death ligand-induced apoptosis. Therefore modulation of cFLIP, may serve as therapeutic approach to overcome apoptosis resistance in tumor therapies or treat allergic reactions.

3.2 cIAP2 is not sufficient to maintain resistance to TNF-induced apoptosis in NF-κB inhibited human keratinocytes

In addition to apoptotic signaling pathways, death receptors can also trigger non-apoptotic pathways like the NF-κB pathway. Activation of NF-κB can promote transcriptional upregulation of anti-apoptotic proteins such as cFLIP and cIAPs. Similar to cFLIP, cIAPs are also described as regulators of cell death by interaction with a variety of inducers and effectors of apoptotic cell death (Deveraux and Reed, 1999; Salvesen and Abrams, 2004; Vucic, 2008). Therefore the question should be answered: whether cIAPs are the crucial mediators of NF-κB-maintained resistance to TNF-induced apoptosis in human keratinocytes?

3.2.1 Constitutive inhibition of NF-κB mediates TNF-induced cell death in HaCaT cells

TNF activates NF-κB signaling pathways in human keratinocytes. This NF-κB activation may be relevant for resistance of keratinocyte to TNF-induced apoptosis. To test the impact of NF-κB activity in TNF resistance, HaCaT cells expressing either a dominant negative IKKβ-Kinase Death (KD) or constitutively active IKKβ-EE were generated (Diessenbacher et al., 2008). Western blot analysis confirmed expression of the respective molecules in transduced HaCaT cells (Fig. 12 A). Examination of cell death induction following stimulation with TRAIL and TNF demonstrated that the sensitivity to TRAIL was not modified by expression of IKKβ mutants. In contrast, resistance to TNF-mediated cell death is abolished in IKKβ-KD-expressing HaCaT cells. Overexpression of IKKβ-EE had no effect on the extent of TNF-induced cell death. (Fig. 12 B). These results demonstrate that TNF-mediated apoptosis is regulated by NF-κB in human keratinocytes.

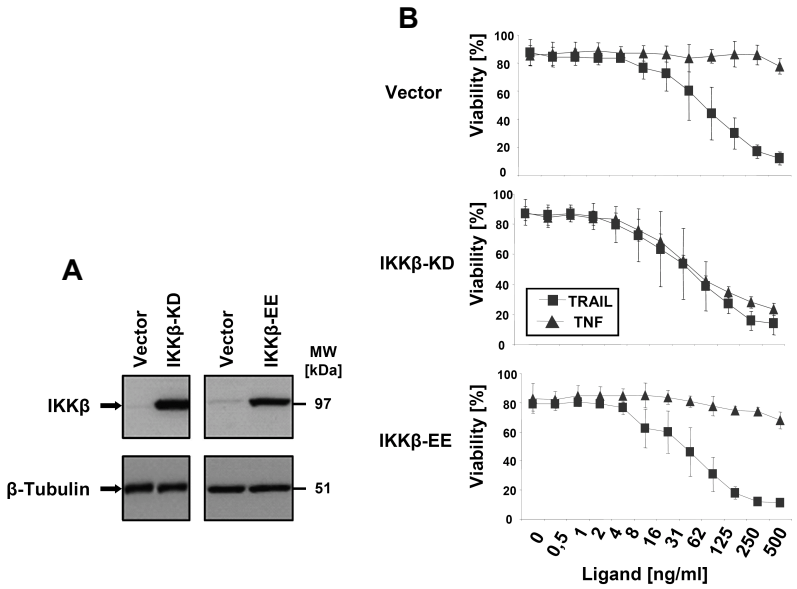


Figure 12: Overexpression of IKKβ-KD, but not IKKβ-EE leads to sensitization to TNF-mediated cell death in HaCaT cells. A) Overexpression of IKKβ mutants in HaCaT keratinocytes. Total cellular lysates of HaCaT cells expressing IKKβ-KD, IKKβ-EE or control vector were analyzed by western blotting for indicated molecules. **B**) Dose-dependent induction of TNF- or TRAIL-mediated cell death in IKKβ mutant-expressing HaCaT cells. Pools of infected cells were either left untreated or stimulated with increasing concentrations of TRAIL or TNF for 16-24 h. Viability was examined by crystal violet staining. Mean +/- SEM of a total of four independent experiments is shown. Data were kindly provided by Philip Diessenbacher.

3.2.2 NF-κB inhibition modulates expression of cIAP2, which lead to repressed cIAP2 recruitment in the TNF-induced signaling complex

Inhibition of NF-κB sensitizes human keratinocytes to TNF-induced apoptosis. It is widely accepted that NF-κB target genes are the crucial factors to maintain TNF resistance.

Therefore, TNF-dependent expression levels of potential NF-κB target genes were analyzed in HaCaT cells expressing control vector (pCF65) or IKKβ-KD in a kinetic manner by western blotting (Fig. 13 A).

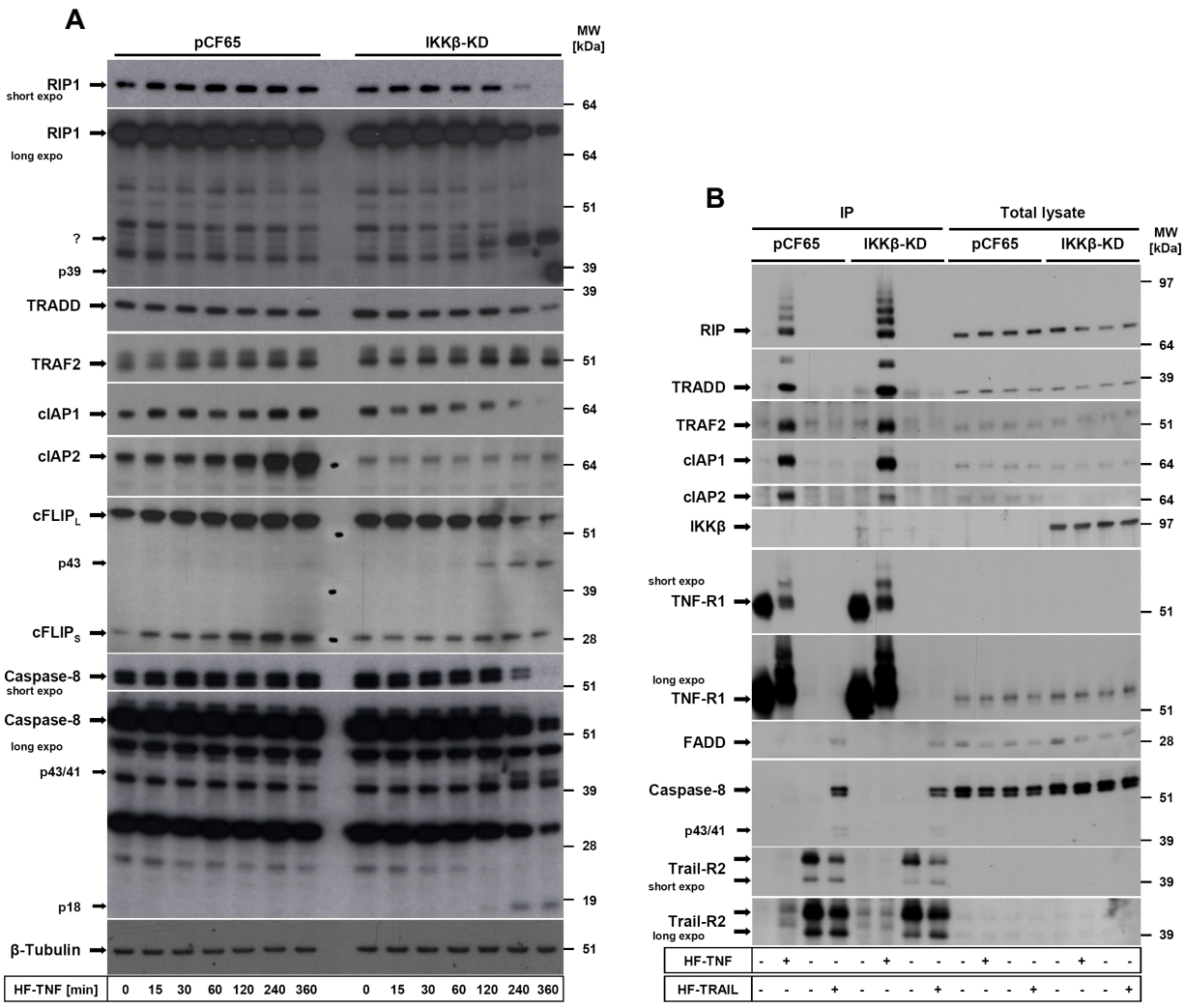


Figure 13: Analysis of cIAP2 modulation by IKKβ-KD and TNF in HaCaT cells. A) Modulation of cIAP2 expression and Caspase-8 activation in NF-κB- and TNF-dependent manner. Cells expressing IKKβ-KD or control vector were incubated for indicated times with 1 mg/ml HF-TNF and subsequently analyzed by Western blotting. **B**) TNF and TRAIL receptor signaling complexes in control or NF-κB-inhibited HaCaT. Control vector and IKKβ-KD expression HaCaT cells were stimulated with 2.5 mg/ml HF-TNF for 15 min or 2.5 mg/ml TRAIL for 30 min. Receptor complexes (IP) were precipitated via FLAG antibody as described in Materials and Methods. Precipitation of TNF and TRAIL receptors following lysis (-) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of total cellular lysates (Total lysate) or ligand affinity precipitates (IP) were subsequently analyzed by Western blotting for indicated molecules.

In control cells TNF stimulation led to a marked upregulation of cIAP2 and cFLIP$_S$, and a slight increase in cFLIP$_L$ expression. This was potentially caused by the TNF-mediated NF-κB activation. Interestingly, expression levels of none of the other presumptive NF-κB target genes, cIAP1 and TRAF2, were changed by TNF stimulation. Inhibition of NF-κB substantially repressed protein level of cIAP2 in IKKβ-KD expressing HaCaT. Furthermore, TNF was unable to induce upregulation of cIAP2, indicating that TNF is not able to

reconstitute cIAP2 expression in NF-κB inhibited cells. Expression levels of cIAP1 and TRAF2 were not affected by inhibition of NF-κB. In contrast to control cells, in IKKβ-KD HaCaT processing of Caspase-8 (p43/41 and p18), $cFLIP_L$ (p43) and RIP1 (p39 and an unknown cleavage fragment) were detected, indicating that NF-κB activity is required to suppress TNF-mediated induction of apoptosis. These in turn raised the question whether NF-κB activity also influences formation of death receptor complexes, which were required for apoptosis induction. Therefore HaCaT cells transduced with control vector (pCF65) or IKKβ-KD were stimulated with TNF and TRAIL followed by the isolation of the receptor complexes (Fig 13 B). The precipitates of TNF signaling complexes contained in control or NF-κB-inhibited HaCaT cells comparable amounts of TNF-R1, RIP1, TRADD, TRAF2, and cIAP1. RIP1, TRADD and TNF-R1 were thereby modified independent of NF-κB. In contrast, the amount of cIAP2 present in the TNF receptor complex of IKKβ-KD-expressing cells was drastically reduced. The known TRAIL DISC components like TRAIL-R2, FADD, and Caspase-8 were unchanged in the presence or absence of IKKβ-KD. TRAIL-induced cleavage of Caspase-8 in the DISC also was not influenced by the repression of NF-κB. These data supported the hypothesis that steady-state levels of cIAP2 are specifically regulated by NF-κB in HaCaT keratinocytes and suggested that the difference that determines NF-κB-mediated resistance to TNF might rely on the expression of cIAP2.

3.2.3 cIAP2 is not altering recruitment of components in the TNF-induced signaling platform

cIAP2 is an NF-κB target gene and is recruited to the TNF signaling complex. To understand the NF-κB-dependent function of cIAP2 for TNF signaling, TNF receptor complexes were precipitated in control and NF-κB-inhibited HaCaT cells under conditions when cIAP2 expression was induced (Fig. 14). Stimulation with 4-HT induced cIAP2 in control and IKKβ-KD expressing cells. RIP1, TRADD, and TRAF2 were found to be recruited to the TNF receptor complex irrespective of the presence or absence of cIAP2. The modifications of RIP1, TRADD and TNF-R1 were also not influenced by the induction of cIAP2. cIAP1 and cIAP2 were specifically recruited to the TNF signaling complex, and cIAP2 levels in the complex were increased in cells stimulated with 4-HT. This experiment could not reveal a critical function of cIAP2 in the formation of the TNF-induced membrane bound complex.

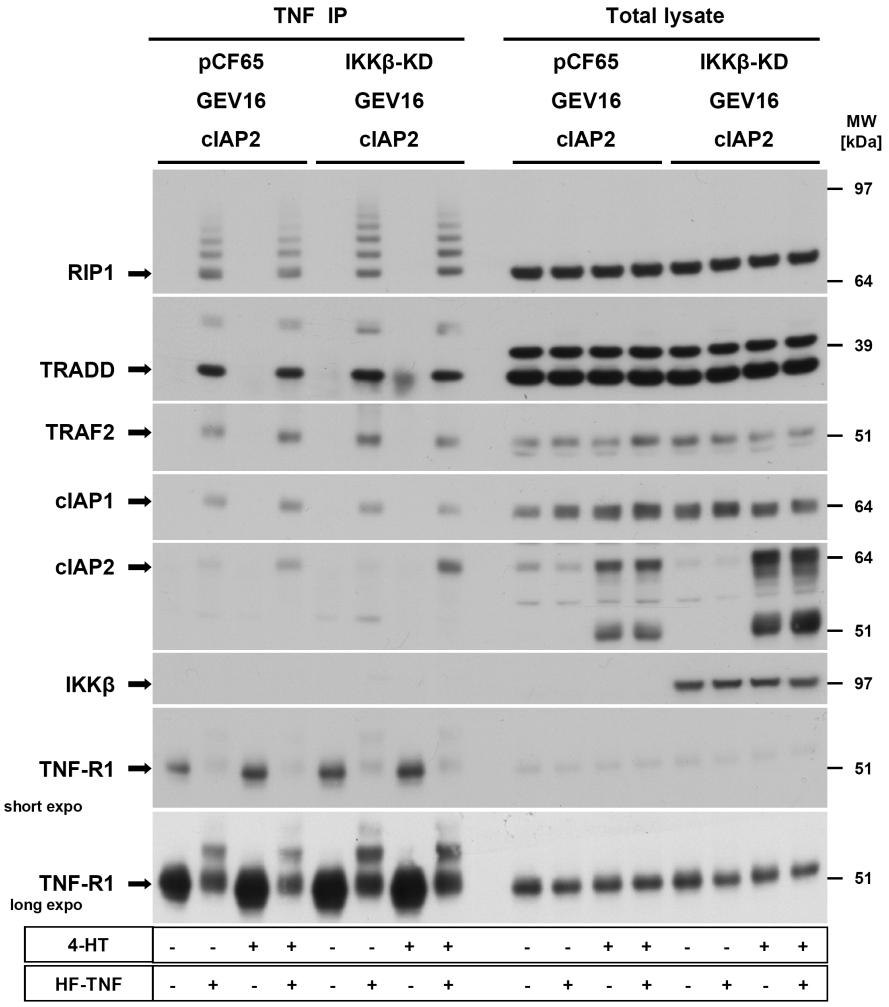


Figure 14: TNF receptor signaling complexes in control and NF-κB-inhibited HaCaT cells in presence and absence of cIAP2. HaCaT cells transduced with inducible lentiviral construct of cIAP2 together with GEV16 and IKKβ-KD or control vector were treated with 50 nM 4-HT or diluent alone for 4 h. Cells were subsequently stimulated with 2.5 mg/ml HF-TNF for 15 min. Receptor complexes were precipitated via FLAG antibody as described in Materials and Methods. Precipitation of TNF receptor following lysis (-) served as internal specificity control when compared with ligand affinity precipitates (TNF IP; +). Equal amounts of total cellular lysates (Total lysate) or ligand affinity precipitates (TNF IP) were subsequently analyzed by Western blotting for indicated molecules.

In summary, inhibition of NF-κB dramatically sensitized human keratinocytes to TNF- but not to TRAIL-induced cell death. The NF-κB target gene cIAP2 was thereby strongly repressed. However, sensitization to TNF-mediated cell death was largely independent of cIAP2.

3.3 cIAPs confer resistance to death ligand-mediated cell death

New evidence for the crucial function of cIAPs for regulation of death receptor-mediated cell death came from recent studies using IAP antagonists. These antagonists decreased cIAP levels and thereby sensitized various tumor cells to TRAIL or TNF (Bertrand et al., 2008; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al.,

2007). Following this, in frame of this study regulative functions of cIAPs in death receptor-mediated cell death in the skin were investigated using the IAP antagonist Compound A (CompA).

3.3.1 CompA induces rapid degradation of cIAPs in skin cells

CompA is a synthetic IAP antagonist designed to inhibit XIAP function. A recent study discovered that CompA induced rapid auto-ubiquitylation and proteasomal degradation of cIAP1 and thereby sensitized various cells to TNF-mediated cell death (Vince et al., 2007). Given these, it was an aim of this study to analyze: how do cIAPs regulate CD95-mediated cell death in skin tumor cells? To exclude a XIAP-dependent phenotype using CompA, several human keratinocyte and SCC cell lines were screened for XIAP expression. Primary keratinocytes, SCC25 and MET1 but not HaCaT and A5RT3 cells expressed XIAP (Fig. 15 A). Based on these results HaCaT, A5RT3 and MET1 cells were chosen for following characterization of CompA.

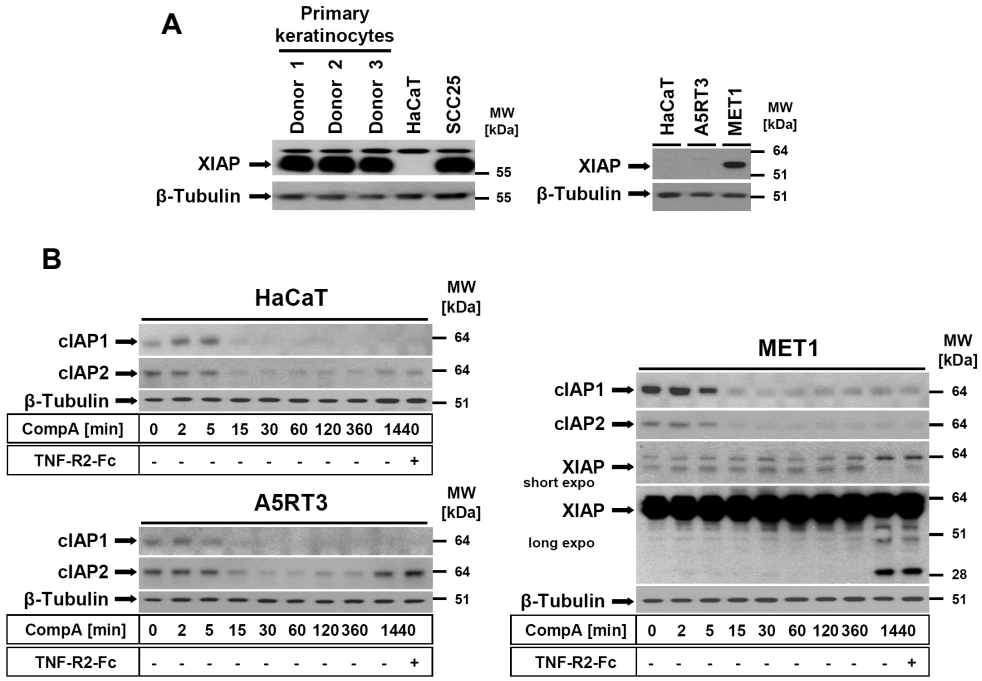


Figure 15: CompA induces rapid degradation of cIAPs. A) Cell type specific expression of XIAP. Protein expression of XIAP was analyzed by Western blotting of total cellular lysates from different donors of primary keratinocytes, HaCaT, SCC25, A5RT3 and MET1 cells. β-Tubulin served as internal control. Data were kindly provided by Philip Diessenbacher and Peter Geserick. **B**) CompA leads to down-regulation of cIAP1 and cIAP2 in HaCaT and SCC cells. For biochemical analysis, HaCaT, MET1 and A5RT3 cells were treated with CompA (100 nM) in presence or absence of TNF-R2-Fc (10 µg/ml) for indicated time points. Western blot analysis was performed for expressions of cIAP1, cIAP2, XIAP and β-Tubulin, which served as internal loading control. Data were generated in collaboration with Peter Geserick.

To analyze whether CompA repressed cIAP expressions HaCaT, A5RT3 and MET1 cells were treated with CompA in a kinetic manner. To exclude a TNF-dependent side effect, TNF-R2-Fc co-stimulation was used as control. As demonstrated a rapid degradation of cIAP1 was detected following CompA stimulation in all treated cells (Fig 15 B). In addition, less pronounced but significant degradation of cIAP2 was observed upon CompA treatment in these cells. Interestingly in HaCaT and A5RT3, but not in MET1 cells cIAP2 re-expression was observed after 24 h stimulation in presence of CompA. In contrast to both other cell lines, MET1 cells also expressed XIAP, which is blocked in function but not degraded by CompA. Cleavage of XIAP was detectable within 24 h of treatment with CompA. These results demonstrated that CompA efficiently repress cIAP1 and cIAP2 in HaCaT, MET1 and A5RT3 cells.

3.3.2 CompA sensitizes HaCaT and MET1 but not A5RT3 cells to CD95- and TRAIL-mediated cell death

Loss of cIAPs sensitized various cells to TNF- and TRAIL mediated cell death (Bertrand et al., 2008; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Vogler et al., 2009). In order to investigate the role of cIAPs in CD95-mediated cell death, the sensitivity of HaCaT, MET1 and A5RT3 cells were characterized in presence and absence of CompA. To exclude TNF-dependent cell death caused by CompA-induced TNF secretion, TNF-R2-Fc co-stimulation was used as a control. HaCaT and MET1, but not A5RT3 cells, were sensitized to TRAIL- or CD95L-mediated cell death after CompA stimulation independent of an autocrine TNF secretion loop (Fig. 16). Interestingly, in MET1 cells a basal sensitivity of ~20% to CompA alone was detectable. The fact that HaCaT cells did not express XIAP, confirmed the suggestion that the CompA-mediated sensitization to death ligands is cIAP-dependent. These data suggest that cIAPs are critical regulators of CD95L- and TRAIL-induced cell death in the skin.

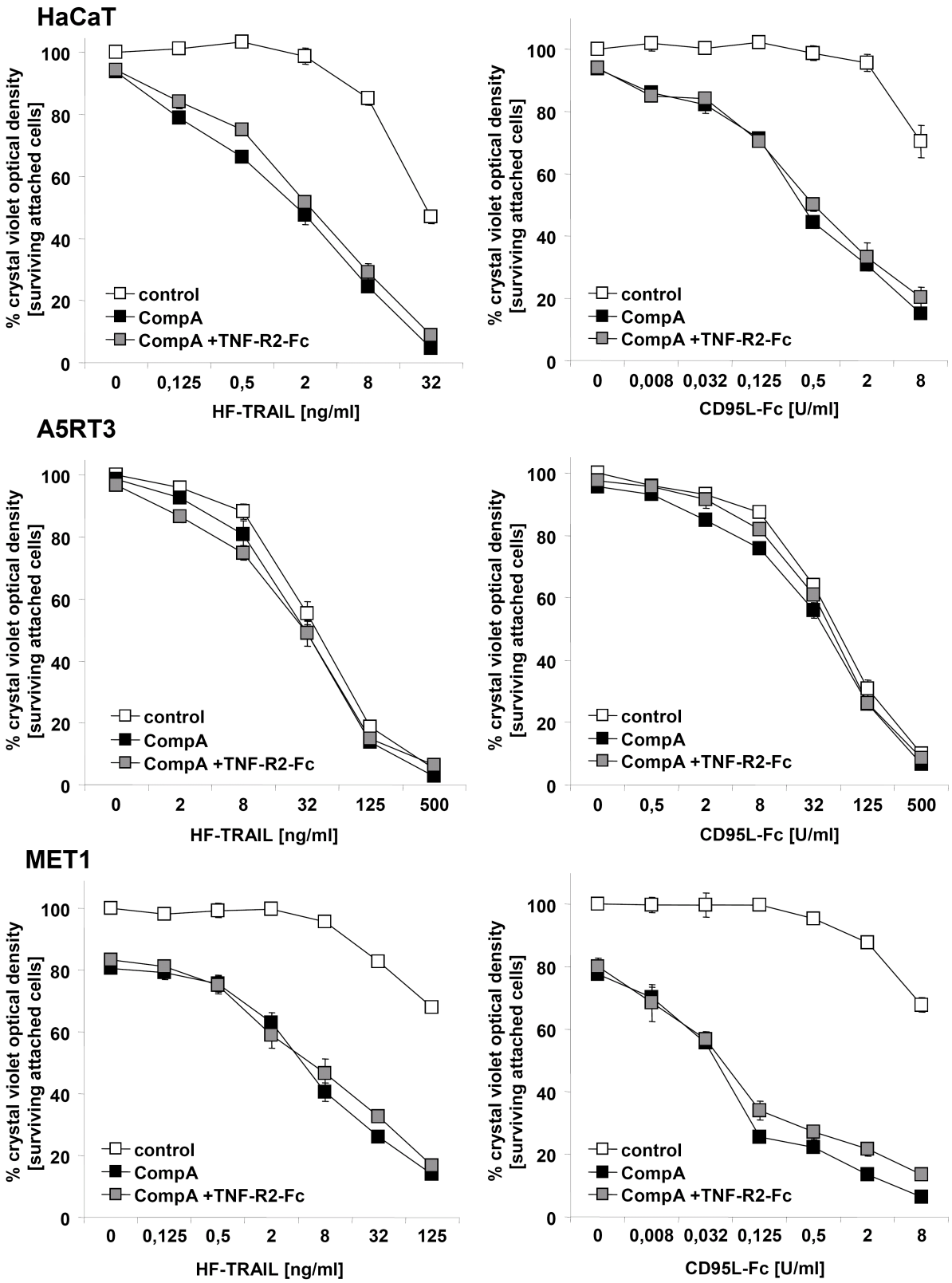


Figure 16: CompA sensitizes HaCaT and SCC cells to CD95- and TRAIL-mediated cell death independent of autocrine TNF secretion. HaCaT, MET1, or A5RT3 cells were either pre-treated with 100 nM CompA alone or in combination with 10 µg/ml TNF-R2-Fc for 30 min and then stimulated with indicated concentrations of HF-TRAIL or CD95L-Fc. Viability of cells was analyzed by crystal violet assay after 18-24 h as indicated in Material and Methods. Mean +/- SEM is shown of tree independent experiments.

3.3.3 cIAPs are negative regulators of CD95-induced cell death

To confirm, that cIAPs are critical regulators of death receptor-mediated cell death, endogenous expression levels of cIAPs were manipulated independent of CompA. Repression of cIAP levels by knockout or siRNA provided sensitization to TRAIL- and CD95L-induced cell death in mouse embryonic fibroblasts and HaCaT cells (Geserick et al., 2009). Given this it had to be clarified, whether the overexpression of cIAP1 and cIAP2 could be sufficient to protect keratinocytes from CD95L/CompA-induced cell death. Therefore HaCaT cells were lentiviral transduced to express GFP, Flag-tagged cIAP1 or cIPA2 in an inducible manner. The respective proteins were strongly inducible within 24 h. Overexpression of cIAP1, but not cIAP2, were fully degraded by treatment with CompA (Fig. 17 A).

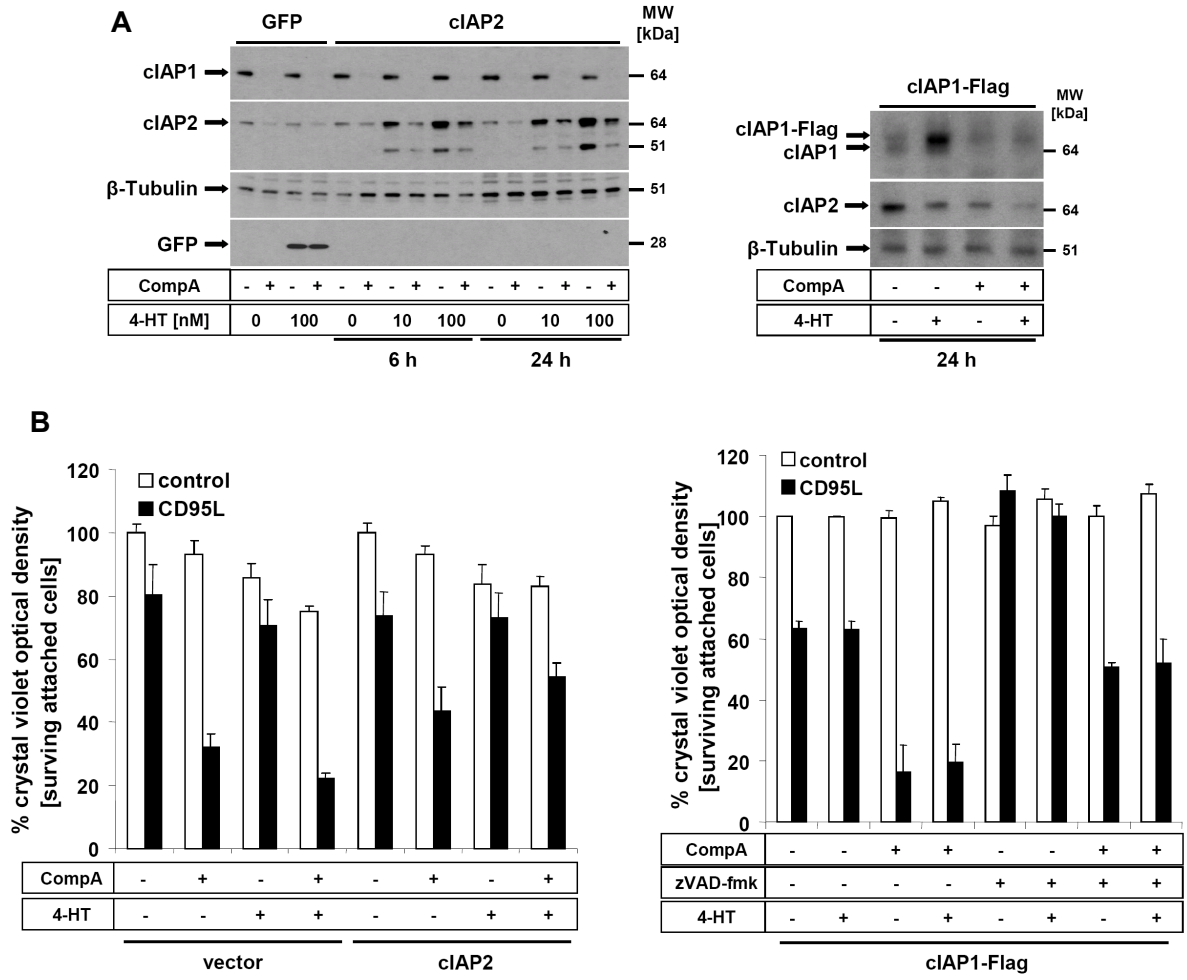


Figure 17: Induced cIAP2 but not cIAP1 expression partially protects from CompA/CD95L-induced cell death in HaCaT cells. A) HaCaT cells transduced with lentiviral control vector, inducible Flag-tagged cIAP1 or inducible cIAP2 were incubated with CompA and indicated concentrations of 4-HT for 6 or 24 h. Expression of induced molecules were subsequently analyzed by Western blotting. β-Tubulin served as internal control. B) Cells were induced with 100 nM 4-HT as indicated followed by treatment with 2.5 U/ml CD95L for 24 h in presence or absence of 100 nM CompA. Viability was examined by crystal violet assay after 24 h. Mean + SEM of three independent experiments is shown.

Inducible expression of cIAP1 did not alter sensitivity to CD95L nor did it protect against the CompA-mediated sensitization to CD95L death. In contrast, overexpression of cIAP2 conferred minor protection from CD95L-induced cell death in the presence of the CompA (Fig. 17 B). Taken together, these data support the hypothesis that cIAPs play an important role in limiting CD95L-mediated toxicity in both human and mouse cell lines.

3.3.4 CompA/death receptor-mediated cell death is neither entirely caspase-dependent nor caspase-independent

TRAIL-R- and CD95-mediated apoptosis is initiated by DISC-activated Caspase-8. To investigate whether in the presence of CompA death receptor-mediated cell death occurred in a caspase-dependent manner the broad spectrum caspase inhibitor zVAD-fmk was used. HaCaT cells were sensitive to TRAIL- and CD95L-induced cell death (Fig. 18). The sensitivity to death ligands was increased in the presence of CompA. zVAD-fmk completely blocked cell death when cells were stimulated with TRAIL or CD95L alone. In contrast TRAIL- or CD95L-mediated cell death in the presence of CompA was only partially blocked by zVAD-fmk. This indicated that in presence of CompA death receptors can induce a caspase-dependent as well as a caspase-independent form of cell death.

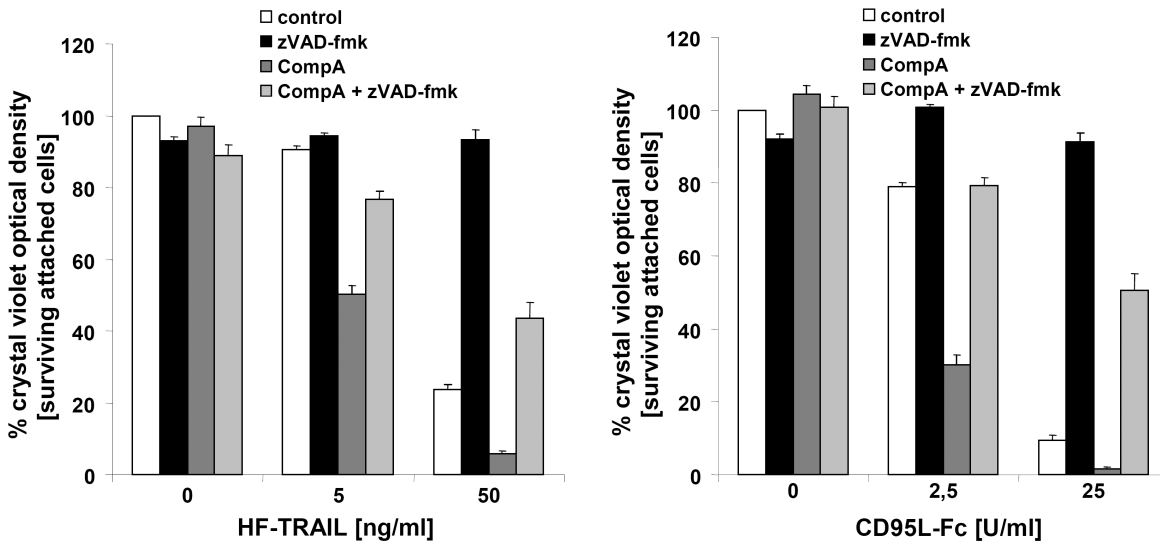


Figure 18: CompA sensitizes HaCaT cells to death ligand-mediated cell death in a caspase-dependent and caspase-independent manner. HaCaT cells were pre- or co-stimulated with zVAD-fmk (10 µM; 1 h) and CompA (100 nM, 30 min). Subsequently, cells were stimulated with indicated concentrations of TRAIL or CD95L. Viability of cells was analyzed by crystal violet assay after 18-24 h. Mean + SEM are shown for three (TRAIL) or six (CD95L) independent experiments.

3.3.5 cIAPs inhibit recruitment of RIP1 into the CD95 DISC and suppress formation of the receptor-independent complex II

CD95-induced apoptosis requires Caspase-8 activation in the DISC. Given this, the question arose whether cIAPs negatively regulate CD95-mediated cell death at the receptor complexes. To tackle this question DISC and receptor-independent complex II were isolated and analyzed in the presence and absence of CompA. To investigate the kinetic formation of both signaling platforms, HaCaT cells were stimulated with CD95L, CompA and/or zVAD-fmk at different time points. As shwon in Fig. 19 zVAD-fmk stabilized thereby both the DISC and the complex II. Stimulation of CD95 led to the formation of SDS-and β-mercaptoethanol-insoluble CD95-based complexes of high molecular weight. The recruitment of FADD, cFLIP, Caspase-8 and RIP1 into the DISC were detected following CD95L stimulation.

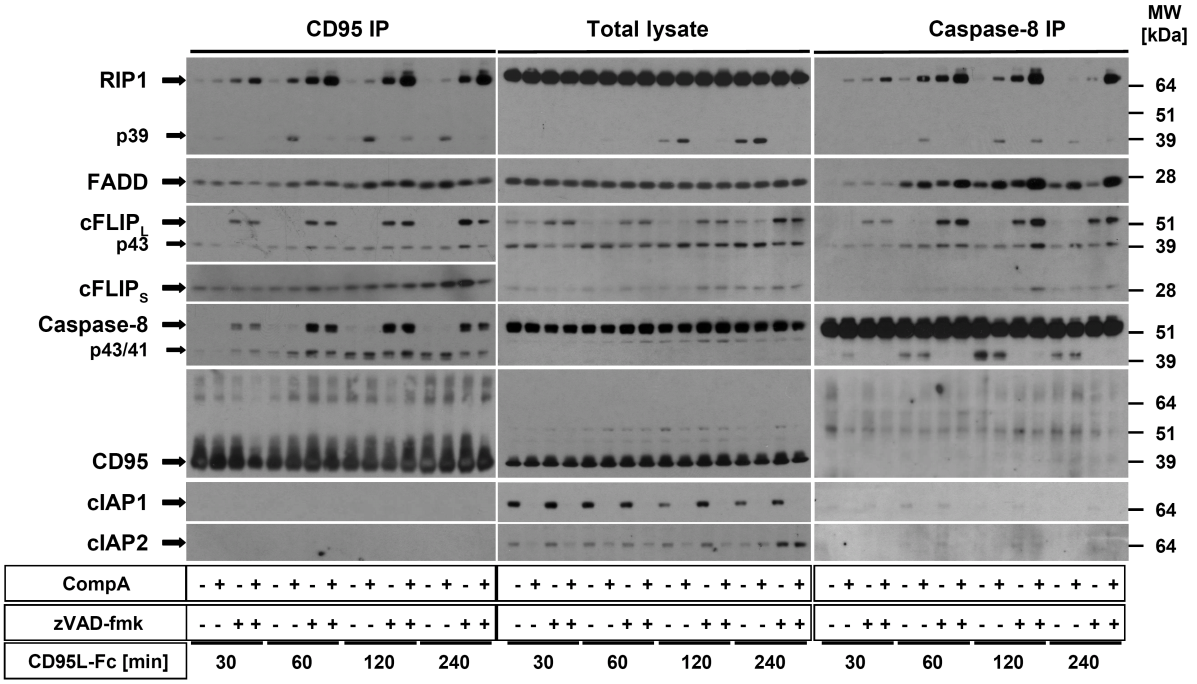


Figure 19: Induction of the ligand-induced receptor bound CD95 complex (DISC) and the intracellular Caspase-8-containing complex (complex II) in presence or absence of CompA. The CD95-DISC was precipitated from parental HaCaT cells either pre-stimulated with CompA (100 nM, 1 h) or in combination with zVAD-fmk (10 µM, 1 h) and subsequently stimulated with CD95L-Fc (250 U/ml) for the indicated times. The CD95-DISC (left panel) was precipitated using ligand affinity precipitation indicated in Materials and Methods. Equal amounts of the CD95-IP or the complex II were subsequently analyzed by Western blotting for indicated molecules. Equal amounts of total cellular lysates were assayed in parallel to investigate input for the assays.

RIP1 recruitment was strongly increased upon treatment with CompA. The qualitative recruitment of RIP1 was almost identical whether cells were treated with zVAD-fmk or not, except that RIP1 was cleaved in the absence of caspase inhibitor. The complex II contained FADD, RIP1, cFLIP and Caspase-8, but in contrast to the DISC no CD95 receptor. RIP1, FADD and cFLIP associations to Caspase-8 were increased upon treatment with CompA.

Interestingly, both the DISC recruitment and the complex II formation increased over the first hour and then remained at a relatively steady level during the entire experimental time frame. These data demonstrated that CompA treatment led to an increased recruitment of RIP1 into the DISC and the complex II and suggested an essential role for both cIAPs and RIP1 in death receptor-mediated cell death in HaCaT cells.

3.3.6 Loss of cIAPs intensifies accumulation of RIP1 in CD95-induced complexes also in non-sensitized A5RT3 cells

To further investigate how CompA facilitated CD95-mediated cell death in HaCaT and MET1 but not in A5RT3 cells, the DISC and the receptor-independent complex II were examined (Fig. 20).

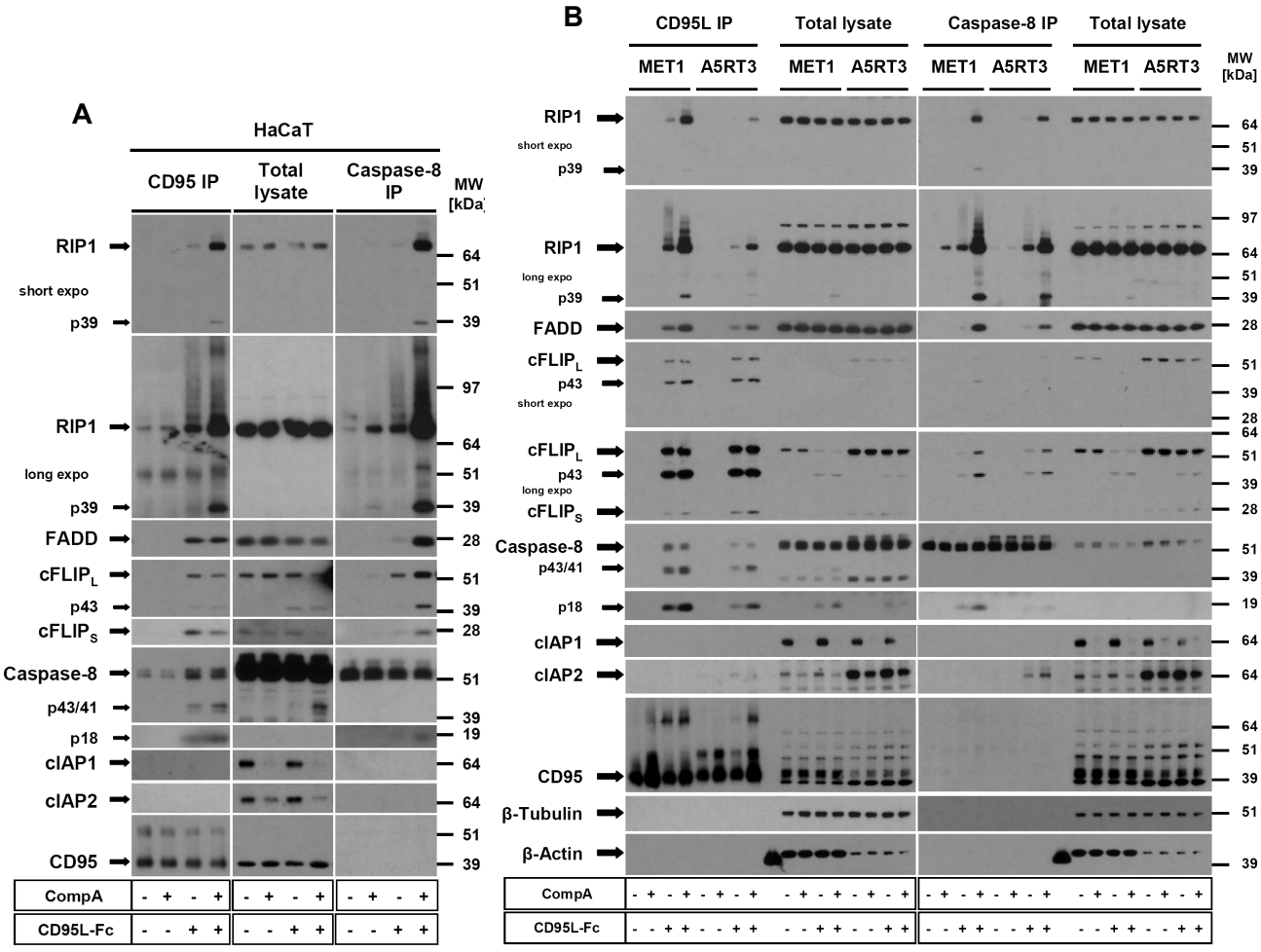


Figure 20: Induction of CD95L-induced DISC and intracellular complex II in presence or absence of CompA. The CD95 DISC was precipitated from HaCaT, MET1 or A5RT3 cells pre-incubated with 10 µM zVAD-fmk and 100 nM CompA for 1 h and subsequently treated with 250 U/ml CD95L-Fc for 2 h. The CD95-induced complexes were precipitated as described in Materials and Methods. Precipitation of receptor complexes after lysis (-) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of the DISC (CD95L IP) or the complex II (Caspase-8 IP) were subsequently analyzed by Western blotting. Equal amounts of total cellular lysates of MET1 and A5RT3 cells were loaded on the same gels to allow comparison of signal strength between CD95L-IP, complex II, and total cellular lysates.

Recruitments of FADD, cFLIP, Caspase-8, and RIP1 after CD95L stimulation were detected in the DISC as well as the complex II in all three cell types. Stimulation with CompA led to enrichment of Caspase-8 cleavage fragment p18 in the DISC. Given the differential sensitivity, recruitment of FADD, FLIP, and Caspase-8 to the DISC was remarkably similar in MET1 and A5RT3 cells, either in presence or absence of CompA. In contrast, RIP1 recruitment was dramatically increased in the CD95 DISC of MET1 in absence of cIAPs, whereas RIP1 recruitment was significant weaker in A5RT3 cells, although still increased by CompA treatment. Examination of the complex II, revealed a similar stimulation-dependent interaction of FADD, cFLIP, and RIP1 with Caspase-8 and substantial increased complex II formation in absence of cIAPs compared with CD95L-treated cells alone. These data suggested that loss of cIAPs increased the DISC recruitment of RIP1 or repressed RIP1 degradation, which translated to an increased level of RIP1 in the complex II. Resistance of A5RT3 cells for CompA-mediated sensitization to death ligands could potentially depend on decreased RIP1 recruitment into the DISC and the complex II cells or higher expression of cIAP2, compared to MET1 cells.

3.4 RIP1 is a key mediator for non-apoptotic cell death in absence of cIAPs in the skin

RIP1 is supposed to be critical for CD95-induced necrosis (Degterev et al., 2005; Holler et al., 2000). In addition, a more recent study suggests that RIP1 can interact with Caspase-8 and thereby activate CD95-mediated death (Morgan et al., 2009). Given this, it was of particular interest to study the role of RIP1 in CD95-mediated signaling pathways in human keratinocytes.

3.4.1 RIP1 is critical for CompA-mediated sensitization to CD95-induced cell death in HaCaT cells

cIAPs inhibit death receptor-mediated cell death and are required for ubiquitylation of RIP1 in the TNF-R1 pathway (Bertrand et al., 2008). Since RIP1 recruitment to CD95-DISC was highly upregulated by loss of cIAPs, the question arose whether RIP1 was essential for CompA-dependent sensitization to death receptor-mediated cell death. To tackle this question, HaCaT cells with decreased levels of RIP1 were generated using stable short hairpin RNA (shRNA) expression (Fig. 21 A) and subsequently tested for sensitivity to TRAIL and CD95L-induced cell death in presence and absence of CompA. While control cells were sensitized to TRAIL and CD95L-mediated cell death by CompA, RIP1 knock-down cells were substantially more resistant to these death ligands in presence of CompA (Fig. 21 B). These

observations indicated that RIP1 is a crucial molecule for CompA-dependent sensitization to death receptor-mediated cell death.

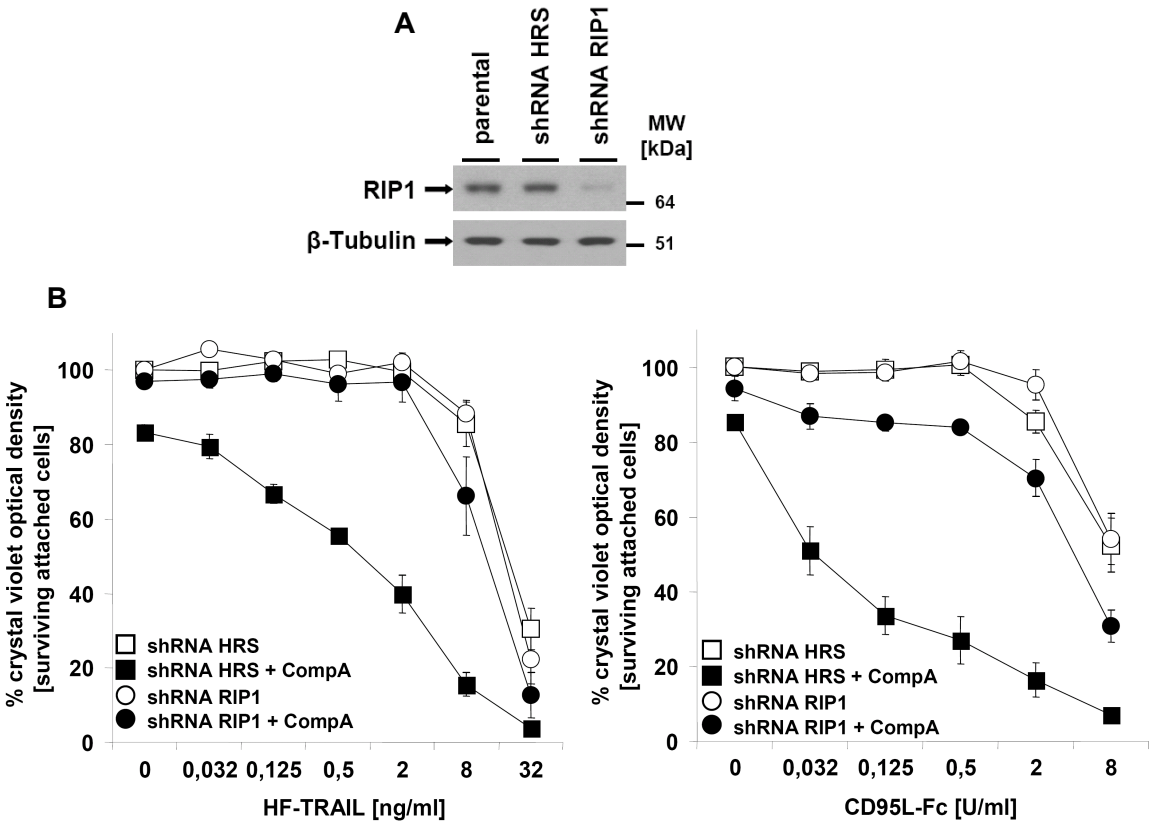


Figure 21: Stable knockdown of RIP1 protects HaCaT cells from CompA-mediated sensitization to death ligand-induced cell death. A) HaCaT cells were retrovirally transduced with either hyper random sequence shRNA (HRS) or RIP1-specific-shRNA and selected for 3 days with puromycin (3 µg/ml). Knockdown efficiency of RIP1 was controlled by Western blot analysis for RIP1. Reprobing of the membrane with antibody to β-Tubulin serves as an internal control for protein loading. **B**) Transduced HaCaT cells as shown in (A) were pre-stimulated for 30 min with 100 nM CompA or diluents alone and subsequently stimulated with indicated concentrations of HF-TRAIL or CD95L-Fc for 18-24 h and assayed by crystal violet assay. +/- SEM of three (TRAIL) or four (CD95L) independent experiments are shown.

3.4.2 RIP1 kinase activity modulates a caspase-independent form of cell death in absence of cIAPs

The essential role of the kinase activity of RIP1 in necrotic pathways has recently been discovered (Degterev et al., 2008, Hitomi et al., 2008). To determine whether the kinase activity of RIP1 was required for CompA-mediated sensitization to death receptor-induced cell death, HaCaT cells were treated with the RIP1 kinase inhibitor Necrostatin-1. While Necrostatin-1 alone was unable to protect cells from death ligand and CompA, co-treatment of Necrostatin-1 and zVAD-fmk resulted in complete protection from cell death (Fig. 22). These data indicated that in absence of cIAPs, a caspase-dependent and a latent RIP1 kinase-dependent cell death pathway can proceed in keratinocytes. To fully block both

types of cell death, inhibition of both caspases and RIP1 kinase activity together is necessary.

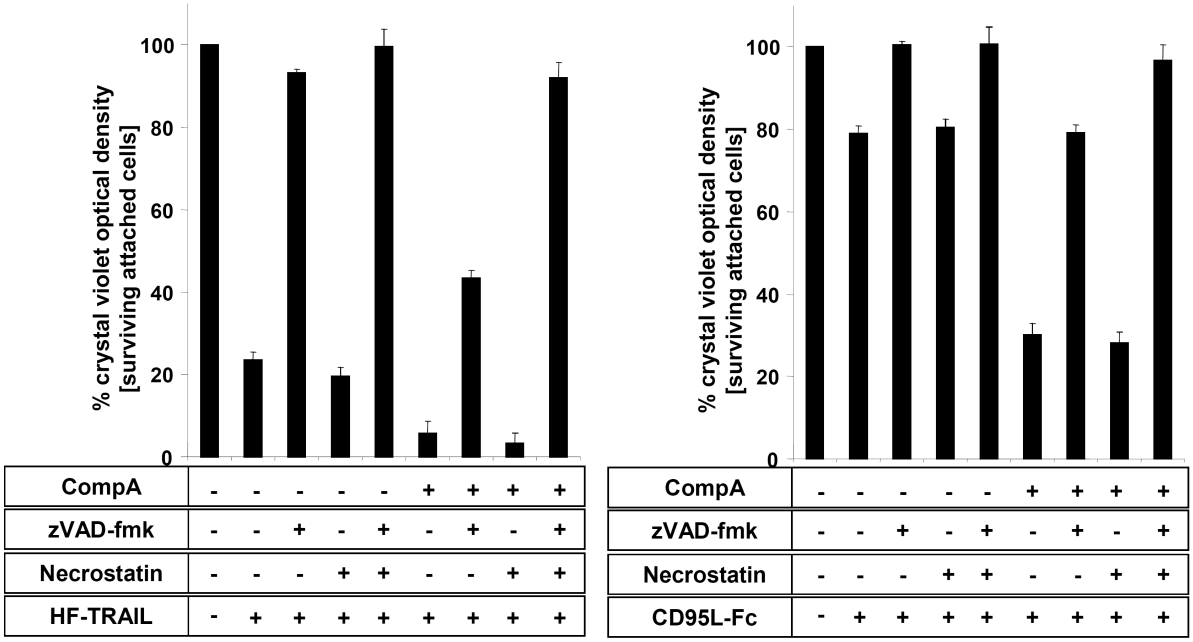


Figure 22: Combined inhibition of caspase and RIP1 kinase activity protects HaCaT cells from TRAIL- and CD95L-mediated cell death in absence of cIAPs. HaCaT cells were either separately pre-stimulated or co-stimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 100 nM CompA for 30 min, followed by stimulation with 50 ng/ml HF-TRAIL or 2.5 U/ml CD95L-Fc for 18-24 h and subsequent crystal violet assay. Mean + SEM of three (TRAIL) or six (CD95L) independent experiments is shown.

3.4.3 RIP1 kinase activity facilitates formation of the complex II in absence of cIAPs

RIP1 and its kinase activity are critical for CD95-induced non-apoptotic cell death in presence of CompA (Fig. 22). Additionally, RIP1 is increasingly recruited to the DISC and the complex II upon CompA stimulation (Fig. 19 and 20). This made the particular aspects of the relationship of signaling platforms and RIP1 kinase activity interesting to investigate. Therefore the DISC and the complex II formations of parental HaCaT cells were analyzed in presence of zVAD-fmk, CompA, Necrostatin-1, and upon various combinations of these agents (Fig. 23). Due to known stabilization of the DISC and the complex II by zVAD-fmk, comparisons should be made between similarly treated samples. Comparable levels of RIP1 were retained in the DISC in presence or absence of Necrostatin-1 (Fig. 23, compare lane 12 with lane 16 for the CD95 IP, RIP1 vs. FADD). Comparison of lane 8 with lane 14 for the CD95 DISC and lane 25 with lane 31 for the complex II indicated that Necrostatin-1 did not detectably change RIP1 association with the CD95 DISC, if DISC-associated cleavage of RIP1 was taken into account. In contrast, level of RIP1 in the complex II was decreased by

Necrostatin-1 (Fig. 23, compare lane 29 with 33, low exposure RIP1). Associations of cFLIP and Caspase-8 with receptor complexes were independent of RIP1 kinase activity. These data suggested that RIP1 kinase activity contributes to the maturation and/or translocation of a RIP1-containing DISC into a RIP1-containing complex II.

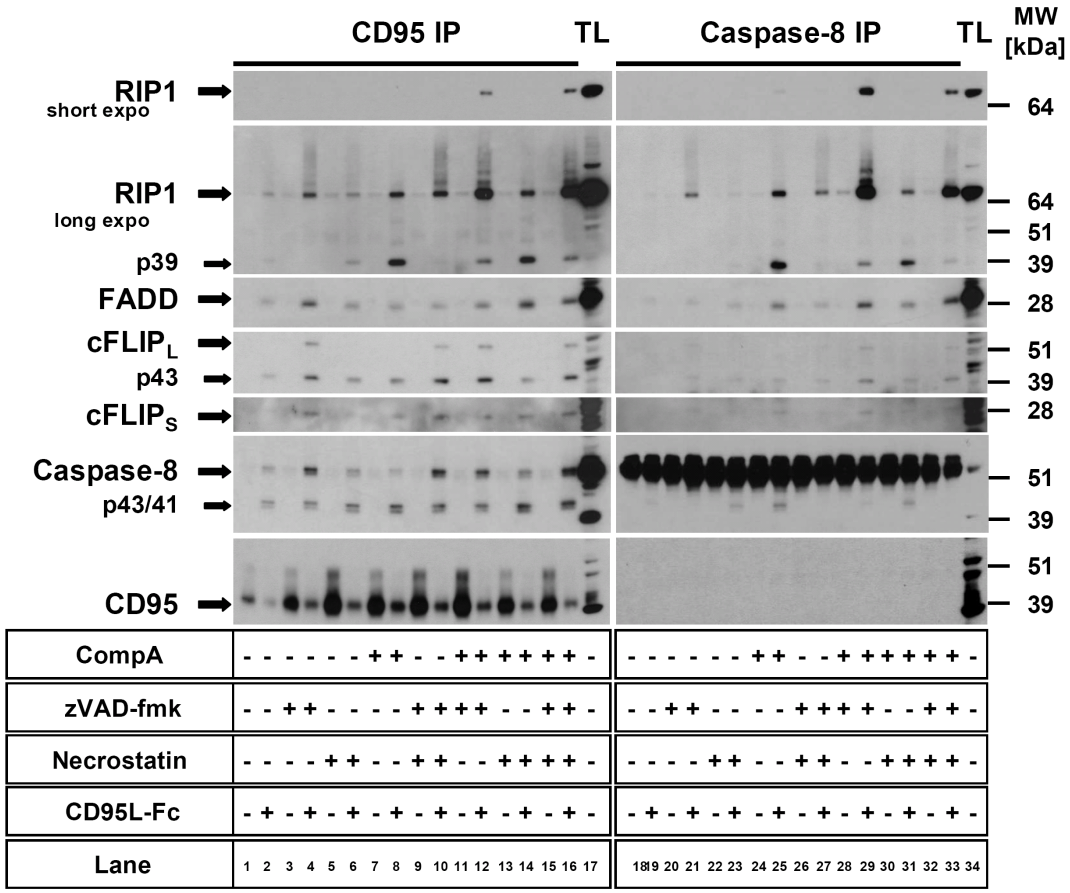


Figure 23: RIP1 kinase activity-dependent DISC and complex II formation in HaCaT cells. Cells were either pre- or co-stimulated with 10 µM zVAD-fmk, 50 µM Necrostatin-1, and 100 nM CompA for 1 h and subsequently stimulated with 250 U/ml CD95L-Fc for 2 h. The CD95 DISC (left) or the complex II (right) were precipitated as described in Materials and Methods. Equal amounts of the DISC (CD95L IP) or Caspase-8-interacting proteins (complex II) were subsequently analyzed by Western blotting.

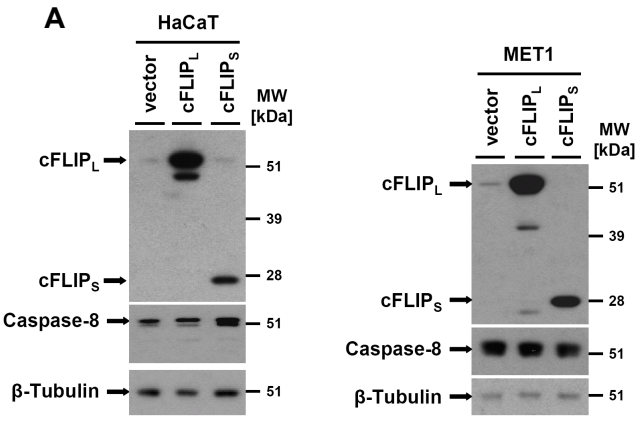
3.5 cFLIP$_L$ but not cFLIP$_S$ confers resistance to death ligand-induced cell death in absence of cIAPs

In this study it is demonstrated that cFLIP isoforms interfere with Caspase-8 activation in the DISC and thereby blocked CD95-induced apoptosis (see 3.1). It is also shown that cIAPs confrere resistance to CD95-mediated cell death by inhibition of RIP1 recruitment into the DISC and the complex II. They interfered thereby with a RIP1-dependent induction of caspase-dependent as well as a caspase-independent cell death upon death ligand stimulation (see 3.3). Therefore the question was raised, whether cFLIP isoforms can

influence cIAP-dependent CD95 signaling pathways? It was hypothesized that cFLIP isoforms partially confer resistance to CompA-mediated sensitization of keratinocytes to CD95L.

3.5.1 cFLIP isoforms differentially contribute to resistance to CD95-mediated cell death in absence of cIAPs

To answer the question whether cFLIP antagonized CompA-sensitization, HaCaT and MET1 cell lines expressing different cFLIP isoforms were generated (Fig. 24 A). Interestingly, the sensitivity to CompA alone was increased in cells overexpressing $cFLIP_S$ but not $cFLIP_L$. Furthermore, $cFLIP_S$ overexpression was not sufficient to protect HaCaT and MET1 cells from CD95L-induced cell death in absence of cIAPs. Blocking of caspase activity by addition of zVAD-fmk failed to protect cells from death. However, Necrostatin-1 treatment prevented CD95L-mediated cell death in cells overexpressing $cFLIP_S$. In contrast, $cFLIP_L$ was highly effective in blocking CD95L-induced death in CompA stimulated HaCaT and MET1 cells (Fig. 24 B). These experiments showed that $cFLIP_L$ and $cFLIP_S$ differentially regulated cell death pathways in absence of cIAPs in a previously unsuspected manner. $cFLIP_L$ protected HaCaT and MET1 cells from caspase-dependent as well as caspase-independent CD95L/CompA-induced cell death. In contrast, $cFLIP_S$ failed to block the caspase-independent form. Additionally, loss of cIAPs alone was sufficient to induce cell death in $cFLIP_S$ expressing cells. These data suggested an essential role of the caspase-like domain of $cFLIP_L$ in limiting caspase-independent cell death.



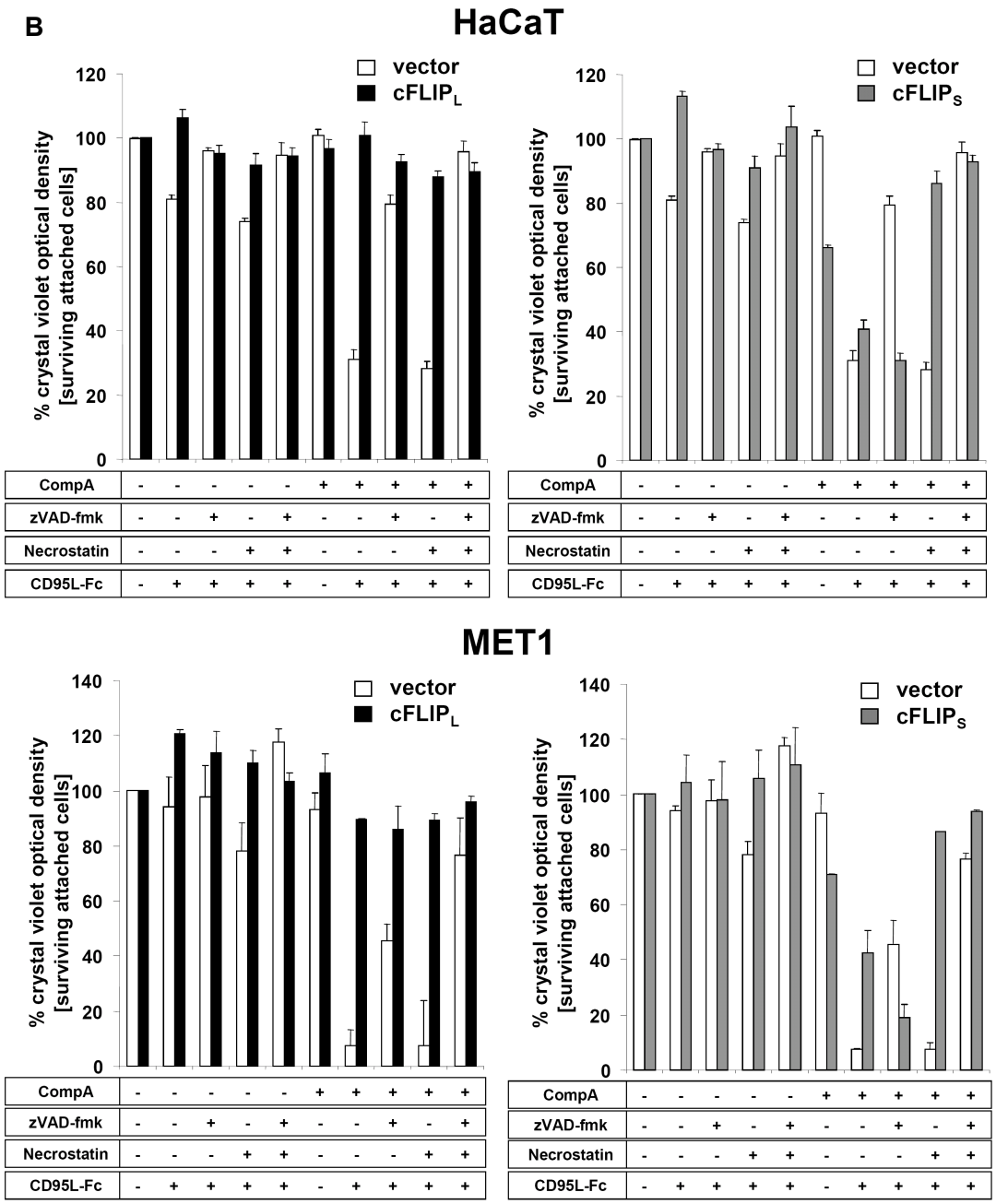


Figure 24: cFLIP$_L$ but not cFLIP$_S$ blocks CD95L/CompA-induced cell death in HaCaT and MET1 cells. A) HaCaT and MET1 cells were transduced with control vector, cFLIP$_L$ or cFLIP$_S$. Total cellular lysates were analyzed for cFLIP, Caspase-8, and β-Tubulin by Western blotting. **B**) HaCaT and MET1 Cells were pre-stimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 100 nM CompA for 30 min, or diluent alone. Subsequently, cells were stimulated with 2.5 U/ml CD95L. Viability of cells was analyzed by crystal violet. Mean + SEM of three independent experiments is shown. Data were generated in collaboration with Peter Geserick.

3.5.2 cFLIP$_L$ but not cFLIP$_S$ represses CD95-induced recruitment of RIP1 in the DISC and the complex II in absence of cIAPs

Both cFLIP isoforms were recruited into and provided their functions at the signaling platforms. To elucidate the molecular mechanism of the cFLIP isoform phenomenon in

absence of cIAPs, the CD95 DISC and the complex II were precipitated from HaCaT cells expressing control vector, $cFLIP_L$, or $cFLIP_S$ (Fig. 25).

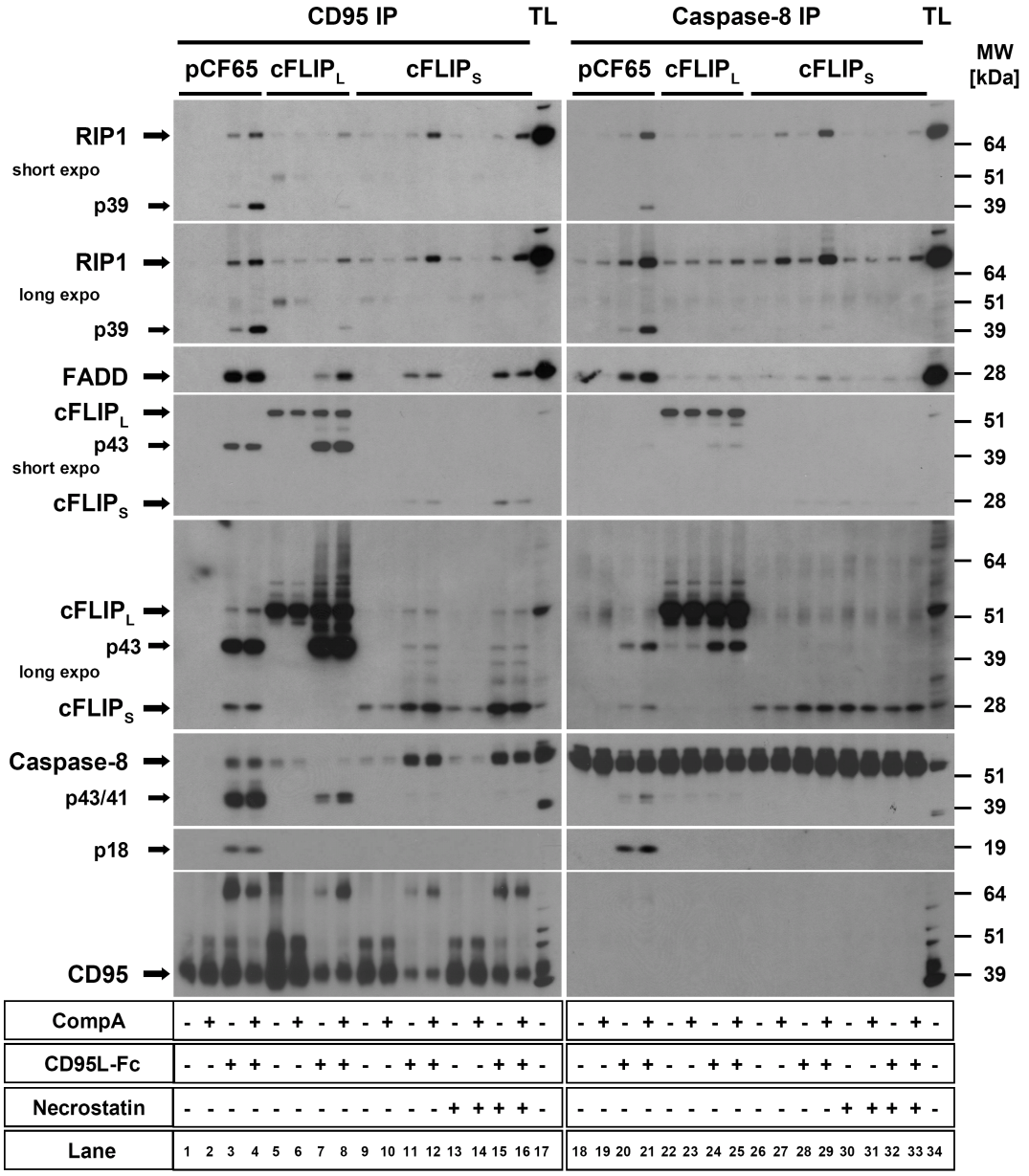


Figure 25: $cFLIP_L$, but not $cFLIP_S$ blocks formation of the complex II in presence or absence of CompA. Transduced HaCaT cells were either pre-stimulated with 50 µM Necrostatin-1 or diluent for 1 h as indicated and afterwards treated with 250 U/ml CD95L-Fc for 2 h. Subsequently, the CD95L DISC (left) was precipitated using ligand affinity precipitation as described in Materials and Methods. Precipitation of receptor complexes after lysis (-) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of the DISC (CD95L IP) or the complex II (Caspase-8 IP) were subsequently analyzed by Western blotting for indicated molecules. Equal amounts of total cellular lysates (TL) were loaded on the same gels to allow comparison of signal strength between IP and total cellular lysates.

In absence of cIAPs, significant increased RIP1 levels were detected in the CD95 DISC of control and $cFLIP_S$ overexpressing cells (Fig. 25 lanes 4, 12 and 16). The fact that RIP1 was cleaved in control cells only suggested a caspase dependency, which is blocked by $cFLIP_S$

(Fig. 25 lanes 3 and 4). $cFLIP_L$ repressed the recruitment of RIP1 in the CD95 DISC. Caspase-8 and cFLIP were recruited and cleaved as shown in R12, R13 and R16. Enrichment of the cleaved Caspase-8 fragment p18 in the DISC of control vector transduced cells (Fig. 25 lanes 3 and 4) suggested a pronounced caspase-dependent cell death in parental HaCaT. In the complex II, increased amounts of RIP1, FADD and p43 cFLIP were detected in control cells upon degradation of cIAPs (Fig. 25 lane 21). In contrast, $cFLIP_L$ inhibited the formation of the complex II (Fig. 25 lanes 22-25). Interestingly, CD95L stimulation of $cFLIP_S$ overexpressing cells led to increased receptor-independent RIP1/Caspase-8 interaction in absence of cIAPs (Fig. 25 lane 29). Even in absence of CD95L stimulation increased RIP1/Caspase-8 interaction was observed following CompA treatment (Fig. 25 lane 27). In contrast to control cells, FADD was not as strong enriched in the complex II of $cFLIP_S$ cells, suggesting a possibility of a death receptor stimulation-independent formation of the complex II. Inhibition of RIP1 kinase activity with Necrostatin-1 blocked association of RIP1 and Caspase-8 and decreased thereby the complex II formation (Fig. 25 lanes 30-33). cIAPs limited RIP1 recruitment into the DISC and maturation of the RIP1-containing complex II. In absence of cIAPs, $cFLIP_L$, but not $cFLIP_S$, was able to block this increased recruitment and prevent cell death. In absence of cIAPs, overexpressed $cFLIP_L$ was able to block this increased recruitment and prevented cell death, whereas overexpressed $cFLIP_S$ was not. $cFLIP_S$ completely blocked Caspase-8 activation and caspase activity in the DISC but was nevertheless insufficient to block CD95L/CompA-induced cell death. These results point to a critical role of the caspase-like domain of $cFLIP_L$ in limiting RIP1 in the CD95 DISC and thereby inhibiting formation and/or translocation of the complex II.

The results of this study indicated the essential roles of cFLIP, cIAPs and RIP1 in regulation of death receptor-induced signaling pathways.

4 Discussion

4.1 cFLIP is a central regulator of death receptor-induced Caspase-8 activation

Apoptosis is a critical mechanism for cell homeostasis and can occur via several pathways. It plays an important role in many physiological processes, including development and maintaining of proper functioning of immune system (Gerl and Vaux, 2005; Krammer, 2000). Dysregulation of apoptosis is an important pathogenic mechanism and can cause AIDS, autoimmunity or cancer (Krammer, 2000). Thus, understanding of regulation of death receptor signaling pathways, like apoptosis, is of essential importance. It is suggested that balance of pro- and anti-apoptotic proteins is critical for death ligand resistance of normal and tumor cells (Karst and Li, 2007; Meng et al., 2006). Malignant melanoma is a highly aggressive cancer with increasing incidence worldwide. Its capacity to metastasize at early stages and its resistance to chemotherapeutic agents results in overall poor prognosis of melanoma patients once metastasis has occurred (Miller and Mihm, 2006). Similar to other cancer cell types, melanoma cells acquire apoptosis resistance during tumor progression. This apoptosis resistance can be mediated by anti-apoptotic molecules like cFLIP. In melanoma cells, the relevance of cFLIP is controversially discussed (Griffith et al., 1998; Nguyen et al., 2001; Zhang et al., 1999). Given these, a further characterization of the function of cFLIP in death receptor mediated signaling pathways of melanoma cells was necessary. $cFLIP_L$ and $cFLIP_S$ were revealed as crucial negative regulators of death receptor-mediated apoptosis in human melanoma cells (Geserick et al., 2008). While upregulation of cFLIP isoforms mediates resistance to CD95L and TRAIL-induced death, repression of cFLIP is sufficient to overcome TRAIL or CD95 resistance in a subset of melanoma cells. This study demonstrated that cFLIP isoforms block apoptosis induction by interference with Caspase-8 activation on the DISC in melanoma cells.

In addition, treatment of primary endothelial cells with the contact allergen nickel repressed levels of cFLIP and thereby decreased their resistance to TRAIL-induced cell death (Schmidt et al., 2009). These suggested that cFLIP is also an important anti-apoptotic key factor for death receptor-induced apoptosis in HUVEC cells. The bivalent cation nickel is a widely distributed noxious agent and an inducer of contact allergy reactions (Hostynek, 2006). Interestingly, Ni^{2+} increased surface expression of both TRAIL-R1 and TRAIL-R2, whereas TRAIL-R3 levels remained unchanged and TRAIL-R4 expression was strongly induced in endothelial cells. Early studies suggested that TRAIL resistance can be regulated by expression of TRAIL receptors (Kimberley and Screaton, 2004). TRAIL-R1 and TRAIL-R2 contain functional death domains and therefore induce cell death. In contrast, TRAIL-R3 and

TRAIL-R4 lack functional death domains, which may counteract apoptosis-inducing capacity of TRAIL. Current models suggest for TRAIL-R4 either co-recruitment with TRAIL-R2 (Merino et al., 2006) or direct binding of TRAIL-R4 to TRAIL-R2 in a ligand-independent manner (Clancy et al., 2005). The overall anti-apoptotic function of TRAIL-R4 is undisputed nowadays. Due to this, nickel-mediated induction of TRAIL-R4 expression cannot be reason for decreased endothelial cell resistance to TRAIL-induced apoptosis. TRAIL-R1/TRAILR2 modulation alone is suggested to not be responsible for nickel-dependent sensitization to TRAIL-induced apoptosis in primary endothelial cells (Schmidt et al., 2009). This hypothesis is supported by previous studies with pharmacological agents, which lead to sensitization to TRAIL-mediated apoptosis independent of TRAIL-R2 upregulation in different tumor cells (Inoue et al., 2004; Inoue et al., 2006; Koschny et al., 2007). Observations that nickel incubation decreased cFLIP levels raised the question whether modulation of cFLIP is responsible for Ni^{2+}-induced sensitization to TRAIL-induced apoptosis in human endothelia cells. In absence of TRAIL-R stimulation nickel-induced repression of cFLIP has no consequence for viability of HUVEC. Low amount of remaining cFLIP proteins seems to be sufficient to counteract basal TRAIL-R activity (Schmidt et al., 2009). It is suggested that high affinity of cFLIP isoforms for DISC recruitment is enough to inhibit Caspase-8 activation at the receptor complex under these conditions (Kataoka, 2005; Leverkus et al., 2003). Contribution of this study was to analyze TRAIL-dependent activation of Caspase-8 in context of cFLIP repression in primary human endothelial cells. Upon TRAIL-R stimulation cFLIP is increasingly recruited in the DISC. In line with the data obtained from melanoma cells, thus blocks Caspase-8 recruitment to, cleavage within, and subsequent release of active Caspase-8 from the DISC. Due to repression of cFLIP by nickel stimulation or knock down using siRNA, available cFLIP proteins in the DISC become limited and are no longer able to sufficient interfere with Caspase-8. As a result, Caspase-8 is increasingly recruited in the DISC, cleaved to its active form and rapidly released into cytoplasm, where it is exerting its pro-apoptotic function.

Both isoforms of cFLIP are efficiently recruited in the DISC upon death ligand stimulation in melanoma and endothelial cells which is consistent with previous studies (Chang et al., 2002; Lavrik et al., 2007; Neumann et al., 2010). $cFLIP_L$ thereby permit autocatalytic cleavage of Procaspase-8 to its p43/p41 fragments in the receptor complex. In contrast, $cFLIP_S$ blocks Caspase-8 cleavage completely but does not preclude its recruitment in the DISC (Krueger et al., 2001). Despite different modulation of Caspase-8 cleavage, both isoforms of cFLIP inhibit sufficiently full Caspase-8 activation and thereby death ligand-induced apoptosis. However, it can be speculated that both isoforms of cFLIP may differentially regulate Caspase-8-dependent non-apoptotic signaling (Siegmund et al., 2007) due to modulation of Caspase-8 cleavage. Molecular mechanisms of cFLIP isoforms in

regulating non-apoptotic death receptor signaling pathways are still unclear (Falschlehner et al., 2007; Lavrik et al., 2005). It is speculated that $cFLIP_L$ or its cleaved fragments may modulate the important apoptosis-independent Wnt signaling pathway (Naito et al., 2004). In addition, concerning the suggested interaction of $cFLIP_L$ cleavage fragments with RIP1 (Dohrman et al., 2005) and TRAF2 (Kataoka and Tschopp, 2004) together with the postulated interaction of cFLIP p22 with IKKγ (Chaudhary et al., 1999; Golks et al., 2006) support the hypothesis that different cFLIP isoforms might be able to differentially regulate death receptor-induced non-apoptotic signaling pathways such as NF-κB or MAPK. These may result in activation of distinct sets of target genes upon death ligand stimulation that potentially facilitate tumor progression or metastasis, as reported in a pancreatic tumor model (Trauzold et al., 2006). In order to carefully evaluate tumor therapy conditions it is necessary to not only enhance apoptosis induction but in addition prevent activation of pro-invasive and pro-inflammatory signaling pathways. Nevertheless it is suggested that interference with cFLIP expression in combination with TRAIL agonist stimulation may represent an attractive therapy at least for melanoma treatment (Chawla-Sarkar et al., 2004; Geserick et al., 2008; Hamai et al., 2006; Ivanov and Hei, 2006). On the other hand, the contradictive data concerning cFLIP and CD95-induced NF-κB activation arrogates further investigation of cFLIP proteins in death receptor-mediated signaling pathways, before there modulation can be used in therapeutic approaches. So far as it known, cFLIP is supposed to decrease (Kreuz et al., 2004; Wachter et al., 2004), not influence (Legembre et al., 2004), or increase (Golks et al., 2006) CD95-induced NF-κB activity. A direct link between CD95 stimulation and NF-κB activation got recently new evidence (Neumann et al., 2010). Authors of original paper showed that the p43 cleavage fragment of $cFLIP_L$ interacts also with the IKK complex which leads to its activation. This pathway differs from the previously published p22-mediated NF-κB activation, in which Procaspase-8 cleaves cFLIP independently of CD95 stimulation (Golks et al., 2006). Interestingly, Neumann and colleagues could not detect RIP1, a well known mediator of death receptor-induced NF-κB activation (Dohrman et al., 2005; Kataoka and Tschopp, 2004; Kreuz et al., 2004; Misra et al., 2005), to be associated with the IKK complex upon those conditions. Taken together, these contradictive data demonstrate that death receptor signaling pathways are regulated in a highly complex manner which is not fully understood to date. This is also supported by the observation that cFLIP protein expression alone did not generally correlate with TRAIL sensitivity in various melanoma cells (Geserick et al., 2008; Zhang et al., 1999). Thus, activation and further outcome of death receptor-mediated signaling pathways can differ depending on the cellular context. Exposure of death receptors on the cell surface (Geserick et al., 2008), intensity of stimulation of these receptors (Neumann et al., 2010), expression levels of death receptor pathway-associated molecules (Thorburn, 2004), ratio of pro- and anti-apoptotic proteins (Karst and Li, 2007;

Meng et al., 2006) and many other unknown regulative signals influence regulation and execution of death receptor-induced signaling pathways in various cells (Lee et al., 2006; Leverkus et al., 2008; Van Herreweghe et al., 2010; Wertz and Dixit, 2010). Crucial regulators of these pathways are cFLIP proteins. Further understanding of death receptor-induced signaling pathways requires complete understanding of cFLIP function. Therefore, future studies have to address associations of cFLIP with interacting molecules in addition to Caspase-8.

4.2 cIAP2 alone is not sufficient to mediate resistance to TNF-induced cell death

Death ligands as TNF are essential cytokines with various effects (Ashkenazi and Dixit, 1998; Locksley et al., 2001). TNF induces apoptotic or non-apoptotic signaling pathways including NF-κB, depending on the cellular context (Wajant et al., 2003b). Over the past decade, it has been widely accepted that NF-κB is involved in maintenance of apoptosis resistance to multiple stimuli (Burstein and Duckett, 2003; Karin and Lin, 2002). This mechanism may play a crucial role for tumor development and progression (Wajant, 2009). Therefore, understanding of NF-κB-mediated resistance to TNF may help to develop efficient TNF-based cancer therapies. Dramatic sensitization to TNF-mediated apoptosis has been observed when NF-κB activation is inhibited by expression of a dominant-negative IKKβ mutant in HaCaT cells. These data are in line with reports showing that death-inducing function of TNF is masked whenever activation of NF-κB by TNF is possible (Wajant et al., 2003b). Given the importance of TNF signaling in human keratinocytes (Banno et al., 2004), it was of particular interest to investigated regulatory mechanisms relevant for TNF-induced apoptosis. Over-expression studies suggested that NF-κB target genes - TRAF1, TRAF2, cIAP1, and cIAP2 - are relevant for TNF-mediated apoptosis (Wang et al., 1998). Following data indicate that neither cIAP1 nor cIAP2 are able to inhibit caspases directly (Eckelman and Salvesen, 2006). Roles of cIAP1 and cIAP2 in TNF-induced cell death were unclear when the current project was started. To clarify the existing contradictive findings, expression of a large panel of potential NF-κB target genes were examined in human keratinocytes (Diessenbacher et al., 2008). cIAP2 is induced dramatically by TNF in keratinocytes which is in line with data obtained using other cell types (Varfolomeev et al., 2005). In addition, cIAP2 expression level was significantly modulated in an NF-κB-dependent manner. Thus, cIAP2 were analyzed to be a candidate for regulation of TNF resistance in keratinocytes. However, several experiments revealed that cIAP2 alone is not crucial for maintaining TNF resistance, at least in keratinocytes. Also formation of the TNF signaling complex could not be influenced by cIAP2 expression alone. It has been suggested earlier, that cIAP1, cIAP2 and XIAP may

be able to compensate each other (Harlin et al., 2001). It was postulated, that IAP2 levels are regulated by ubiquitin ligase activity of cIAP1 (Conze et al., 2005; Vaux and Silke, 2005a). Despite of the negative results for cIAP2 in this set of experiments, recent studies (Bertrand et al., 2008; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007) and subsequent data of this work could finally reveal the critical roles of cIAP1 and cIAP2 in regulating death receptor-mediated signaling pathways by using IAP antagonists. To answer the question why inhibition of NF-κB sensitizes human keratinocytes to TNF requires further investigations in the future. Nowadays it can be only speculated, that other NF-κB regulating molecules like A20 (Heyninck and Beyaert, 2005; Shembade et al., 2010), A20 Binding Inhibitor of NF-κB (ABIN1) (Heyninck et al., 2003; Verstrepen et al., 2009; Wagner et al., 2008), Heme-Oxidized IRP2 ubiquitin Ligase-1 (HOIL-1) and HOIL-1 Interacting Protein (HOIP) (Haas et al., 2009; Kirisako et al., 2006; Tokunaga et al., 2009) may be involved in this process.

4.3 Both cIAP1 and cIAP2 together confer efficient resistance to death ligand-mediated cell death

IAP antagonists have been designed to mimic the natural IAP inhibitor SMAC/DIABLO. They prevent XIAP from inhibiting caspases and thereby inducing apoptosis in cancer cells (Li et al., 2004; Schimmer et al., 2004). It is shown that IAP antagonists, which sensitize cells to TNF (Bertrand et al., 2008; Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007) and TRAIL-induced cell death (Fakler et al., 2009; Fulda et al., 2002; Vogler et al., 2009), also can dramatically alter sensitivity to CD95L. During the last decade CD95L was intensively investigated for therapeutic exploitation in tumor treatment (Ehrenschwender and Wajant, 2009; Gerspach et al., 2009b). In line with the literature, the pharmacological inhibitor CompA induces rapid degradation of cIAP1 (Vince et al., 2007) in skin cells. Additionally cIAP2 is also repressed in these cells. Interestingly, CompA sensitizes HaCaT and MET1, but not A5RT3 cell lines to CD95 and TRAIL-mediated cell death. Increased cell death is independent of a previously suggested TNF-loop (Petersen et al., 2007; Vince et al., 2007). The fact that HaCaT cell do not express XIAP suggests that loss of cIAPs causes the increased sensitization to CD95L in HaCaT cells. The relevance of cIAPs in regulating CD95-mediated cell death is confirmed by examination of cIAP modulation studies. Repression or complete knock out of cIAPs sensitizes cells to CD95L (Geserick et al., 2009), while over-expression of cIAPs partially protects from CD95L-induced cell death. CD95L induces apoptosis in keratinocytes, which is in line with findings in many other cell types (Mahmood and Shukla, 2010; Nagata and Golstein, 1995). In presence of cIAPs, CD95L induces apoptosis can be blocked by inhibition

of caspase activity. Loss of cIAPs strongly increase CD95L induced apoptosis. Interestingly, blocking of the CD95-mediated apoptotic pathway, in addition revealed a cryptic alternative pathway in absence of cIAPs. This non-apoptotic pathway is independent of caspase activity and thereby cannot be blocked by zVAD-fmk. Early studies have shown that blocking of the death receptor-mediated apoptotic pathway, can induce an alternative cell death pathway (Holler et al., 2000; Matsumura et al., 2000; Vercammen et al., 1998). This pathway is variously called programmed necrosis or necroptosis and can occur in absence of FADD and Caspase-8 (Bell et al., 2008; Ch'en et al., 2008). A recent study has identified extracellular pH as a possible mechanism to switch death receptor-induced apoptotic cell death to necrotic cell death in absence of pharmacological caspase inhibitors such as zVAD-fmk (Meurette et al., 2007). In the current study it is demonstrated that cIAPs inhibit both a caspase-dependent apoptotic as well as a caspase-independent necrotic cell death upon CD95 stimulation.

cIAPs are recruited to the proximal TNF-R1 signaling platform (Vaux and Silke, 2005b). Interestingly, cIAP1 and cIAP2 could not be detected in the DISC and the receptor-independent complex II of CD95L stimulated HaCaT and MET1 cells. Exclusively in A5RT3 cells, cIAP2 recruitment in both complexes was observed. High levels of cIAP2 in A5RT3 cells may explain its detection in both complexes and may also explain resistance of A5RT3 cells to CompA-induced sensitization to death ligands. cIAP are suggested to be recruited by TRAF2 in the TNF complex (Mace et al., 2010) to suppress Caspase-8 activation (Micheau and Tschopp, 2003; Wang et al., 1998). In contrast with the literature, TRAF2 could also not be detected in the CD95 DISC (Siegmund et al., 2007). Lack of TRAF2 can be the reason why cIAPs were not detectable in the DISC. These negative results of detection of cIAPs and TRAF2 in both complexes can not exclude their recruitment. Further experiments with less stringent agents may reveal a weak interaction of cIAPs and TRAF2 with the CD95 receptor complex. On the other side, cIAPs may regulate recruitment of other important DISC-associated molecules including RIP1 outside of the CD95 receptor complex.

In summary, it is hypothesized that cIAP1 and cIAP2 together are essential regulators of death receptor-mediated cell death. Depletion of just one cIAP can be partially compensated by the second molecule. A recent study could show that upregulation of cIAPs is associated with a progressive course of chronic lymphocytic leukaemia (Grzybowska-Izydorczyk et al., 2010). Therefore, repression of both cIAPs for example by IAP antagonists may be an efficient therapeutic approach to sensitize tumors to death ligands.

4.4 RIP1, a main target of cIAP regulation, is a central key factor in activating different death receptor-induced signaling pathways

Examination of the CD95-mediated DISC and the complex II formation further revealed a cIAP-dependent regulation of RIP1 similar to TNF (Mahoney et al., 2008; Varfolomeev et al., 2008). The current study showed that recruitment of RIP1 to the CD95 DISC and subsequent formation of the intracellular complex II are strongly repressed by cIAPs. The precise physiological relevance of complex II formation after death ligand stimulation remains elusive to date (Lavrik et al., 2008; Varfolomeev et al., 2005). Loss of cIAPs leads to dramatically enrichment of RIP1 at the CD95 DISC. Following CD95 induction, RIP1 can provide both apoptotic (Morgan et al., 2009) as well as necrotic (Degterev et al., 2005; Holler et al., 2000) cell death pathways. Increased RIP1 recruitment leads to more efficient activation of Caspase-8 in the DISC as suggested recently (Morgan et al., 2009). This would explain the observed enhancement of apoptotic death in cells treated with CompA. This hypothesis is supported by enrichment of Caspase-8 cleavage fragment p18 in the DISC whenever cIAPs are repressed. Previously, it was suggested that cIAPs can suppress Caspase-8 activation (Micheau and Tschopp, 2003; Wang et al., 1998). In contrast, a recent study demonstrated that cIAP1 and cIAP2 bind but do not inhibit caspases (Eckelman and Salvesen, 2006). The data of the current study suggest that cIAPs suppress caspase activation indirectly via modulation of RIP1. Inhibition of caspase activity upon CD95 stimulation unmasks an alternative necrotic cell death pathway in absence of cIAPs. In line with data published for TNF (Chan et al., 2003; Vercammen et al., 1997) and TRAIL (Holler et al., 2000) this necrotic CD95 pathway is RIP1-dependent and can be repressed by knock down of RIP1. The specific RIP1 kinase inhibitor Necrostatin-1 confirms the necessity of the kinase activity of RIP1 for the necrotic phenotype as was described previously (Degterev et al., 2008; Vandenabeele et al., 2008). Recent studies demonstrated that the kinase activity of RIP1 is necessary for RIP1 interaction with RIP3, a further kinase required for TNF-mediated necrosis (Cho et al., 2009; Feng et al., 2007; He et al., 2009; Zhang et al., 2009). Activated RIP3 regulates subsequently several metabolic enzymes (Zhang et al., 2009). These enzymes are proposed to mediate an excessive increase in energy metabolism which is part of the execution mechanism of TNF-induced necrosis (Moquin and Chan, 2010; Vandenabeele et al., 2010). Future experiments have to be performed in order to investigate this metabolic pathway in more details, which in turn may clarify why necrotic cell death is so energy consuming comparing with apoptosis.

A5RT3 cells are resistance to CompA-mediated sensitization to CD95. Reasons for this resistance could not be reveled in frame of this project. Given the differential sensitivity, recruitment of FADD, cFLIP isoforms and Caspase-8 in the DISC is remarkably similar in sensitive MET1 and non-sensitive A5RT3 cells, either in presence or absence of CompA. In

contrast, RIP1 enrichment in the CD95 DISC of A5RT3 is much less pronounced compared to MET1 cells in absence of cIAPs. Therefore it can be speculated, that the total amount of recruited RIP1 in the DISC of A5RT3 cells is below a certain threshold for increased RIP1-dependent cell death induction. However, in contrast to the DISC, RIP1 levels associated in the complex II of A5RT3 and MET1 cells were significantly less different. This finding indeed provides a strong evidence for a downstream effect below RIP1 underlying the observed resistance of A5RT3 cells to CompA-mediated sensitization. Future studies may reveal whether modulation of RIP3 or further downstream factors in the cell death pathway can efficiently counteract loss of cIAPs and provide resistance to death ligands.

Interestingly, not only total amount of RIP1 is increased in both signaling platforms in absence of cIAPs, the pattern of RIP1 modification also was altered in all tested cells. Several studies have shown that RIP1 is a direct target of the E3 ligase activity of cIAPs (Bertrand et al., 2008; Park et al., 2004; Varfolomeev et al., 2008). This change in RIP1 modification is potentially a change in ubiquitylation pattern caused by the loss of cIAPs. Ubiquitination has emerged as an important secondary protein modification that regulates various biological processes (Fang and Weissman, 2004). It is proposed that RIP1 can be K-63-polyubiquitinated on K-377 (Ea et al., 2006) by cIAP1 and cIAP2 (Bertrand et al., 2008). This event enables RIP1 to act as scaffold for assembly of TAK1 as well as the IKK complex and causes the activation of NF-κB pathways (Wu et al., 2006). Consequently, it can be speculated that loss of cIAPs can lead to suppression of NF-κB activation and subsequently increase cell death upon CD95 stimulation. Future investigations may address this question. However, K-63 linkage of RIP1 mediated by cIAPs cannot explain the dramatically increase of RIP1 recruitment in the CD95 DISC and the complex II. Intriguingly, it is also shown that cIAPs can assemble K-48 chains to RIP1 (Bertrand et al., 2008; Park et al., 2004). K-48 polyubiquitination leads to proteasomal degradation of RIP1, which would limit RIP1 in the DISC. Nevertheless, in frame of this project it could not be discovered how cIAPs limit RIP1 in CD95 signaling complexes. Future studies may reveal molecular mechanisms and clarify whether cIAPs inhibit RIP1 recruitment into the DISC or whether they promote RIP1 removing from the DISC.

A further point of RIP1 regulation is that RIP1 is not only modified in signaling platforms but also cleaved by Caspase-8 as suggested (Cho et al., 2009; Vandenabeele et al., 2010). This fact leads to the question what is the impact of caspase activation on RIP1-dependent cell death. Cleavage of RIP1 under apoptotic conditions can be a mechanism to ensure that cells undergo apoptotic death (Declercq et al., 2009). It is proposed that RIP1 is inactivated by proteolytic cleavage by Caspase-8 (Cho et al., 2009). In presence of caspase inhibitor zVAD-fmk cleavage of RIP1 is prevented as shown in frame of this project. Zhang et al., 2009 postulate that this blocking of RIP1 cleavage by zVAD-fmk allows necrosis to

proceed. This is supported by the observation in a subset of cell lines, where loss of Caspase-8 function results in activation of a necrotic cell death pathway that requires RIP1 (Ch'en et al., 2008; Festjens et al., 2007; Vandenabeele et al., 2010). Significant increase of full-length RIP1 in the CD95 DISC is obvious when keratinocytes treated with zVAD-fmk. However, cell death is completely blocked by zVAD-fmk in presence of cIAPs. Expression of natural caspase inhibitors like cFLIP isoforms does not increase RIP1 recruitment in the CD95 DISC whenever cIAPs are present. These suggest an off-target effect of zVAD-fmk for enrichment of RIP1 in the CD95 receptor complex independent of caspase inhibition. Therefore the mechanism of RIP1 recruitment in these complexes as well as its cleavage remains elusive. It is tempting to speculate that enrichment of full-length RIP1 in the CD95 DISC alone is not sufficient to increase cell death. Further regulation of RIP1 is required for cell death induction. This regulation can be mediated by the loss of cIAPs and by following change of RIP1 modification. In addition, it is suggested that regulation of RIP1 by cleavage further facilitates CD95-induced apoptotic cell death. Whereas inhibition of RIP1 cleavage may causes an activation of a necrotic cell death pathway. It remains a challenge for future study to verify this hypothesis.

In human keratinocytes, RIP1 is critical for CD95-induced cell death in absence of cIAPs. As previously mentioned, its kinase activity is required for activation of the necrotic pathway. RIP1 is increasingly recruited in the DISC and the complex II upon loss of cIAPs. Enrichment of distinct kinases can cause their autoactivation (Eswarakumar et al., 2005). Given these, particular aspects of relationships of signaling platforms and RIP1 kinase activity were interesting to analyze. In this study it was demonstrated that RIP1 kinase activity contributes to formation of the intracellular complex II in HaCaT cells. Inhibition of RIP1 kinase activity with Necrostatin-1 abolishes interaction of RIP1 and Caspase-8. Thus suggest that kinase activity of RIP1 facilitates maturation of the cytoplasmic complex II. It can be only speculated what are the molecular mechanisms underlying this process. Future studies my reveal whether RIP1 kinase activity facilitates translocation of the RIP1-containing DISC into the RIP1-containing complex II or facilitates new formation of the secondary complex. As a part of TNF-mediated necrotic pathway, it is postulated that deubiquitinated RIP1 dissociates from the TNF complex I and recruits RIP3, FADD and Caspase-8 (He et al., 2009). This newly formed complex is called necrosome (Vandenabeele et al., 2010). Presence of Necrostatin-1 blocks interaction of RIP1 and RIP3 and thereby inhibits necrosome formation. This can be similar in the CD95-induced necrotic pathway. Then, the CD95-induced intracellular complex II could be the equivalent to the TNF-induced necrosome. To get higher evidence for this hypothesis, it has to be shown in future studies that RIP3 is associated with the CD95-induced complex II. Afterwards it can be analyzed whether interaction of RIP1 and RIP3 may stabilize this secondary complex.

4.5 Different cFLIP isoforms have distinct signaling capabilities when cIAPs are repressed

Expression of anti-apoptotic molecules has been suggested to correlate with tumor progression (Valente et al., 2006). Hence, upregulation of apoptosis inhibitors can maintain a threshold toward CD95-mediated apoptosis and in addition promote CD95-mediated tumor growth (Chen et al., 2010). Both cFLIP and cIAPs confer resistance to CD95-mediated cell death and are described for activating survival pathways (Neumann et al., 2010; Varfolomeev and Vucic, 2008). Combined inhibition of cFLIP and IAP family member as Survivin (Schultze et al., 2006) or XIAP (Wilson et al., 2009b) mediates apoptosis in different cancer cells. Given these, it was of particular interest to assess the association of cFLIP and cIAPs together in regulation of death receptor-induced cell death. In particular the question should be answered whether cFLIP isoforms may confer resistance to CompA-mediated sensitization to CD95L. Surprisingly, cFLIP isoforms differentially contribute to resistance to CD95-mediated cell death in HaCaT and MET1 cells in absence of cIAPs. While both isoforms block apoptotic cell death by inhibition of caspase activation, $cFLIP_S$ is insufficient to prevent activation of RIP1-dependent necrotic cell death. In contrast, $cFLIP_L$ effectively blocks both CD95-induced apoptosis and necrosis. Inhibitory potential of $cFLIP_L$ is possibly associated with its ability to repress RIP1 accumulation in the CD95 DISC and complex II. These data point to different hypotheses. Both cFLIP isoforms block fully activation of Caspase-8. However, in contrast to $cFLIP_S$, $cFLIP_L$ allows partial cleavage of Caspase-8. Given these, it can be speculated that the partially cleaved Caspase-8 fragments p43/p41 and/or the caspase-like domain of $cFLIP_L$ may be involved in cleavage and thereby limitation of RIP1 in the CD95 complexes. Alternatively, $cFLIP_L$ might simply be better than $cFLIP_S$ in inhibiting RIP1 recruitment because of steric hindrance. So, $cFLIP_L$ was reported to mediate binding to RIP1 (Kataoka, 2005). In particular, cleaved $cFLIP_L$ fragment p12 may bind RIP1 and thereby inhibit RIP1 association in CD95 complexes. Indeed, the interaction of cFLIP p12 and RIP1 has to be demonstrated first. Finally, it is also possible that cFLIP isoforms act independently of receptor complexes as previously suggested in lymphoid cell (Golkset al., 2006). For example, it is possible that non-ubiquitylated forms of RIP1 bind to FADD independently of DISC formation. This could subsequently lead to necrosome formation and thereby facilitate necrotic cell death. This hypothesis is supported by the intriguingly finding, that CompA treatment induces spontaneous complex II formation in $cFLIP_S$-expressing HaCaT cells independent of death receptor stimulation. The current study using isoforms of cFLIP has the obvious limitation of ectopic over-expression of both isoforms. HaCaT cells express low levels of endogenous cFLIP as compared with primary keratinocytes (Leverkus et al., 2000) and therefore represent an ideal model for these experiments (Wachter et al., 2004). However, over-expression experiments can result in non-physiological responses. In

case of cFLIP, lack of any clear data about physiological relevance of different isoforms makes it challenging to interpret such experiments. Even in case of viral FLIP (vFLIP) isoforms, which are highly similar to $cFLIP_S$, it is unknown what are the intracellular protein levels during virus infection. Also it is unknown how endogenous cFLIP affects vFLIP effects. Of note, vFLIP of Human Herpesvirus type 8 (HHV8) dramatically increases its expression during late stages of HHV8-induced Kaposi sarcomas (Sturzl et al., 1999). Thus, a major shift of ratio of $cFLIP_L$ to $cFLIP_S$, such as that which occurs in our over-expression experiments, may be of patho-physiological relevance. In contrast to HaCaT cells, MET1 cells are p53 wild type and express XIAP. Over-expression of $cFLIP_L$ or $cFLIP_S$ in MET1 cells resulted in very similar observation compared to HaCaT cells. This suggests a general phenomenon in respect to isoform-specific effects of cFLIP. In summary these data show a remarkable and previously unsuspected specificity concerning the mechanism of death inhibition by cFLIP isoforms. A novel and differential functions of $cFLIP_L$ and $cFLIP_S$ is demonstrated in absence of cIAPs.

4.6 Does modulation of cIAPs have physiological relevance?

Although IAP antagonists have attracted great interest as cancer therapeutics, it could be questioned whether these findings have any physiological relevance. The cytokine TNF-like Weak inducer of apoptosis (TWEAK) binds to the Fibroblast growth factor-inducible 14 (FN14) receptor and promotes rapid degradation of cIAPs in an analogous manner to IAP antagonists (Vince et al., 2008; Wicovsky et al., 2009). TWEAK signaling duplicates demonstrated effects of the IAP antagonist CompA (Geserick et al., 2009) indicating that such signals have a physiological relevance. TWEAK and other ligands such as TRAIL, CD95L, or TNF are likely to be present in the same physiological scenario (Vince and Silke, 2006). Thus, stimulation of FN14 (Geserick et al., 2009) or possibly other receptors such as CD40 may enable the recruitment of cIAPs, which in turn may triggers death receptor-mediated apoptotic to necrotic cell death. Alteration in death receptor response may lead to major physiological and pathophysiological consequences during tumorigenesis or inflammatory response in multicellular organisms (Kerstan et al., 2009; Leverkus et al., 2008; Wicovsky et al., 2009).

4.7 Perspectives

The current study and resulting publications clearly demonstrate crucial functions of cFLIP, cIAPs and RIP1 in regulation of death receptor-mediated signaling pathways. Despite this remarkable progress, significantly more questions arise (Fig. 26).

- How is recruitment of RIP1 in death receptor complexes controlled? It was suggested that RIP1 is constitutively coupled with ubiquitin moieties by E3 ligase activity of cIAPs in the cytoplasm (Bertrand et al., 2008). Thus, localization and constitutive or induced modification of RIP1 may regulate its affinity to death receptor-induced complexes. Alternatively, cIAPs may induce RIP1 removal from the DISC. Further investigation in cIAP-mediated polyubiquitination of RIP1 may help to solve these questions.
- How do both cFLIP isoforms influence activation of MAPK and transcription factors upon death ligand stimulation? Future experiments have to solve the contradictory discussion about repression and activation of NF-κB promoted by cFLIP (Neumann et al., 2010; Wachter et al., 2004). This may clarify, whether repression of cFLIP suppresses or facilitates tumor progression in potential therapeutic approaches.
- Why do cFLIP isoforms differentially regulate necrotic cell death? Both cFLIP proteins block apoptotic cell death, whereas only $cFLIP_L$ is competent to block necrotic cell death. Does $cFLIP_L$ simply compete with RIP1 for recruitment in the DISC and/or the complex II and thereby block necrosis? Or does $cFLIP_L$ promoted partially activation of Procaspase-8 which in turn mediates cleavage (and potentially inactivation) of RIP1 and thereby interfere with necrotic cell death?
- $cFLIP_S$ neither activates Procaspase-8 nor does it block RIP1 recruitment in the DISC and complex II. In addition, $cFLIP_S$ expression induces spontaneous formation of the cytoplasmic complex II in absence of cIAPs independent of death ligand stimulation. How does $cFLIP_S$ stabilize this complex II formation? And more generally, what is the physiological relevance of this cytoplasmic complex? Is this complex associated with the described necrosome and is RIP3 involved (Vandenabeele et al., 2010)?
- A number of viruses express $cFLIP_S$ homologous isoforms (e.g. vFLIP). Do high levels of $cFLIP_S$ together with low levels of cIAPs favor elimination of virus? Can the observed shift of death receptor-induced apoptotic to necrotic cell death be important for host response to virus infection? Alternatively, do vFLIP-expressing viruses require cIAPs for resistance to necrosis? Answering these questions would be interesting for the potential usage of IAP antagonists for treatment of virus-induced cancer types.

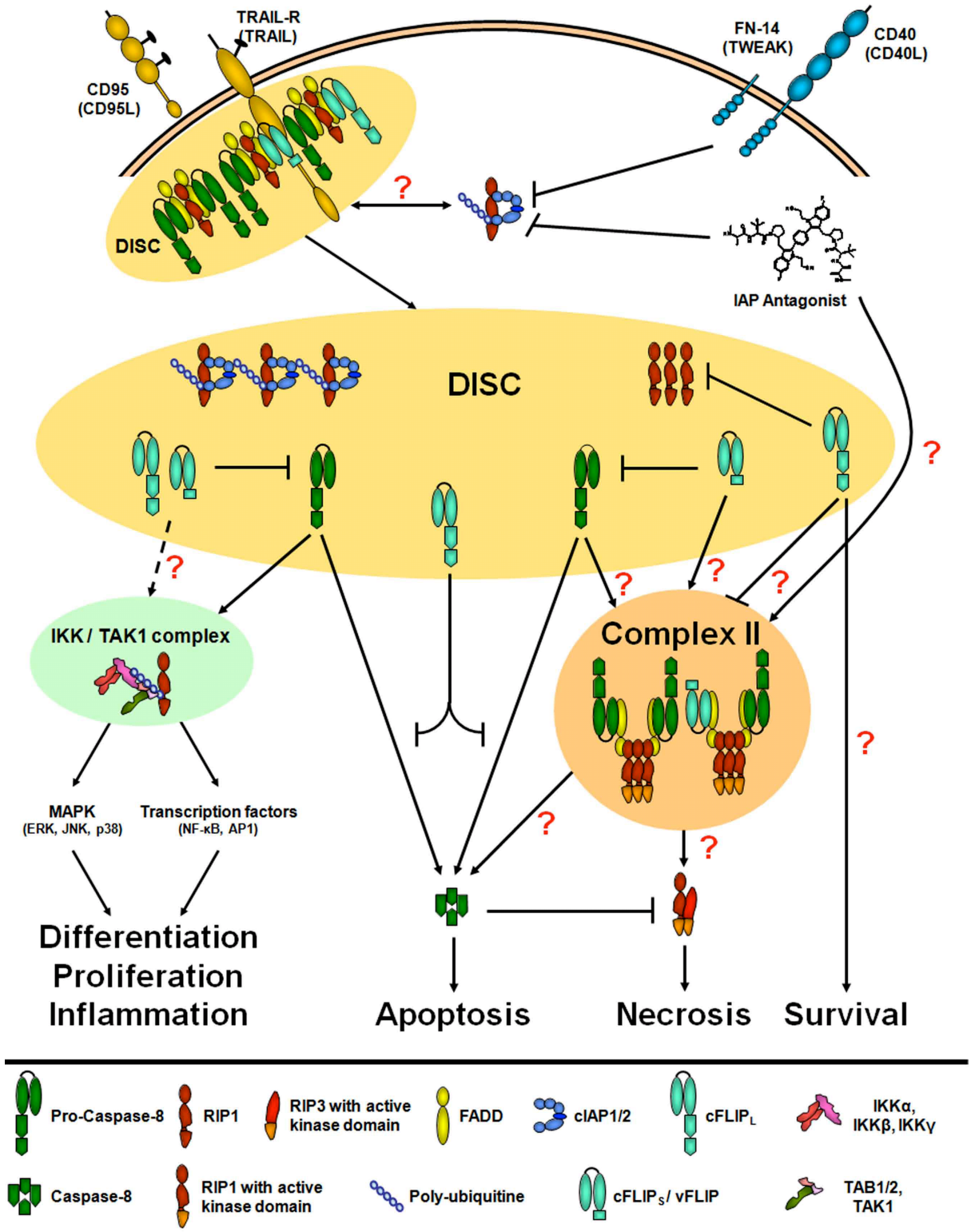


Figure 26: Roles of cIAPs, RIP1 and cFLIP isoforms in death ligand-induced apoptotic and necrotic signaling pathways. Recruitment to and degradation of RIP1 in the DISC and/or in the secondary complex is critically regulated by cIAPs. The intracellular localization of cIAP-mediated ubiquitinylation of RIP1 is so far unknown. Enrichment of modified RIP1 in the DISC mediates formation of the IKK/TAK1 complex. This complex promotes activation of MAPK and/or NF-κB pathways and thereby cell death resistance. Under this condition all cFLIP isoforms repress activation of Caspase-8, but their relevance for MAPK and NF-κB activation is controversial discussed to date. In contrast, synthetic compounds (IAP antagonist) or activation of other signaling pathways (e.g. TWEAK or CD40L) can antagonize the function of cIAPs and promote death ligand-triggered cell death. This cell death can be either apoptotic via activation of caspases or necrotic via activation of RIP1. The necrotic pathway is unmasked only in absence of both active caspases and cIAPs. Increased

accumulation of unmodified RIP1 in the DISC promote increased formation of the RIP1 containing cytoplasmic complex II. Formation of the secondary complex is increased by autoactivation of RIP1. $cFLIP_L$ but not $cFLIP_S$ is able to block formation of the complex II by limiting RIP1 recruitment. In addition, high levels of $cFLIP_S$ lead to the spontaneously formed complex II independent of death ligand stimulation, in absence of cIAPs. Admittedly, the physiological relevance of the cytoplasmic complex in providing apoptosis and/or necrosis is still unresolved. It is suggested that RIP1 binds RIP3 which subsequently induce the necrotic cell death pathway.

Death ligand-based cancer therapeutic approaches are under current investigation (Gerspach et al., 2009a). Nevertheless, to develop efficient and side-effect free therapeutic approaches complete understanding of death receptor-induced signaling pathways is required. However, the increasing complexity of these signaling pathways will be challenging for further verification.

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6 Appendix

6.1 Abbreviations

% (v/v) - percent by volume
% (w/v) - percent by mass
4-HT - 4-Hydroxytamoxifen
aa - amino acids
ABIN1 - A20 Binding Inhibitor of NF-κB
AIF - Apoptosis-Inducing Factor
Bcl-2 - B-cell lymphoma 2
BIR - Baculoviral IAP Repeat
CARD - Caspase Activation and Recruitment Domain
Caspase - Cysteinyl-Aspartate Specific Protease
CD95L = FasL (FS7-associated cell surface antigen Ligand)
cFLIP - cellular FLICE-Inhibitory Protein
cIAP - cellular Inhibitor of Apoptosis Protein
CIAP - Alkaline Phosphatase from calf intestine
CompA - Compound A (IAP antagonist)
DD - Death Domain
DED - Death Effector Domain
DISC - Death Inducing Signaling Complex
D-MEM - Dulbecco´s Modified Eagle Medium
DNA - deoxyribonucleic acid
dNTPs - Deoxynucleoside Triphosphate Set
E1 - ubiquitin activating enzyme
E2 - ubiquitin conjugating enzyme
E3 - ubiquitin ligase enzyme
E.coli - Escherichia coli
EDTA - ethylenediaminetetraacetic acid
Expo - exposition
F - Flag (tag)
FADD - Fas-Associated Death Domain protein
FBS - Fetal Bovine Serum
Fc - Fragment crystallizable
Fig - Figure
GFP - Green fluorescent protein
HaCaT - kuman adult low calcium temperature (keratinocytes)
HEK 293T - Human Embryonic Kidney 293 large T transformed
HF - His-Flag (tag)
HOIL-1 - Heme-oxidized IRP2 ubiquitin ligase-1
HOIP - HOIL-1 interacting protein
HRP - Horseradish peroxidase
HUVEC - Human Umbilical Vein Endothelial Cells
IgG - Immunoglobulin G
IκB - Inhibitor of NF-κB
IKK - Inhibitor of κB Kinase
IP - Immunoprecipitation
K - lysine
KD - Kinase Death
kDa - kilo Dalton
MAPK - Mitogen-Activated Protein Kinase
MW - Molecular Weight
NEMO - NF-κB Essential Modulator
NF-κB - Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
NIK - NF-κB-Inducing Kinase
PAGE - Polyacrylamide Gel Electrophoresis

PBS - Phosphate-Buffered Salines
PCR - Polymerase chain reaction
PVDF - Polyvinylidene fluoride
R - Receptor
RING - Really Interesting New Gene
RIP1 - Receptor-Interacting Protein 1
SDS - Sodium Dodecyl Sulfate
SEM - Standard Error of Mean
ShRNA - Small hairpin RNA
SiRNA - Small interfering RNA
SMAC/DIABLO - Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI
TAB1 - TAK Binding protein 1
TAE - Tris-acetate-EDTA
TAK1 - Transforming Growth Factor-β (TGF-β)-Activated Kinase 1
TL - Total Lysate
TNF - Tumor necrosis factor
TRADD - TNF Receptor Associated Death Domain
TRAF2 - TNF Receptor Associated Factor-2
TRAIL - TNF-related apoptosis-inducing ligand
U - Unit
UBA - Ubiquitin-Associated (domain)
UV - Ultra Violet
XIAP - X-linked IAP
ZVAD-fmk - z-Val-Ala-DL-Asp(OMe)-fluoromethylketone

6.2 Curriculum vitae

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6.3 Erklärung

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Erklärung

Hiermit erkläre ich, dass die von mir eingereichte Dissertation zum Thema:

Apoptotic and non-apoptotic death receptor signaling pathways and their regulation by cFLIP, cIAPs and RIP1 in the skin

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, der 22.06.2010

Diplom Biologe Mike Hupe